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Growth Inhibition, Enhancement of Intercellular Adhesion, and Increased Expression of Carcinoembryonic Antigen by Overexpression of Phosphoinositides-specific Phospholipase C β1 in LS174T Human Colon Adenocarcinoma Cell Line

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By using a retrovirus-derived system we generated derivatives of the human colon adenocarcinoma cell line LS174T (ATCC CL 188) that stably overexpress a full-length cDNA encoding the β1 isoform of bovine phosphoinositides-specific phospholipase C (PI-PLC). This was confirmed by the elevated levels of catalytic activity to release phosphoinositides from phosphatidylinositol (PI-PLC) or phosphatidylinositol-bis-phosphate (PIP_2-PLC), and the enhanced expressions of messenger RNA and protein. PI-PLC β1 overexpresser clones grew to form cell clumps floating in liquid medium, whereas the pMV7-introduced control clones displayed morphologic characteristics that were very similar to those of the parent LS174T cell line. Three individual PI-PLC β1 overexpresser cell lines displayed increased doubling time (18.0 h, 21.5 h, and 23.8 h) when compared with 4 individual pMV7-introduced control cell lines (13.1 h, 10.7 h, 12.9 h, and 9.3 h). Anchorage-independent growth ability in soft agar medium was dramatically suppressed by overexpression of PLC β1, and the ability of PLC-overproducer clones to form aggregates when cultured in liquid medium was dramatically enhanced when compared with that of pMV7-introduced control clones. Tumorigenicity of PLC β1-overproducers was much weaker than that of vector-transduced control clones. The spontaneous release of carcinoembryonic antigen from PLC β1-overproducer clones was much higher than that from pMV7 control clones. The ability of PLC β1-overproducer clones to form aggregates during suspension culture was much stronger than that of the control clones. These results provide the first evidence that elevated levels of endogenous PI-PLC β1 suppress tumor cell growth, but enhance the ability to form cell aggregates and to release carcinoembryonic antigen, an intercellular adhesion molecule.

Key words: Phospholipase C β1 — Overexpression — Carcinoembryonic antigen — Cell aggregation — Colon adenocarcinoma

Phosphoinositides-specific phospholipase C (PI-PLC) is one of the key enzymes in the signal transduction pathways from various kinds of agonist-mediated stimuli to cells. Hydrolysis of inositol-phospholipids by the enzyme generates diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which, in turn, activate phosphatidylcholine-specific phospholipases C and D by way of activation of protein kinase C (PKC) and Ca_2 release, respectively.[1,2] PI-PLCs are ubiquitous enzymes and thus far, three major PI-specific subtypes (β, γ, and δ) have been identified in mammalian tissues.[3–5] All three isoforms have similar hydrolytic properties towards three common phosphoinositides, i.e., phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bis-phosphate (PIP_2). There is also evidence that specific isoforms are coupled to different receptors and that they are activated by different mechanisms [for reviews, see Refs. 2, 3, and 5]. Only PLC γ isoforms have both SH2 and SH3 intracellular domains, certain tyrosine residues of which are phosphorylated by several receptor tyrosine kinases, such as epidermal growth factor and platelet-derived growth factor, which subsequently results in IP turnover.[6–10] On the other hand, β isoforms of PLC are activated by several agonists which bind to G-protein-linked 7 membrane-spanning receptors, such as muscarinic (M1, M3, and M5), serotogenic (5-HT1c), and adrenergic (α1B) receptors.[3] The agonist-induced conformational changes in these receptors dissociate the heterotrimeric proteins into G_α and $G_{\beta\gamma}$ subunits, both of which can activate PLC β isoforms in either a pertussis toxin-insensitive or sensitive manner.[11–15] Although four distinct PLC δ isoforms are known, the mechanism by which these isozymes are coupled to membrane receptors remains unclear.

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We have recently become interested in the possibility that PLC might play an important role in the origin and growth of human colon cancer. We have found that bile acid, which has been implicated as a promoter in colon carcinogenesis, can enhance the activity of PLC in extracts of normal human colon mucosa and colon tumors.[16] In addition, we found that human colon tumors frequently display increased levels of PLC γ1 isoform and decreased levels of δ1 when compared with normal colonic mucosa, but only low levels of β1 isoform. Furthermore, a series of human colon tumor cell lines express high levels of PLC γ1, only low levels of PLC β1, and undetectable levels of PLC δ1.[17] Because a recent series of studies has also demonstrated elevated contents of PLC γ1 as protein and/or catalytic activity in primary human tumors such as colon, breast cancer, renal cell carcinoma and nonsmall lung carcinoma as compared with the corresponding normal tissues,[18–21] the elevation of PLC γ1 might be universal in tumor tissues.

At present, little is known about the role of signal transduction through G-protein-linked receptors in colon carcinogenesis. A recent series of studies, however, showed that receptors that couple through G proteins to activation of PI-PLC β isoforms (serotonin 1c, α1B adrenergic) can effectively transform NIH 3T3 fibroblasts.[22–24] Therefore, it is likely that the PLC β isoform when overexpressed in cells might function as a protooncogene. We therefore took a direct molecular approach to address the precise role of PLC β isoform in human colon cancer.[25] We generated derivatives of the LS174T human colon cancer cell line that stably overexpress large amounts of PLC β1. The LS174T cell line was employed since it has been extensively characterized with respect to its growth properties.[26] Contrary to our expectation, our findings indicate that overexpression of PLC β1 in LS174T cells can cause a marked suppression of growth in cell culture, suppression of tumorigenicity in nude mice, and augmented ability to form aggregates in suspension culture. We also found that the PLC β1 overexpressers express and release increased levels of carcinoembryonic antigen molecule, an intercellular homotypic adhesion molecule.

