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Enhanced Phosphoinositide Metabolism in Colorectal Carcinoma Cells Derived From Familial Adenomatous Polyposis Patients

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Abstract The production of the second messenger molecules diacylglycerol and inositol 1,4,5-trisphosphate is mediated by activated phosphatidylinositol-specific phospholipase C (PLC) enzymes. We report the enhancement of the phosphoinositide metabolism pathway in KMS-4 and KMS-8 cells, both of which are human colorectal carcinoma cell lines derived from familial adenomatous polyposis patients. In these cells, the cellular contents of diacylglycerol and inositol 1,4,5-trisphosphate were constitutively increased and the PLC activity in vitro was significantly high, as compared with those in normal colon cells or in other sporadic colorectal carcinoma cells. Northern and Western analyses showed the high expression levels of both PLC-γ1 and PLC-δ1 in KMS-4 and KMS-8 cells. Moreover, we detected the enhancement of protein–tyrosine kinase activity and tyrosine phosphorylation of PLC-γ1 in these KMS cells. These results suggest the involvement of activated phosphoinositide signaling pathways in the colorectal tumorigenesis of familial adenomatous polyposis.

Key words: phospholipase C, inositol trisphosphate, diacylglycerol, tyrosine kinase

The stimulation of a variety of cell surface receptors results in the activation of phosphoinositide-specific phospholipase C (PLC) isoforms and the rapid hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which generates the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DG). IP_3 releases Ca^{2+} from intracellular stores, whereas DG activates protein kinase C [Berridge and Irvine, 1989; Nishizuka, 1988]. Much evidence has accumulated that cellular transformation by some types of oncogenes and receptor activation by some growth factors enhance phosphoinositide metabolism by stimulating the activity of related enzymes [Ullrich and Schlessinger, 1990].

Molecular cloning of PLC cDNA has implied the existence of four different types of PLC: PLC-α, -β, -γ, and -δ [Rhee et al., 1989; Meldrum et al., 1991]. Among them, PLC-β, -γ, and -δ have two conserved regions (X and Y), which are involved in their catalytic activity [Emori et al., 1989]. Additionally, PLC-γ contains a region homologous to the N-terminal regulatory domain of a nonreceptor type of tyrosine kinase in *src* family oncogenes [Stahl et al., 1988; Suh et al., 1988]. Activation of PLC-β isoforms requires cellular proteins, including GTP binding proteins [Taylor et al., 1990], their βγ subunits [Camps et al., 1993]. The activity of PLC-γ isoforms is regulated by either receptor- or nonreceptor-type protein–tyrosine kinase [Wahl et al., 1988; Nishibe et al., 1990].

Colorectal cancer is a common malignancy in humans, where it is accompanied by allelic deletions in chromosomes 5q, 17p, and 18q, and by frequent mutations of the *ras* oncogene [Fearon

Abbreviations used: DG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; FAP, familial adenomatous polyposis; FBS, fetal bovine serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphatidylinositol-specific phospholipase C; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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and Vogelstein, 1990]. It has been recently reported that the *DCC* (deleted in colorectal cancers) gene in chromosome 18q is important for the development of colorectal cancers [Fearon et al., 1990a]. The *MCC* (mutated in colorectal cancers) and *APC* (adenomatous polyposis coli) genes in chromosome 5q, the latter of which is tightly linked to the autosomal dominant disease familial adenomatous polyposis (FAP), have also been identified [Kinzler et al., 1991a,b; Groden et al., 1991]. Although their roles in colonic carcinogenesis are still unknown, a sequence similarity between *APC* and the m3 muscarinic acetylcholine receptor encompassing codons 454–479 of *APC* [Kinzler et al., 1991b] suggests that the *APC* protein may interact with intracellular signal transduction systems including phosphoinositide metabolism mediated by PLC. In this study, we examined phosphoinositide metabolism of KMS-4 and KMS-8 cells, which are colorectal carcinoma cell lines established from FAP patients, and observed the enhanced phosphoinositide signaling in these cells, as compared with phosphoinositide metabolism in various cell lines derived from normal colon tissues and sporadic colorectal carcinomas.

METHODS

Duplicate measurements were made in experiments indicated below. All tests were independently performed several times with comparable results within a 10% error margin, and representative data are shown.

