Tyrosine Phosphorylation of a M, 63,000 Protein Induced by an Endogenous Mitogen in Human Colon Carcinoma Cells, but not in Normal Colonocytes

Brigitte Marian, Sidney Winawer, and Eileen Friedman

Gastroenterology Service and Laboratory of Gastrointestinal Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Transformation of normal human colonocytes makes them sensitive to new mitogenic signals. Long-chain diglycerides (LCDGs) found in the human colon are mitogens selective for colon tumor cells, inducing mitogenesis in premalignant cells from each of 13 adenomas and in malignant cells from two of four carcinoma cells, but having no mitogenic effects on normal colonocytes (E. Friedman, P. Isaksson, J. Rafter, B. Marian, S. Winawer, and H. Newmark, Cancer Res., 49: 544-548, 1989). Parallel to this biological activity pattern, LCDGs induce protein phosphorylation only in adenomas and carcinomas. Immunoblotting with an anti-phosphotyrosine monoclonal antibody demonstrated that the LCDG mitogen, dimyristin, at concentrations found within the body, induced a 6-fold increase of tyrosine phosphorylation of an M, 63,000 protein found in the particulate fraction of colon carcinoma cells. Tyrosine phosphorylation was maximal 0.5 min after addition of the LCDG, then fell, but remained elevated 40% over constitutive levels for at least 6 h. The M, 63,000 tyrosine phosphoprotein was found in each of four colon carcinoma cell lines and an adenoma, but not in normal colonocytes, suggesting that the tyrosine kinase is activated only in tumor cells. Constitutive levels of the M, 63,000 substrate were enhanced 2-fold by incubation of cells for 20 h with sodium orthovanadate, a tyrosine phosphatase inhibitor. This result suggested that carcinoma cells continually phosphorylate and dephosphorylate this tyrosine kinase substrate during growth. Thus, the colon tumor cell mitogen, dimyristin, utilizes a signal transduction pathway, containing the M, 63,000 tyrosine kinase substrate, which is already in use during cell growth, possibly by other mitogens or growth factors.

INTRODUCTION

In an earlier study, we identified a class of endogenous colonocyte mitogens, falcidiglycerides composed of oleic, myristic, or palmitic acid residues (1). They are probably derived from partial lipolysis of triglycerides in dietary fat. These diglycerides, when added in micelles to primary cultures of adenomas, carcinomas, or normal colonocytes at the concentration range found in vivo, induced proliferation of all of the adenomas and one-half of the carcinomas assayed (1). Mitogenesis of normal colonocytes was not observed, suggesting that these endogenous diglycerides might induce selective growth of premalignant and malignant cells. The biological effects of diglycerides were not always mimicked by TPA,4 suggesting that all mitogenic signals on normal mucosa, following a 30-min room temperature incubation with 1 mM concentrations each of EDTA and EGTA (7), by vigorous shaking by hand and pelleting at 200 x g for 30 s to remove single cells. The crypts were washed twice with phosphate-buffered saline to remove EDTA and EGTA. HT29-H1-1 is a TPA- and diglyceride-sensitive cloned colon carcinoma cell line derived from the HT29 cell line. Initial experiments to determine basal phosphorylation levels were performed in DMEM (6). Cells were prelabeled in phosphate-free DMEM with 0.2 mCi/ml 32P for 3 h, and then treated with 50 ng/ml TPA, 50 ng/ml 4a-PDD, 33 µM dimyristin, or no additions for 5 min. Then cultures were precipitated with 10% trichloroacetic acid, and total cpm incorporated per µg of protein was determined. Protein concentration was determined by the procedure of Lowry et al. (8). Control and 4a-PDD cultures gave very similar results in all cases. Preparation of Cellular Fractions and Cell Lysates. Parallel cultures of subconfluent HT29-H1-1 cells were treated with dimyristin or TPA in DMEM containing 10 mg/ml polysorbate 80 and 1 mg/ml bovine serum albumin (essentially fatty acid free; Sigma) to allow the dimyristin to be dispersed in micelles by a 10-s sonication immediately before addition. After treatment, cells were washed with cold phosphate-buffered saline, swelled for 5 min on ice in 20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 1 mM EGTA-1 mM NaF-100 µM NaVO4, 20 µM leupeptin-24 µM phenylmethylsulfonyl fluoride-2 mM benzamidine (buffer A), and then homogenized in buffer A containing 0.3 M sucrose. Large membrane fragments and nuclei were pelleted at 4,000 x g for 5 min, washed twice with buffer A containing sucrose, and stored at -80°C. The supernatant was clarified by centrifugation at 10,000 x g for 60 min to yield a cytosol fraction. Membrane proteins were extracted from the membrane-nuclear pellet with 1% NP40 in buffer A. Primary cultures were immediately lysed with 1% NP40 in buffer A. Na2VO4 was prepared as a 0.1 M stock solution in water and stored in small aliquots at -20°C.
Phosphorylation of Endogenous Substrates. Cell lysates were dialyzed for 2 h at 4°C against a 1000-fold volume of 20 mM Tris-HCl, pH 7.2-7.5-0.25 mM EGTA-1 mM NaF to remove NP40 and EDTA. Five µg of protein were reacted with 5 or 20 µCi [γ-32P]ATP in a final volume of 30 µl containing 1.2 mM CaCl2-8 mM MgCl2-4 mM NaF-100 µM ATP. The reaction was allowed to proceed for 5 min at room temperature and was stopped by the addition of concentrated SDS-sample buffer containing 20 mM EDTA and 20 mM NaF, then heat denatured in a boiling water bath for 2 min, and separated by discontinuous gel electrophoresis on 7.5% acrylamide. Phosphorylated proteins were detected by autoradiography on Kodak XAR-5 X-ray films.

