Altered Levels of Protein Kinase C and Ca\(^{2+}\)-dependent Protein Kinases in Human Colon Carcinomas

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ABSTRACT

Protein kinase C (PKC) is a Ca\(^{2+}\)- and phospholipid-dependent protein kinase which is implicated in tumor promotion, since it has been demonstrated to be a high affinity receptor for tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate. Colon carcinogenesis appears to proceed through distinct stages of initiation and promotion. The present studies show that PKC and calcium-dependent protein kinase specific activities are reduced in human colon carcinomas when compared to their normal adjacent colon mucosa. There were significantly higher Ca\(^{2+}\)-dependent protein kinase and PKC specific activities observed in both the cytosolic and particulate fractions of the normal mucosa relative to the corresponding values obtained with the carcinoma fractions. The average specific activity ratios were 5.1 (normal cytosolic/carcinoma cytosolic) and 3.7 (normal particulate/carcinoma particulate) for PKC. PKC activity was reduced in the carcinoma tissues with respect to both protein and tissue weight. The percentage of Ca\(^{2+}\)-dependent protein kinase and PKC activities that were present in the particulate fraction of each of the samples varied considerably among tissues, and in general there was no systematic difference between the carcinoma and normal mucosa samples. However, in the carcinoma samples that contained an extensive admixture of benign adenomatous tissue, the particulate fractions consistently contained greater than 60% of the total Ca\(^{2+}\)-dependent protein kinase and PKC activities. The present studies indicate that colon carcinogenesis is associated with alterations in cellular levels of protein kinase activities.

INTRODUCTION

PKC is a Ca\(^{2+}\)- and phospholipid-dependent protein kinase (1). This membrane-associated enzyme is implicated in tumor promotion, since it has been demonstrated to be a receptor for TPA and related tumor promoters, and since binding of these compounds to this enzyme system activates the protein kinase (2). Furthermore, PKC is ubiquitous in eukaryotes (3) and its in vivo activity is normally modulated by alterations in membrane structure and phospholipid turnover; thus, it appears to play a key role in transmembrane signaling (4, 5). In fact, a number of growth factors and hormones appear to act via PKC activation, since they stimulate phosphatidylinositol turnover and thus cause the production of DAG, an endogenous activator of PKC (6). Although TPA-mediated tumor promotion has been most widely studied in the mouse skin model, this phenomenon has also been demonstrated in the upper gastrointestinal tract of rodents (7).

PKC is normally found in both the cytosolic and membrane compartments of cells at levels that vary from tissue to tissue and also with the physiological and embryological state of a tissue (8–10). Treatment of cells with TPA results in a translocation of PKC from the cytosol to the membrane fraction and subsequent down regulation of enzyme activity (11–13), as well as down regulation of phorbol ester receptor binding (14). For example, in a human mammary carcinoma cell line, there is an early and reversible process of translocation which is followed, after prolonged exposure to TPA, with a complete loss of cellular PKC activity, presumably due to proteolysis of the translocated membrane-bound PKC (15, 16). Recent studies indicate that certain transformed cells have elevated levels of DAG (17, 18), suggesting that transformed cells may undergo continuous PKC stimulation. Thus, the levels of PKC and possibly its compartmentalization might be altered in certain tumors, but to our knowledge this has not been previously examined directly.

Colon carcinogenesis appears to proceed through distinct stages of initiation and promotion (19–22). Bile acids which are normally present in the gastrointestinal tract have been shown to act as tumor promoters in colon carcinogenesis in vivo (23, 24) and to enhance cell transformation in vitro (25). Thus, bile acids appear to be endogenous colon tumor promoters. In view of these considerations, it was of interest to compare the levels and compartmentalizations of PKC in the normal colon mucosa and in colon tumors of patients with colon cancer. In this report, we show that PKC and the total level of calcium-dependent protein kinase-specific activities are present at reduced levels in human colon carcinomas when compared to their normal adjacent colon mucosa.

MATERIALS AND METHODS

- ATP, bovine serum albumin, histone Type III-S, phenylmethylsulfonyl fluoride, Tris-HCl, DEAE-Sephacel, NP-40, soybean trypsin inhibitor Type I-S, and phosphatidylserine were purchased from Sigma Chemical Co. (St. Louis, MO). [\(^{32}\)P]ATP was purchased from American Corp. (Arlington Heights, IL). Phosphocellulose paper, grade P81, was from Whatman, Inc. (Clifton, NJ). Hydrofluor was from National Diagnostics (Somerville, NJ), and TPA was from L. C. Co., Inc. (Schaumburg, IL). Leupeptin was a gift from the United States-Japan Cooperative Cancer Research Program (courtesy of Dr. Walter Troll, New York University Medical Center, New York, NY). Stock solutions of TPA (1 mg/ml) were made up in dimethyl sulfoxide and stored at −20°C. Human colon tumors and their corresponding normal mucosa (near the histologically normal margins of resection) were collected from the operating rooms of Columbia-Presbyterian Hospital and immediately rinsed with cold phosphate-buffered saline. In order to ensure that only intact tumor tissue and normal mucosa were used for analysis, ulcerated and necrotic tissues were dissected away from the tumor and the submucosa and muscularis were removed from the normal tissue. Portions of the same tissues were processed for routine histological examination in order to establish the precise diagnosis. Within 30 min after resection, the tissue samples were frozen in liquid nitrogen and stored at −70°C until analysis.