Cell Lines and Culture Condition

LoVo and DLD-1 (colorectal adenocarcinomas), SW837 (rectal adenocarcinomas), WiDr (rectosigmoidal adenocarcinoma), and FHs74Int (established from human fetal normal small intestine) were obtained from the Japanese Cancer Research Resources Bank. COLO205 (colorectal adenocarcinoma), and CCD841CoN [Thompson et al., 1985] and FHC [Siddiqui and Chopra, 1984] (both established from human fetal normal colonic mucosa; morphology is epithelial like) were purchased from the American Type Culture Collection. KMS-4 and KMS-8 cells were colorectal adenocarcinoma cells derived from different FAP patients established by Namba [Namba et al., 1983]. LoVo, SW837, WiDr, FHs74Int, CCD841CoN, and FHC cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS); DLD-1, COLO205, KMS-4, and KMS-8 cells were grown in RPMI1640 medium containing 10% FBS. FHs74Int, CCD841CoN, and FHC cells, which were derived from normal small intestine or normal colon tissues, exhibited the cell contact–dependent, growth-inhibitory property and did not form any colony in soft agar. All cells were cultured at 37°C in a humidified 5% CO_2 incubator, and logarithmically growing cells were used for each experiment.

Analysis of IP_3 and DG Content

Cells in 100 mm dishes were washed with phosphate-buffered saline (PBS) and then extracted immediately with 1 ml of ice-cold 10% (w/v) trichloroacetic acid followed by 1 ml of ice-cold distilled water. The extracts were combined and clarified by centrifugation at 5,000*g* for 15 min at 4°C. The supernatant was washed four times with water-saturated diethylether and neutralized to pH 7.4 with 10% (w/v) $NaHCO_3$. Aliquots of the supernatant were used for IP_3 analysis using an IP_3 assay kit (Amersham Co.). For analysis of the DG content, cells were washed with PBS and lipids were extracted with 3 ml of chloroform/methanol (1:2, v/v). The extracts were washed with 0.5 ml of 1 M KCl and the neutral lipids contained in the organic phase were dried under a stream of nitrogen. Aliquots were analyzed for DG content using a DG assay kit (Amersham Co.) and for phosphorus content [Kato et al., 1987].

PLC Activity

Cells were suspended in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/NaOH buffer (pH 7.4) containing 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by brief sonication two times for 5 sec. The lysates were then centrifuged at 100,000*g* for 1 hr at 4°C. The supernatant fractions were used as enzyme preparations. PIP_2-hydrolyzing activity was measured as described [Homma et al., 1988] in a reaction mixture consisting of 50 mM Hepes/NaOH (pH 6.8), 100 μM EDTA, 1 mg/ml bovine serum albumin, and 100 μM PIP_2 containing 20,000 dpm of [^{3}H]PIP_2, 50 μM phosphatidylethanolamine, 100 μM Ca^{2+}, and the enzyme preparation (10 μg of protein). The reaction was carried out at 37°C for 10 min and stopped by adding 2 ml of chloroform/methanol (2:1 by volume). IP_3 was extracted with 1 N HCl and measured by counting the radioactivity of

the aqueous phase in a liquid scintillation counter. Levels of IP_3 production measured in the absence of enzyme source were subtracted from values obtained in the presence of enzyme source to correct for nonspecific production. One nanogram of purified PLC-γ1 catalyzed hydrolysis of 10 pmol of PIP_2 (400 dpm) per minute under this condition.

Expression Level of PLC Isoforms

For detection of PLC isoforms by specific antibodies, the high-speed supernatants described above were applied to a HiTrap-heparin column (1 ml, Pharmacia LKB Biotechnology) connected to an high-performance liquid chromatography system, and the proteins were eluted by 30 ml of a linear gradient of 0.1 M–0.5 M NaCl at a flow rate of 1 ml/min. An aliquot (10 μl) of each fraction was assayed for PIP_2-hydrolyzing activity. Another aliquot (20 μl) was subjected to SDS-PAGE [Laemmli, 1970] and the proteins were transferred onto nitrocellulose membranes (S & S, BE85). The filters were independently incubated with four different antibodies, of which specificities were confirmed previously [Homma et al., 1990], and then with alkaline phosphatase–conjugated anti-rabbit IgG. Immunoreacting bands were visualized with 5-bromo-4-chloro-3-indol phosphate and nitroblue tetrazolium as alkaline phosphatase substrates. Positive bands were not detected when preimmune sera were used.