Immunoblotting. Membrane extracts or cytosols for the cell line studies, and cell lysates from primaries, were adjusted to equal protein in SDS-sample buffer containing 10 mM NaF and 100 µM Na3VO4. Equal amounts of protein (20 µg) were fractionated by SDS-PAGE on 7 to 12% acrylamide gradient gels until the bromophenol blue had moved 9 cm and then transferred to a PVDF filter (Immobilon-P; Millipore) by electrophoresis at 30 V for 16 h in transfer buffer (86.7 g glycine-18 g Tris base-0.55 g NaVO4-1.2 liters methanol/6 liters). Proteins binding sites on the filter were blocked by incubation for 6 h with 10% nonfat dry milk in 10 mM Tris-HCl, pH 7.2-50 mM NaCl-0.5 mM EDTA-100 µM Na3VO4-0.05% Tween 20. The filters were then incubated with monoclonal antibody to phosphotyrosine (PY69; ICN Biologicals) (9) at 1 µg/ml in the above buffer overnight at 4°C. The mouse immunoglobulins were detected by incubation with alkaline phosphatase-coupled goat anti-mouse antibodies (Bio-Rad) followed by detection with 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium. In each experiment, a parallel filter was stained with Coomassie blue to verify that equal amounts of protein had been loaded and transferred in each lane. To quantitate the results, negatives of the blots were analyzed by densitometric scanning. For KOH treatment studies, subconfluent HI-1 cells were labeled for 3 h with 0.2 mCi/ml [32P]Pi, and then treated with dimyristin or untreated (see Fig 4 legend), and NP40 extracts were analyzed on SDS-PAGE gradient gels. The gels were fixed, incubated with 1 M KOH at 55°C for 2 h, neutralized, rinsed with 10% acetic acid-10% isopropanol alcohol, redried, and exposed to XAR-5 film.

Additional Materials. Dimyristin (Serdy Research Laboratories, London, Ontario, Canada) was stored as a 19.5 mM stock solution in dimethyl sulfoxide, added to media or buffers containing 10 mg/ml polysorbate 80, and dispersed by sonication immediately before use. TPA (PL Biochemicals) was stored as a 10 µg/ml stock solution in dimethyl sulfoxide in the dark at -20°C and diluted just before use. ATP was from Sigma. [32P]Pi-γ-ATP (3000 Ci/mmol) was purchased from NEN (Boston, MA).

RESULTS

Initial Phosphorylation Experiments. Fecal diglycerides induced mitogenesis of all premalignant cells and many malignant cells but did not induce proliferation of normal colonocytes (1). In an initial experiment to determine whether protein phosphorylation might play a role in the diglyceride mitogenic signal, the specific activity of cellular phosphorylated proteins was determined after treatment with TPA, dimyristin, or a TPA analogue inactive in tumor promotion (see "Materials and Methods"). Four resected carcinomas exhibited an 8-fold increase in their basal phosphorylation levels, compared to normal cells from two patients and cells from four adenomas (benign tumors). The diglyceride and TPA increased basal phosphorylation levels 3- to 5-fold in adenoma cells, and 1- to 2-fold (with much variation between different specimens) in resected carcinoma cells. No increase was observed in normal cells. Therefore, there was a correlation between induction of protein phosphorylation solely in tumor cells by dimyristin and the selectivity of its mitogenic action for only tumor cells.