Extraction of Ca\(^{2+}\)-Dependent Protein Kinase and PKC Activities from Carcinomas and Adjacent Normal Mucosa. Frozen tissue samples were homogenized in Buffer A (25 mM Tris-HCl at pH 7.5, 5 mM EDTA, 5 mM ethyleneglycol bis(\(\beta\)-aminoethyl ether)-N\(_2\),N\(_4\),N\(_6\),N\(_8\)-tetraacetic acid, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 50 μg/ml

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3 The abbreviations used are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; DAG, diacylglycerol; NP-40, nonidet P-40.
soybean trypsin inhibitor Type I-S, 15 mM \( \beta \)-mercaptoethanol, and 0.25 mM sucrose) with 30 strokes in an automatic Dounce homogenizer at a ratio of 1 g tissue per 5 ml Buffer A. Tissue homogenates were centrifuged at 100,000 \( \times g \) for 1 h at 4°C. The supernatant fraction, which represents the "cytosolic" fraction, was then stored at 4°C, and the pellet was solubilized in Buffer A containing 0.1% NP-40 (5 ml buffer per g tissue) by stirring for 12-16 h at 4°C. The solubilized pellet was centrifuged at 10,000 rpm at 4°C for 20 min, and the resulting supernatant represents the solubilized "particulate" fraction. Protein concentrations were determined for the cytosolic and particulate fractions with the Bio-Rad protein assay solution, using bovine serum albumin as a standard. At the concentration used, 0.1%, NP-40 in Buffer A had no effect on the Bio-Rad protein assay. Ca\(^{2+}\)-dependent protein kinase activity was assayed directly from the cytosolic and particulate fractions. Cytosolic and particulate fractions were further purified by DEAE-Sephacel chromatography in order to measure PKC activity. In each case, 1 ml of the fraction was applied to a 0.5-ml DEAE-Sephacel column which was equilibrated in Buffer A. The column was then washed with 10 ml of Buffer A, and PKC activity was eluted with 1.5 ml of Buffer A containing 0.3 mM NaCl. DEAE-Sephacel eluates were used to determine PKC activity in cytosolic and particulate fractions, as described below.

Assays of PKC and Ca\(^{2+}\)-dependent Protein Kinase Activities. PKC was assayed by measuring the TPA- and phosphatidylinositol-dependent phosphotransferase reaction between [\( ^{32} \)P]ATP and histone Type III-S, in a reaction mixture containing 20 mM Tris-HCl at pH 7.5, 5 mM \( \beta \)-mercaptoethanol, 10 mM MgCl\(_2\), 200 nM TPA (or none), 1 mM ethyleneglycol bis(\( \beta \)-aminoethyl ether)-N\(_2\),N\(_4\),N\(_6\),N\(_8\)-tetraacetic acid, 80 \( \mu \)g/ml phosphatidylinositol (or none), 70 \( \mu \)M [\( ^{32} \)P]ATP (250-400 cpm/pmol), 0.67 mg histone Type III-S/ml, and about 5-20 \( \mu \)g of cell extract protein which was prepared from the tissue samples, as described above. Using a purified preparation of rat brain PKC (26), we determined that the residual amount of NP-40 which was present in PKC assays containing the particulate fractions (0.0087% NP-40) had no detectable effect on PKC activity. Reactions were initiated by the addition of PKC and incubated at 30°C for 10 min. Time course studies indicated that the reactions were linear during this time period. Reactions were terminated by transferring 40-\( \mu \)l aliquots onto phosphocellulose paper, and the radioactivity which was incorporated into histone Type III-S in a TPA- and phosphatidylinositol-dependent manner was determined as previously described (26). All assays were done in triplicate and varied by less than 15%. The values given in the tables have been corrected for the blank values, i.e., by the subtraction of the radioactivity obtained in the absence of added Ca\(^{2+}\) in the case of the Ca\(^{2+}\)-dependent protein kinase activity and by the subtraction of the values obtained in the absence of added TPA and phosphatidylinositol in the case of the PKC activity.