Poly (A^+) RNA was obtained from various cells and an aliquot (5 μg) of each sample was subjected to electrophoresis. The RNA was transferred to a nylon filter and hybridization was performed using ^{32}P-labeled rat PLC cDNA fragments for PLC-β1, -γ1, -γ2, and -δ1 [Homma et al., 1989]. After 12 hr of hybridization, the filter was washed twice at room temperature in 2 × SSC (1 × SSC: 0.15 M sodium chloride and 0.015 M sodium citrate) containing 0.1% (w/v) SDS, and then three times at 65°C. The filter was exposed to Kodak X-Omat film with an intensifying screen at −80°C. The filters were further reprobed with ^{32}P-labeled β-actin cDNA to confirm the amount of mRNA and the transcript levels were quantified by densitometer.

Detection of Protein–Tyrosine Phosphorylation

Cells were incubated with phosphate-free Eagle's minimum essential medium containing 10% FBS, previously dialyzed against the same medium, for 1 hr and then labeled with ^{32}P-orthophosphate (0.2 mCi/ml) for 2 hr. Labeled cells were lysed with 1% (w/v) NP-40 in a buffer consisting of 50 mM Hepes/NaOH (pH 7.4), 1 mM PMSF, 0.1 mM sodium orthovanadate, 20 mM sodium fluoride, 100 μg/ml of leupeptin, and 100 μg/ml of aprotinin on ice for 30 min. The high-speed supernatant was precleared with Protein-G agarose (Oncogene Science), and then incubated with a monoclonal antibody PY20 (ICN Co.) in the presence of Protein-G agarose. For detection of tyrosine phosphorylation of PLC-γ1, the immunoprecipitates obtained by PY-20 were solubilized with 2% SDS and further incubated with anti-PLC-γ1 antibody in the presence of Protein-G agarose, after diluting SDS to 0.1% with the lysate buffer described above. The resultant precipitates were thoroughly washed and analyzed by SDS-PAGE.

RESULTS

In order to examine the changes of phosphoinositide metabolism, we measured the cellular contents of IP_3 and DG in KMS-4 and KMS-8 cells derived from different FAP patients and compared with those in normal colon cells or in other sporadic colorectal carcinoma cells. As shown in Figure 1, the IP_3 content was about 2- and 2.2-fold higher in KMS-4 and KMS-8 cells, respectively, than in CCD841CoN cells. The DG level in both KMS cells was also about 1.7-fold higher than in CCD841CoN cells. The high levels of IP_3 and DG in these KMS cells were also evident, when they were compared with those in other colorectal carcinoma cells. The IP_3 and DG contents in other normal cells, FHs74Int and FHC cells, were almost the same as those in CCD841CoN cells. Since these results indicated the enhanced phosphoinositide signaling pathway, we next examined the PIP_2-hydrolyzing activity in the high-speed supernatants obtained from various cells. As shown in Figure 2, the enzyme activity in KMS-4 and KMS-8 cells was about threefold higher than in CCD841CoN cells and other colorectal carcinoma cells tested. These changes seemed to occur in parallel to the levels of intracellular messengers (Fig. 1).

We further examined the levels of PLC transcripts and activities in KMS-4 and KMS-8 cells. When PLC activities were resolved by heparin column chromatography, two peaks were eluted, at 0.2 M and 0.3 M NaCl (Fig. 3). These active fractions were analyzed by immunoblotting with anti-PLC isoform antibodies [Homma et al., 1990]. As shown in the Figure 3 insert, PLC-γ1