The major proteins constitutively phosphorylated in normal colonocytes and carcinomas were compared by preparing cell lysates with NP40, removing the NP40 and cyclic nucleotides by dialysis, and then allowing endogenous kinases to phosphorylate endogenous substrates by addition of only [32P]γ-ATP, Mg2+ and Ca2+ (see "Materials and Methods"). In SDS-PAGE analysis, three major bands were found in normal colonocytes from each of two patients at M, 47,000, M, 50,000/51,000, and M, 55,000/56,000 (Table 1, Fig. 1, Lane a). Nine carcinomas fell into five general groups, depending on the presence or absence of major phosphoproteins at M, 55,000/56,000, M, 60,000/64,000, M, 70,000, M, 76,000 to 85,000, M, 100,000, and M, 120,000. There were no correlations between tumor histopathology and band pattern. The broad band at M, 60,000 to 64,000 (Fig. 1, Lane b, arrow) was found in each of the nine carcinomas and in neither of the two normal specimens (Table 1). Therefore, carcinoma cells were characterized by constitutive phosphorylation of this group of proteins, suggesting that the protein kinases utilizing these substrates were specifically activated in tumor cells.

Diglyceride Mitogen Induces Tyrosine Phosphorylation of M, 63,000 Carcinoma Protein. HI-1 cells, a subclone of the human colon carcinoma cell line HT29, which was TPA and diglyceride sensitive, was selected as the cell line model for more detailed studies. By use of the in vitro phosphorylation experiment described above, HI-1 cells were shown to express constitutively a M, 60,000 to 64,000 group of dimyristin-enhanced, alkali-resistant phosphoproteins, implicating phosphorylation on tyrosine. Therefore, for all following experiments, a detection system was employed using immunoblotting with a monoclonal antibody to phosphotyrosine. The positions of the major signals found in each sample are shown in Fig. 1. The major phosphoproteins found in resected carcinoma cell lysates are indicated in Table 2.
TYROSINE PHOSPHORYLATION INDUCED BY DIGLYCERIDE MITOGEN

Table 1 Major phosphoproteins in colonic cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>47,000</th>
<th>50,000/51,000</th>
<th>55,000/56,000</th>
<th>60,000–64,000</th>
<th>70,000</th>
<th>76,000–85,000</th>
<th>100,000</th>
<th>120,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 68</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Normal 78</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carcinomas 1,461-L, 1,533, 1,542</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carcinoma 1,461-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carcinomas 1,468 and 1,487-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carcinomas 1,476 and 1,487-2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carcinoma 1,493</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

NP-40 extracts were labeled with [32P]-γ-ATP and analyzed by SDS-PAGE (see “Materials and Methods”). +, phosphoproteins present. One to four bands appeared between M, 76,000 and M, 85,000; + indicates that at least one was present.

Parallel cultures of HI-1 colon carcinoma cells were treated with 0, 10, 25, 50, and 100 μM dimyristin in micelles for 2 min. Particulate and cytoplasmic fractions were then made and analyzed by SDS-PAGE on 7 to 12% gradient gels, followed by Western blotting with monoclonal antibody to phosphotyrosine (see “Materials and Methods”). HI-1 cells constitutively expressed a low level of an M, 63,000 phosphotyrosine protein (Fig. 2, Lane a). The phosphorylation of this protein was increased markedly by treatment with the biologically active diglyceride dimyristin, with an optimum 6-fold increase at 25 μM (Fig. 2, Lane c). In each experiment (Figs. 2 to 7), parallel lanes transferred to Immobilon filters were stained for proteins with Coomassie blue, which demonstrated that each lane contained equal amounts of the same major bands (not shown). Examination of HI-1 cytosols demonstrated that an M, 63,000 tyrosine phosphoprotein was present constitutively which did not increase with dimyristin treatment (Fig. 2, Lanes f and g). This suggested that the activity of the tyrosine kinase was limited to the M, 63,000 substrate in the membrane.