Ca\(^{2+}\)-dependent protein kinase activity was assayed by a modification of the above procedure. The assay mixtures contained 1 mm CaCl\(_2\) instead of TPA and phosphatidylinositol and aliquots of the crude cytosol and particulate fractions (approximately 5-20 \( \mu \)g protein) instead of the purified DEAE-Sephacel fractions used in the PKC assay. The remaining conditions were the same as in the PKC assays. These assays were also done in triplicate and varied by less than 15%.

RESULTS

Table IA shows the Ca\(^{2+}\)-dependent protein kinase specific activities recovered in cytosolic and particulate subcellular fractions which were extracted from human colon carcinomas and their adjacent normal mucosas. This activity is defined operationally as the total protein kinase activity present in these cell extracts which is stimulated by the addition of Ca\(^{2+}\) (see "Materials and Methods"). In each set of carcinoma and adjacent normal tissues studied, there were significantly higher specific activities observed in both the cytosolic and particulate fractions of the normal mucosa relative to the corresponding values obtained with the carcinoma fractions. The average ratios of Ca\(^{2+}\)-dependent protein kinase-specific activities observed were 4.4 (normal cytosolic fraction/carcinoma cytosolic fraction, with a median of 2.1 and a range from 1.5 to 13.6) and 3.5 (normal particulate fraction/carcinoma particulate fraction, with a median of 3.1 and a range from 1.9 to 6.0). In both the normal mucosa and the carcinoma samples, Ca\(^{2+}\)-dependent protein kinase-specific activities were consistently higher in the particulate fractions than in the corresponding cytosolic fractions (Table 1A).

In order to specifically distinguish PKC activity from other Ca\(^{2+}\)-dependent protein kinases, such as calmodulin-dependent protein kinases, cytosolic and particulate fractions obtained from colon carcinomas and normal mucosa were first purified through DEAE-Sephacel columns and then assayed for TPA- and phosphatidylyserine-dependent protein kinase activity (see "Materials and Methods"). Previous studies have established that TPA specifically stimulates PKC activity and that this effect is dependent on the presence of an anionic phospholipid (2).

Table 2A shows the PKC-specific activities recovered from the cytosolic and particulate fractions of colon carcinomas and their adjacent normal mucosa. As in the case of the Ca\(^{2+}\)-dependent protein kinase activities (Table 1A), higher specific activities were observed in both the cytosolic and particulate fractions of the normal mucosa than in the respective fractions of the carcinoma tissue. The average ratios of the PKC specific activities were 5.1 (normal cytosolic fraction/carcinoma cytosolic fraction, with a median of 2.3 and a range from 1.7 to 14.5) and 3.7 (normal particulate fraction/carcinoma particulate fraction, with a median of 3.1 and a range from 1.7 to 8.1). Unlike the Ca\(^{2+}\)-dependent protein kinase activities described in Table 1, which consistently displayed higher specific activities in the particulate than in the cytosolic fractions, PKC-specific activity showed considerable variability in its subcellular distribution, among individual carcinomas and normal mucosa samples.

Whereas Tables 1A and 2A express kinase activities in terms of specific activity, Tables 1B and 2B indicate the total cytosolic and particulate kinase activities for the Ca\(^{2+}\)-dependent protein kinases and PKC, respectively, expressed as pmol/min/g wet weight of tissue. We found that the carcinoma tissues contained considerably less PKC activity per g wet weight of tissue than the adjacent normal tissues. In contrast, the Ca\(^{2+}\)-dependent protein kinase activity per g weight of tissue did not differ significantly between the normal mucosa samples and the carcinoma samples. Thus, PKC activities were reduced in carcinoma tissues with respect to both specific activity in terms of protein (Table 2A) and with respect to tissue weight (Table 2B).

Tables 3 and 4 indicate the percentage of Ca\(^{2+}\)-dependent protein kinase and PKC activities that were present in the particulate fractions of each of the samples. The values varied considerably between tissues (from 20 to 97%) and, in general, there was no systematic difference between the carcinoma and normal mucosa samples. It is of interest, however, that in the carcinoma samples that contained an extensive admixture of benign adenomatous tissue (Table 3, patients 3, 4, 5, and 6, and Table 4, patients 9 and 10), the particulate fractions consistently contained greater than 60% of the total Ca\(^{2+}\)-dependent protein kinase and PKC activities.