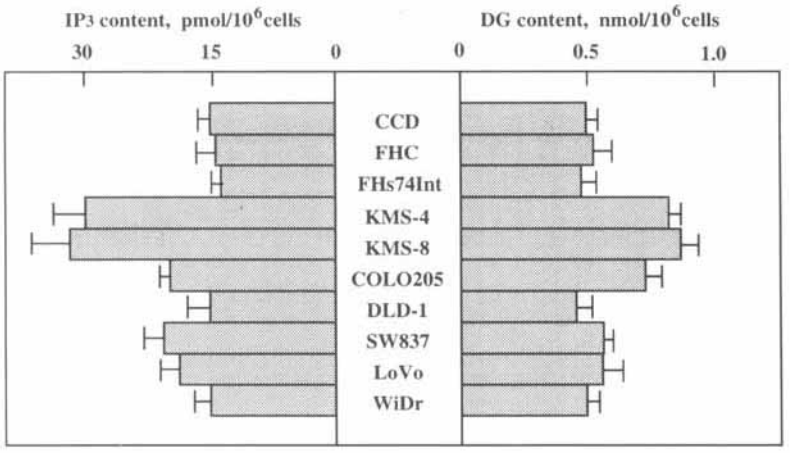

Fig. 1. IP_3 and DG contents in colon carcinoma cells. Cells grown to the exponential phase were harvested, and the IP_3 and DG contents were measured separately as described in Methods. The results show the means ± SD of three independent experiments.

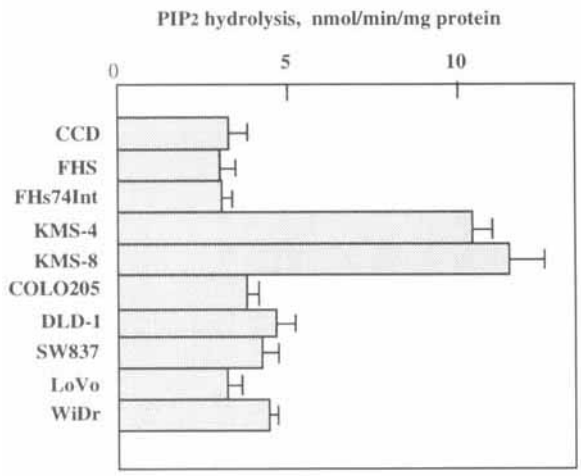

Fig. 2. PLC activity in colorectal carcinoma cells. Aliquots (10 μl) of the high-speed supernatants obtained from various cells indicated were used for the determination of PIP_2-hydrolyzing activity. The results show the means ± SD of three independent experiments.

eluted from the column first in fractions 18–19 and then PLC-δ1 was found in fractions 22–23. Since these positive bands were not observed when antibodies were pretreated with purified PLC-γ1 and PLC-δ1 (data not shown), the above results indicate that PLC-γ1 and PLC-δ1 are the main contributors to the first and the second peaks, respectively. On the other hand, immunoreactive bands were not detected with anti-PLC-β1 and anti-PLC-γ2 antibodies in all fractions (data not shown). Large amounts of PLC-γ1 and PLC-δ1 were detected in KMS-4 and KMS-8 cells, with protein levels severalfold higher than in CCD841CoN cells. The PLC activities in both peaks were also ~threefold higher than in CCD841CoN cells. These results were consistent with those obtained from Northern blot analysis using rat PLC cDNA clones as probes, as shown in Figure 4. When a cDNA clone for PLC-γ1 was used as probe, the transcription levels in KMS-4 and KMS-8 cells were 20- to 30-fold higher than in CCD841CoN cells. The expression levels of PLC-δ1 in KMS-4 and KMS-8 cells were 23-fold higher than in CCD841CoN cells. Since the mRNA level of β-actin was almost equal among cell lines used (data not shown), these differences reflect the changes of transcript levels among these cells. Neither PLC-γ1 nor PLC-δ1 DNA was amplified in these cells by Southern blot hybridization (data not shown). The expression of PLC-β1 and PLC-γ2 was not detected in these cells even after exposing the X-ray films for a week (data not shown).

The mRNA levels of PLC isoforms were varied among sporadic colorectal carcinoma cell lines. Expression of PLC-γ1 and PLC-δ1 was relatively high in COLO205, DLD-1, SW837, and LoVo cells, and COLO205 and LoVo cells, respectively, while neither the PLC-γ1 nor PLC-δ1 gene was amplified in these cells by Southern blot hybridization (data not shown). Although DLD-1 and COLO205 cells expressed PLC-δ1 and PLC-γ1 at relatively high levels, they did not exhibit a significant enhancement of IP_3 levels (Fig. 1) and PLC activities (Fig. 2).