Three other human colon carcinoma cell lines, HT29, SW620, and LS174T, exhibited constitutive expression of the M, 63,000 tyrosine kinase substrate (data not shown). Since the M, 63,000 substrate was consistently found in carcinoma cells, it might have a more general function in signal transduction than response to fecal diglyceride mitogens. If it played a role in carcinoma cell growth, for example, responding to mitogens present in fetal calf serum or to autocrine growth factors, the M, 63,000 substrate should be phosphorylated and dephosphorylated as cells progressed through the cell cycle. To test this hypothesis, HI-1 carcinoma cells were incubated for ap-
Fig. 4. Alkali resistance and phosphotyrosine sensitivity of the M, 63,000 phosphoprotein. NP40 extracts of control and dimeristin HI-1 cells were prepared and analyzed on SDS-PAGE gradient gels (see "Materials and Methods"). Lanes a and b, dimeristin-treated cells were immunoblotted with monoclonal antibody 69 as in Figs. 2 and 3, except that 5 mM phosphotyrosine was present in Lane b. Lanes c and d, 3P-prelabeled HI-1 cells (see "Materials and Methods") were treated for 2 min with 25 μM dimeristin (Lane d) or left untreated (Lane c). Then NP40 membrane extracts were made and analyzed on SDS-PAGE gradient gels. The phosphoserine and phosphothreonine residues were then hydrolyzed by incubating the gel with KOH (see "Materials and Methods"), followed by autoradiography.

Fig. 6. Phosphorylation on tyrosine of an M, 63,000 protein substrate by TPA. Parallel cultures were treated with 0 (Lanes a and f), 5 ng/ml TPA (Lanes b and g), 10 ng/ml TPA (Lanes c and h), 25 ng/ml TPA (Lane d) or 50 ng/ml TPA (Lane e). Particulate (Lanes a to e) and cytosol (Lanes f to h) fractions were prepared (see "Materials and Methods") and analyzed on gradient SDS-PAGE gels, followed by immunoblotting with phosphotyrosine antibody as in Fig. 2.

Fig. 7. Phosphotyrosine proteins in primary cultured colonic epithelial cells. Total cell NP40 lysates (see "Materials and Methods") were analyzed by SDS-PAGE gradient gels, followed by immunoblotting with phosphotyrosine antibody as in Fig. 2. Normal colonocytes from patient 1 (Lanes a to c) and patient 2 (Lanes d to f) and adenoma cells from patient 3 (Lanes g to j) were analyzed after no treatment (Lanes a, d, and g), exposure for 2 min to 25 μM dimeristin (Lanes b, e, and h), or exposure to 50 ng/ml TPA (Lanes c, f, and j).

Approximately one cell cycle, 20 h, with 100 and 400 μM sodium orthovanadate, a tyrosine phosphatase inhibitor (10). Vanadate has been used by other investigators to stabilize transient tyrosine phosphorylations in vivo (11, 12). Treatment of HI-1 cells with vanadate led to a 2-fold increase in phosphorylated tyrosine residues on the M, 63,000 tyrosine kinase substrate (Fig. 3, Lanes a to c), similar to cells treated with diglyceride (com-
pare to Fig. 2). The treated cells remained viable, but rounded up, as they do when treated with dimyristin. Therefore, dimyristin activates a carcinoma signal transduction pathway already functioning during cell growth.

An alternate explanation for these results is that dimyristin treatment acts like sodium vanadate and inhibits a tyrosine phosphatase, leading to elevated tyrosine phosphoprotein levels. This is an unlikely explanation because tyrosine phosphorylation was lost from the M₄₃,₀₀₀ substrate when control or dimyristin-treated cells were extracted in the absence of vanadate or when vanadate was omitted from the blotting buffers.

The presence of the phosphotyrosine analogue phenylphosphate at 5 mM (Fig. 3, Lanes d and e) or phosphotyrosine (Fig. 4, Lanes a and b) in the antibody assay completely eliminated the binding of the anti-phosphotyrosine monoclonal to the M₄₃,₀₀₀ protein. The bands found between M₃₀,₀₀₀ and M₄₀,₀₀₀ are nonspecific, due to the detection system, and also occurred when normal mouse serum was substituted for the anti-phosphotyrosine monoclonal. Both phenylphosphate and phosphotyrosine inhibition of the antiphosphotyrosine antibody, and the increase in the M₄₃,₀₀₀ substrate caused by vanadate inhibition of tyrosine phosphatase, provided further evidence that the M₄₃,₀₀₀ substrate is phosphorylated on tyrosine.