DISCUSSION

The present study indicates that human colon carcinomas have reduced levels of both Ca\(^{2+}\)-dependent protein kinase activity and protein kinase C activity when compared to their
ALTERNED PROTEIN KINASE LEVELS IN HUMAN COLON CARCINOMAS

Table 1  Total calcium-dependent protein kinase activities in cytosolic and particulate fractions of normal colon mucosa and colon tumours

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cytosol Normal</th>
<th>Carcinoma</th>
<th>Particulate Normal</th>
<th>Carcinoma</th>
<th>Cytosol Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>978 ± 88</td>
<td>662 ± 53</td>
<td>6150 ± 492</td>
<td>1964 ± 137</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>4975 ± 100</td>
<td>2410 ± 24</td>
<td>8045 ± 161</td>
<td>4169 ± 125</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>543 ± 60</td>
<td>2410 ± 16</td>
<td>3313 ± 464</td>
<td>1593 ± 44</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>718 ± 29</td>
<td>394 ± 16</td>
<td>9695 ± 388</td>
<td>1629 ± 244</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>&lt;70</td>
<td>&lt;40</td>
<td>5203 ± 156</td>
<td>971 ± 29</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>194 ± 17</td>
<td>&lt;40</td>
<td>4054 ± 203</td>
<td>1373 ± 205</td>
<td>&gt;4.9</td>
</tr>
</tbody>
</table>

Table 2  Protein kinase C activities in cytosolic and particulate fractions of normal colon mucosa and colon tumours

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cytosol Normal</th>
<th>Carcinoma</th>
<th>Particulate Normal</th>
<th>Carcinoma</th>
<th>Cytosol Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2317 ± 209</td>
<td>1902 ± 152</td>
<td>7191 ± 575</td>
<td>3571 ± 250</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>1840 ± 368</td>
<td>18075 ± 145</td>
<td>20917 ± 418</td>
<td>12507 ± 362</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>553 ± 61</td>
<td>&lt;138</td>
<td>1325 ± 186</td>
<td>2549 ± 178</td>
<td>&gt;4.0</td>
</tr>
<tr>
<td>4</td>
<td>574 ± 23</td>
<td>893 ± 116</td>
<td>2145 ± 86</td>
<td>2462 ± 369</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>&lt;140</td>
<td>&lt;138</td>
<td>3469 ± 104</td>
<td>3625 ± 109</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>364 ± 33</td>
<td>&lt;138</td>
<td>4662 ± 233</td>
<td>2197 ± 330</td>
<td>&gt;2.6</td>
</tr>
</tbody>
</table>

Table 3  Cellular distributions of calcium-dependent protein kinases in normal colon mucosa and colon carcinomas (% of particulate)

<table>
<thead>
<tr>
<th>Tumor properties</th>
<th>Cellular distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poorly</td>
<td>Normal: 70%</td>
</tr>
<tr>
<td>Moderate</td>
<td>Normal: 70%</td>
</tr>
<tr>
<td>Well</td>
<td>Normal: 70%</td>
</tr>
</tbody>
</table>

Table 4  Cellular distribution of protein kinase C in normal colon mucosa and colon tumours

<table>
<thead>
<tr>
<th>Tumor properties</th>
<th>Cellular distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate</td>
<td>Normal: 70%</td>
</tr>
<tr>
<td>Well</td>
<td>Normal: 70%</td>
</tr>
</tbody>
</table>

The observed decreases in PKC activity might be due to down regulation of this enzyme in the carcinoma cells, a phenomenon which occurs subsequent to the translocation of the enzyme in TPA-treated cell cultures (15, 27–30). For instance, time-course studies on TPA-treated MCF-7 cell cultures have demonstrated an initial translocation of the PKC enzyme from the cytosolic fraction to the particulate fraction (15). This is followed, shortly thereafter, by an apparent down regulation of the translocated enzyme. This progressive...
decline in PKC activity, initially seen in the cytosol and then in the membrane fraction, leads to an almost complete disappearance of PKC activity in MCF-7 cells after 45 h of TPA treatment (15). Similar decreases in total PKC activity after prolonged exposure to phorbol esters have been reported in other cell types (27–30). The recent demonstration of elevated levels of DAG, an endogenous activator of PKC, in K-ras- and sis-transformed rat kidney cells (18) suggests that in some transformed cells PKC is continuously exposed to high levels of DAG. This might produce effects similar to those of chronic exposure to TPA and thus, result in the down regulation of PKC activity in tumor cells.

Although we did not observe any consistent differences between carcinoma and normal mucosa samples in terms of intracellular distribution of PKC activity, it may be significant that carcinoma samples that contained an admixture of benign adenomatous tissue displayed an apparent shift of PKC activity from the cytosol to the membrane fraction, whereas later stages are associated with an overall decrease in total PKC activity. The decrease in Ca2+-dependent protein kinase activity in colon tumors seen in the present study may reflect PKC activity, calmodulin-dependent protein kinase activity, and other Ca2+-dependent protein kinases. Previous studies have demonstrated an elevation of calmodulin content (31) in transformed cell lines and primary tissues. To our knowledge, however, calmodulin activity has not been measured in human colon carcinomas.

Further studies are required to determine whether the decreased levels of PKC activity seen in colon carcinomas in the present study reflect increased turnover of the enzyme itself or changes at the level of translation or transcription. We are currently analyzing the abundance of PKC mRNA transcripts in colon tumors utilizing PKC complementary DNA clones recently isolated in our laboratory (32).

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