To examine whether the enhanced phosphoinositide metabolism in these cells is coupled to the tyrosine kinase system, ^{32}P-labeled cells were analyzed by immunoprecipitation using anti-

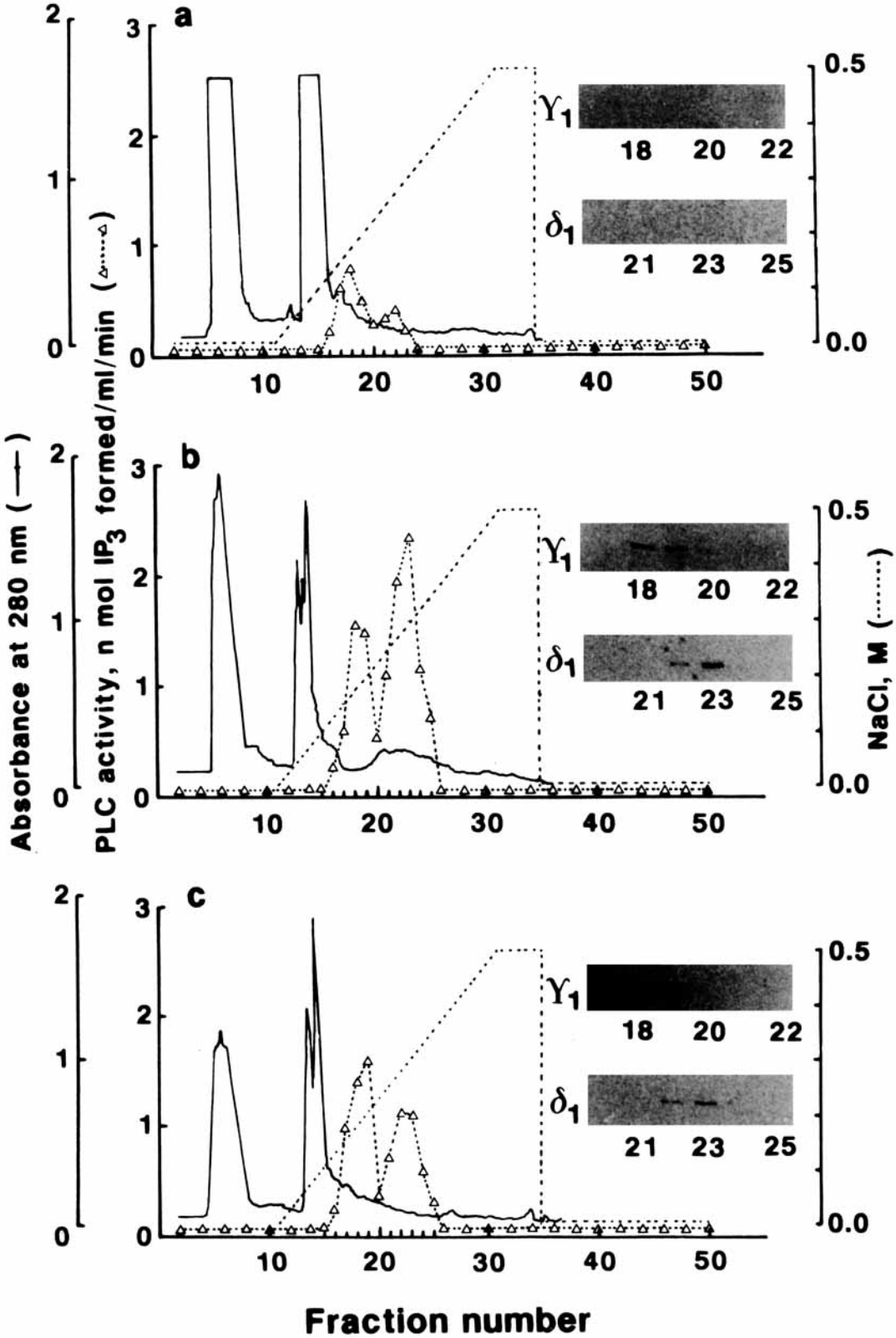

Fig. 3. Separation of PLC isoforms by heparin–Sepharose column chromatography. The high-speed supernatants obtained from CCD841CoN (4 mg protein) **(a)**, KMS-8 (2 mg protein) **(b)**, and KMS-4 (2 mg protein) **(c)** were separated on a heparin column. Each fraction was monitored for absorbance at 280 nm and an aliquot (10 μl) was assayed for PIP_2-hydrolyzing activity. A portion of each fraction was examined for PLC species by Western blotting with anti-PLC-γ1 and anti-PLC-δ1 antisera.