Phosphorylations on serine or threonine residues are sensitive to alkali treatment, while phosphotyrosine is relatively insensitive to alkali (13). HI-1 carcinoma cells were prelabeled with ³²P, and then treated with dimyristin or left untreated, and membrane extracts were then analyzed by SDS-PAGE (see "Materials and Methods"). The SDS-PAGE gels were then treated with KOH (see "Materials and Methods") to decrease radioactivity due to phosphoserine and phosphothreonine labeling. A diffuse alkali-resistant band centering at M₄₃,₀₀₀ was found as membrane extracts of untreated HI-1 cells and enhanced in cells treated with dimyristin (Fig. 4, Lanes c and d). Alkali-stable phosphorylation of an M₄₃,₀₀₀ protein, whose phosphorylation is enhanced by dimyristin treatment, provides a fourth line of evidence that the M₄₃,₀₀₀ phosphoprotein is phosphorylated on tyrosine. Further experiments will compare the phosphoamino acids in the M₄₃,₀₀₀ phosphoprotein from untreated and dimyristin-treated cells.

A time course experiment demonstrated that tyrosine phosphorylation of the M₄₃,₀₀₀ substrate occurred very quickly after addition of the diglyceride. Parallel cultures of HI-1 cells were treated for 0, 0.5, 2, 10, and 30 min (Fig. 5, Lanes a to e) and for 2 and 6 h (Fig. 5, Lanes f and g) with dimyristin micelles at 25 μM concentration. Maximum observed phosphorylation occurred at the earliest time point, 30 s (Fig. 5, Lane b). The phosphorylation remained markedly elevated over control levels for 30 min and decreased to 40% over constitutive levels by 2 and 6 h of treatment. These experiments, taken together, show that dimyristin, a colon tumor cell mitogen found endogenously, induces a very rapid tyrosine phosphorylation of an M₄₃,₀₀₀ membrane-bound protein at biologically effective concentrations (10 to 50 μM) (1).

TPA Also Induces Phosphorylation on Tyrosine of a M₄₃,₀₀₀ Membrane-Bound Protein. TPA can induce mitogenesis of tubular adenoma cells (14) and urokinase secretion from more advanced adenomas and carcinomas (14, 15). However, its biological effects on adenomas and carcinomas do not always parallel those of the fecal diglycerides (1). Both compounds have no mitogenic activity on normal colonocytes (1, 16). When parallel cultures of carcinoma HI-1 cells were treated with a range of TPA concentrations, a dose-dependent induction of tyrosine phosphorylation of an M₄₃,₀₀₀ membrane protein occurred, with an optimum 2-fold induction at 10 ng/ml (Fig. 6, Lane c) and decreasing effects at 5, 25, and 50 ng/ml (Fig. 6, Lanes b, d and e). Examination of the cytosolic fractions from these cells demonstrated the presence of an M₄₃,₀₀₀ tyrosine kinase substrate in the cytosol of untreated cells (Fig. 6, Lane f), which was not increased with 10 or 25 ng/ml TPA treatment (Lanes g and h). 4α-PDD (inactive analogue of TPA) did not affect the level of the M₄₃,₀₀₀ tyrosine kinase substrate (data not shown). Whether TPA and dimyristin induce phosphorylation of the same substrate, and if so, on the same tyrosine residue, is not known. An M₉₀,₀₀₀ phosphotyrosine protein was observed in this experiment and the dimyristin time course. Its phosphorylation was induced with the same kinetics as the M₄₃,₀₀₀ phosphorylation. Since the M₉₀,₀₀₀ protein was not observed in each experiment, such as the dimyristin dose-response curve (Fig. 2), its significance is unknown.

Tyrosine Phosphorylation in Clinical Samples. In an earlier study, diglycerides induced mitogenesis in each of the adenomas, in only one-half of the carcinomas, and in none of the normal specimens tested (1). Therefore, we limited the biochemical study of clinical samples to adenoma cells and to normal cells, which gave uniform biological responses. Normal colonocytes from two subjects and premalignant cells from one villous adenoma were placed into primary culture (see "Materials and Methods") and treated for 2 min with either 25 μM dimyristin or 25 ng/ml TPA. An M₄₃,₀₀₀ tyrosine phosphorylated protein was not detected in normal cells from either subject, even after treatment by either agent (Fig. 7, Lanes a to j). In contrast, the adenoma cells displayed a low constitutive level of M₄₃,₀₀₀ tyrosine kinase substrate which was markedly increased by dimyristin, but not by TPA (Lanes g to j). Thus, the adenoma mitogen, dimyristin, induces tyrosine kinase phosphorylation of an M₄₃,₀₀₀ substrate in adenoma cells, which is not detectable in normal cells, on which the diglyceride is inactive.