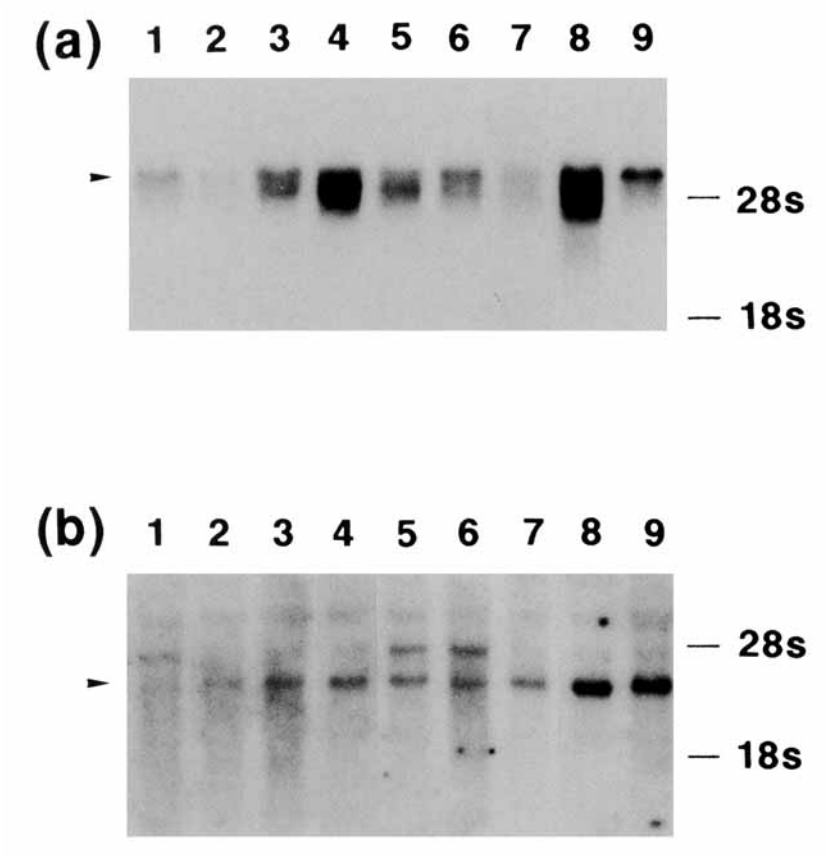

Fig. 4. Northern blot analysis of the mRNAs for PLC isozymes from cultured colorectal carcinoma cells. Poly (A^+) RNAs (5 μg each) were analyzed by Northern blotting analysis using radiolabeled PLC-γ1 **(a)** and PLC-δ1 **(b)** cDNA probes. Ribosomal RNAs were used as size markers. **Lane 1,** CCD841CoN; **lane 2,** FHC; **lane 3,** DLD-1; **lane 4,** COLO205; **lane 5,** SW837; **lane 6,** LoVo; **lane 7,** WiDr; **lane 8,** KMS-8; **lane 9,** KMS-4.

phosphotyrosine (PY-20) antibody. As shown in *Figure 5a*, endogenous tyrosine kinase activities were apparently high in KMS-4 and KMS-8 cells, while the phosphorylation level was low in CCD841CoN cells, suggesting the constitutive activation of tyrosine kinases in KMS-4 and KMS-8 cells. Phosphoproteins with molecular masses of 220, 145, 110, 100, 75, 60, and 45 kDa were prominent in these cells. Among them the phosphorylated 145 kDa protein was shown to be PLC-γ1 (Fig. 5b).

DISCUSSION

We have examined phosphoinositide metabolism in various cell lines derived from normal colon tissues and colorectal carcinomas including KMS-4 and KMS-8 cells which are derived from colorectal carcinomas of FAP patients. We first determined the amounts of IP_3 and DG, PIP_2-hydrolyzing activity in vitro, and expression level of PLC isoforms in these cell lines. KMS-4 and KMS-8 cell lines showed high contents of DG and IP_3, and an extremely high level of PIP_2-hydrolyzing activity, as compared with other colorectal carcinoma cell lines and normal cell lines. Although we used cell lines from fetal normal colonic mucosa as controls, the possibility that the alteration is derived simply from differences between fetal and adult epithelial cells can be ruled out, because activities of PLC and cellular contents of IP_3 and DG in KMS cells were substantially enhanced when they were compared with those in sporadic colorectal carcinoma cells. To analyze the mechanism underlying the high activity, we determined the expression levels of PLC isozymes. Immunoblotting analysis showed an enhanced expression of both PLC-γ1 and PLC-δ1 among PLC isoforms tested in KMS-4 and KMS-8 cells, and this result was confirmed by Northern blotting analysis. The transcriptional and/or translational enhancement of both PLC isoforms seems to be a mechanism to rationalize the high PLC activity observed in these cells. The enhanced expression

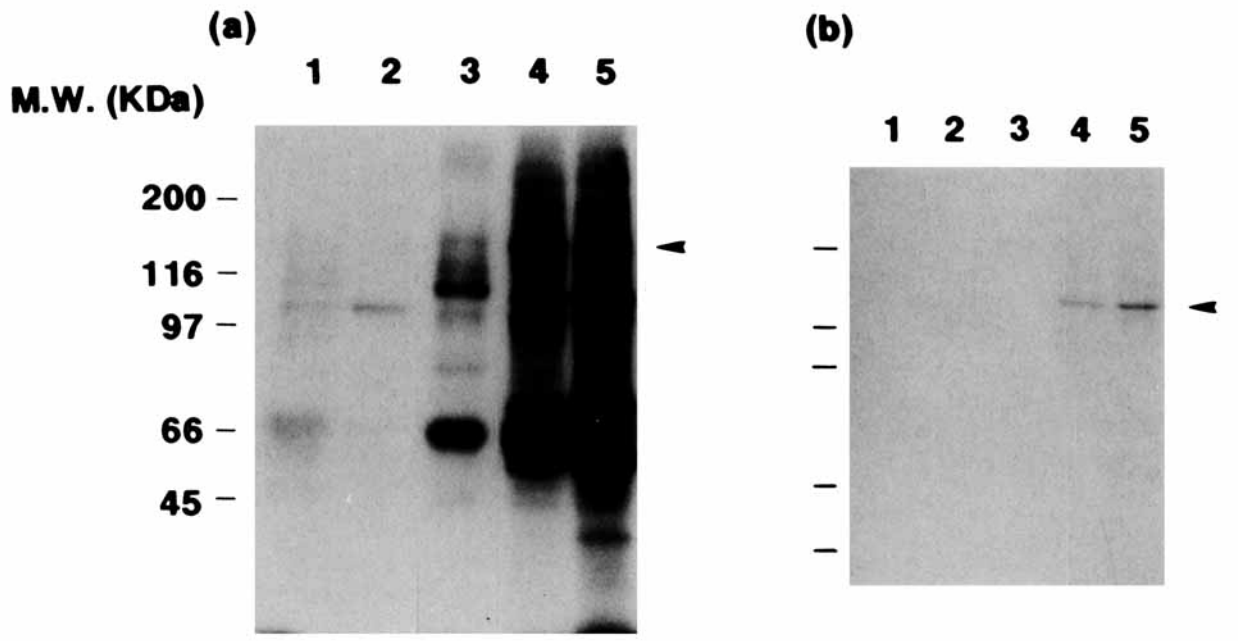

Fig. 5. Phosphotyrosine-containing proteins in colon carcinoma cells. **a:** ^{32}P-labeled cells were lysed and immunoprecipitated with monoclonal anti-phosphotyrosine antibody (PY20) in the presence of protein-G agarose. Immunoprecipitates were solubilized in Laemmli's SDS buffer, separated in 7.5% SDS-polyacrylamide gels, dried, and autoradiographed. **Lane 1,** CCD841CoN; **lane 2,** LoVo; **lane 3,** COLO205; **lane 4,** KMS-4; **lane 5,** KMS-8. **b:** Immunoprecipitates obtained with PY-20 were solubilized and further treated with anti-PLC-γ1 antibody in the presence of protein-G agarose. The final immunoprecipitates were solubilized in Laemmli's SDS buffer, separated in 7.5% SDS-polyacrylamide gels, dried, and autoradiographed. **Lane 1,** CCD841CoN; **lane 2,** LoVo; **lane 3,** COLO205; **lane 4,** KMS-4; **lane 5,** KMS-8.