DISCUSSION

Diglycerides are endogenous mitogens for premalignant colonocytes from adenomas and for one class of carcinoma cells, but not for normal colonocytes (1). Colonic diglycerides also increase the secretion of urokinase from adenoma and carcinoma cells and induce some morphological changes, leading to separation of adjacent cells. By using a monoclonal antibody to phosphotyrosine, we have shown that the biologically active fecal diglyceride dimyristin, within 30 s of application to live cells, activates a tyrosine kinase which phosphorylates an M₄₃,₀₀₀ protein. This modulation occurs in the particulate fraction of colon carcinoma cells, implicating a membrane site of action, which would be consistent with the modulation of cell to cell contact. TPA also induced phosphorylation of an M₄₃,₀₀₀ membrane substrate and, in earlier work, induced a loss of functional gap junctions between colon tumor cells (17).

Our observation of tyrosine kinase activation was unexpected, as diglycerides and TPA are known to activate protein kinase C (2), which phosphorylates threonine and serine residues (18). It is not the only example of such a phosphorylation. TPA and the synthetic diglyceride 1-oleyl-2-acetylglycerol induced phosphorylation on tyrosine of an M₄₂,₀₀₀ protein in chicken embryo fibroblasts (19). This M₄₂,₀₀₀ substrate was also phosphorylated on tyrosine in cells transformed by avian sarcoma viruses and in cells stimulated by epidermal growth factor.

---

4B. Marian, S. Harvey, G. Markus, S. Winawer, and E. Friedman, manuscript in preparation.
platelet-derived growth factor, or somatomedin A. Down-regulation experiments did not clarify the role of protein kinase C, because continuous exposure to TPA for 24 h did not decrease phosphorylation of the M, 63,000 tyrosine kinase substrate when TPA was applied a second time (data not shown). Also tyrosine phosphorylation of the M, 63,000 protein remained elevated when diglyceride was continuously present for 6 h (Fig. 4). Possibly, protein kinase C is only significant in the first stage of signal transduction, binding both diglycerides and TPA, then activating a tyrosine kinase which remains active even in the absence of further protein kinase C action.

Many oncogene products and growth factor receptors phosphorylate themselves and their intracellular protein substrates on tyrosine (3, 4). Constitutive expression of such tyrosine kinases may play a role in the etiology of several human tumors. For example, src tyrosine kinase activity is enhanced severalfold in resected colon carcinomas compared to normal colonocytes (20). Giordano et al. (21) have found that 13 of 18 human tumor cell lines contained tyrosine-phosphorylated proteins, including gastric, colon, and urinary tract carcinomas. No such proteins were found in normal cells from those organs. This group also examined the HT29 cell line for tyrosine kinase substrates, finding two proteins of M, 130,000 and M, 110,000, but not the M, 63,000 tyrosine kinase substrate reported here (21). The reasons for this difference may be due to their use of a different phosphotyrosine antibody and whole cell lysates instead of particulate fractions.

The identity of the tyrosine kinase whose activity is enhanced in colon tumor cells by diglycerides is unknown. It is constitutively activated in each of the four carcinoma cell lines and the one adenoma examined but was not detected in normal colonocytes, suggesting that the activation is tumor specific. Inhibition of tyrosine phosphatases by growth of cells in sodium orthovanadate also increased tyrosine phosphorylation of the M, 63,000 protein. Thus, the diglycerides utilize a signal transduction pathway already in use, possibly by other mitogens or autocrine or exocrine growth factors. Supporting this hypothesis are preliminary studies showing that certain polypeptide growth factors induce tyrosine phosphorylation of the M, 63,000 substrate in carcinoma cells. These results implicate its tyrosine kinase as a central player in growth control of colon tumor cells.

REFERENCES


Tyrosine Phosphorylation of a $M_r$ 63,000 Protein Induced by an Endogenous Mitogen in Human Colon Carcinoma Cells, but not in Normal Colonocytes

Brigitte Marian, Sidney Winawer and Eileen Friedman


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/15/4231

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.