of PLC isoforms, however, does not always account for the high level of IP_3 and DG. Therefore, the other mechanisms might be involved in the activation of PLC isoforms.

It has been demonstrated that the phosphoinositide metabolism is often coupled to the tyrosine kinase system [Ullrich and Schlessinger, 1990] and the phosphorylation of PLC-γ1 by tyrosine kinases directly stimulates its catalytic activity [Wahl et al., 1988; Nishibe et al., 1990]. In addition, the activation of endogenous tyrosine kinases has been observed in some surgical specimens from colorectal carcinomas [Cartwright et al., 1990]. In this study, we showed PLC-γ1 is phosphorylated in KMS-4 and KMS-8 cells by endogenous tyrosine kinase. This may also explain the activation of PLC and the enhanced phosphoinositide metabolism in these cells. The mechanism underlying the enhanced tyrosine kinase activity in two KMS cells is still unknown. Since production of growth factors by human colon carcinoma cell lines was reported previously [Anzano et al., 1989], it is possible that these cells secrete in an autocrine manner growth factors which bind to cell surface receptors and in turn activate intrinsic tyrosine kinases. It is also possible that cellular tyrosine kinases are constitutively activated by intramolecular changes such as an amino acid substitution by point mutation. Further studies are required to clarify the mechanism.

It has been reported that *APC* gene is tightly linked to FAP [Kinzler et al., 1991b; Groden et al., 1991]. We demonstrated the enhanced phosphoinositide metabolism and tyrosine kinase activities in two independent cell lines derived from FAP patients, suggesting that these intracellular changes may be associated with alterations of *APC* gene. Further analysis of the phosphoinositide metabolism and tyrosine kinase activity in other cell lines established from FAP patients [Olive et al., 1993; Untawale et al., 1993] may clarify this possibility. The *APC* gene was found to be deleted or mutated at the early stage in the development of sporadic colorectal carcinomas [Powell et al., 1992]. Thus, it is interesting to compare the deletion and/or mutation sites of *APC* between KMS cells and sporadic colorectal carcinoma cells, and to analyze the relevance of these sites of *APC* to activation of PLC. It remains to determine a particular carcinogenic pathway that occurs in FAP pa-

tients affecting phosphoinositide metabolism unlike that occurring in sporadic cases.

The activation of PLC leads to increased production of DG and IP_3. Because DG is an endogenous activator of protein kinase C, the above results suggest that protein kinase C might be activated in these colorectal carcinoma cells. We observed that the protein kinase C activity located in the membrane fraction was significantly increased in KMS-4 and KMS-8 cells (unpublished observation). Since protein kinase C has been reported to be translocated from the cytosol to the membrane in response to stimulation, the increase in protein kinase C activity in the membranes may indicate an increase in the active state of protein kinase C.

The mRNA level of PLC isoforms was varied among sporadic colorectal carcinoma cell lines. Enhanced expression of PLC-γ1 and -δ1 in COLO205, DLD-1, SW837, and LoVo cells may result in slight increases of the IP_3 and DG levels and PLC activity in vitro, as compared with those in CCD841CoN and FHC cells. It has been reported that PLC-γ1 is highly expressed in more than 70% of human breast carcinoma cases [Artega et al., 1991]. We also observed the high expression of PLC-γ1 in 50% of human leukemic cell lines (unpublished results). It is of interest that transcriptional controls of the PLC-γ1 and PLC-δ1 genes are changed during cell transformation.

In summary, we observed several changes of transcriptional and/or translational control of *the PLC genes* and activity of PLC isoforms in two independent carcinoma cell lines derived from FAP patients. These results suggest that the activation of PLC and subsequent activation of protein kinase C play an important role in signal transduction for the transformed phenotype of these cells.

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