MATERIALS AND METHODS

Cells and culture conditions LS174T human colon adenocarcinoma cell line (ATCC CL 188) was used. The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and kanamycin at a concentration of 50 μg/ml.

Isolation of cell lines stably overexpressing PI-PLC β1 Full length cDNA encoding the β1 subspecies of bovine PI-PLC was kindly provided by Dr. Knopf (Genetic Inst.). A retrovirus-derived cDNA expression vector designated pMV7[25, 27] and a construct designated pMV7-PLC β1 containing the total PLC β1 cDNA were transfected onto subconfluent GPAM12 cells by using Lipofectin (Gibco BRL, Gaithersburg, MD). After 48 h, the medium was harvested, filtered, and used to infect recipient subconfluent LS174T cells with 2 μg/ml polybrene for 48 h. The cells were then trypsinized and replated in culture medium that contained 500 μg/ml of the neomycin derivative G418 (Geneticin, Gibco). Resistant colonies were cloned and maintained in culture medium containing 500 μg/ml G418.

Assay of PI-PLC and PIP_2-PLC activities in tissue culture cells Both total PI-PLC and PIP_2-PLC activities in crude cell extracts were determined *in vitro*.[28] Briefly, the cells were harvested with 2 ml of homogenization buffer [10 m*M* Tris HCl, pH 7.5, 0.25 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 m*M* 2-mercaptoethanol], and homogenized in a Teflon homogenizer at 4°C. Protein concentration was determined by the Lowry method.

The PLC activity present in the cell extracts was assayed immediately after isolation by using mixed micelles of ^{3}H-phosphatidylinositol and phosphatidylinositol or ^{3}H-phosphatidylinositol-bisphosphate and phosphatidylinositol-bisphosphate. Briefly, the samples were suspended at a concentration of 375 μg/ml (PI-PLC) or 37.5 μg/ml (PIP_2-PLC) in the reaction buffer [50 m*M* HEPES, pH 7.0, 100 m*M* NaCl, 1 m*M* $CaCl_2$, 0.15 mg/ml bovine serum albumin (BSA), and 1 mg/ml sodium deoxycholate], and mixed with a substrate mixture of l-α-phosphatidylinositol (ammonium salt) and l-α-phosphatidyl[2-^{3}H]inositol (Amersham Co., Arlington Heights, IL) or a mixture of 1-α-phosphatidylinositol 4,5-biphosphate (sodium salt, Sigma Chem. Co., St. Louis, MO) and L-3-phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate (Amersham Co.) at final concentrations of 100 μM and 10^4 dpm, respectively. Then, the mixture was incubated at 37°C for 60 min (PI-PLC) or 15 min (PIP_2-PLC), and the reaction was stopped by addition of 375 μl of chloroform/methanol (1/2, v/v). The radioactivity in the aqueous fraction after extraction of lipids from the reaction mixture by the methods of Bligh and Dyer[29] was detected with a liquid scintillation counter.

RNA isolation and northern blot analysis Total RNA was extracted from cell lines with guanidium isothiocyanate and layered over cesium chloride (5.7 *M*) in sodium acetate (25 m*M*, pH 5.0).[30] Gradients were centrifuged overnight in a Beckman SW41 rotor at 100,000*g*. Aliquots (20 μg) of the RNA were analyzed by electrophoresis on 1% agarose gels containing 6% formaldehyde and transferred to Hybond-N hybridization transfer membranes (Amersham). UV-treated filters were prehybridized for 5 h at 65°C and hybridized for 16 h at 65°C in Church buffer (1 m*M* EDTA, 250 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1% BSA (fr. V), 7% BSA, pH 7.2].[31] Between 20 and 50 ng of full length cDNA of bovine PI-PLC β1 was labeled with the Multiple DNA labeling system (Amersham) for use as hybridization probes. The blots were washed as follows: 30 min in 1× saline-sodium citrate buffer (SSC) supplemented with 0.1% sodium dodecyl sulfate (SDS) at 65°C and then 30 min in 0.1× SSC at room temperature. Autoradiography was performed at −80°C for 1 to 7 days.

Western blot analysis Cells were washed with phosphate-buffered saline (PBS), and harvested in a buffer (1% Triton X100, 20 m*M* HEPES, pH 7.4/5 m*M* EGTA/1 μg each of aprotinin and leupeptin per ml], and the suspension was sonicated. Samples at a concentration of 1 mg/ml in the buffer were stored at −80°C until use. The samples were subjected to 8% SDS/polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Membranes were sonicated with TN-TX100 buffer (50 m*M* Tris, pH 7.5, 200 m*M* NaCl, and 0.2% TX100), then incubated with a 1:300 dilution of mouse anti-bovine PI-PLC β1 monoclonal antibody (Upstate Biotechnology, Inc., NY) in TN-TX100 supplemented with 3% BSA fr. IV (TN-TX-BSA) at room temperature for 90 min followed by incubation with anti-mouse IgG second antibodies conjugated with alkaline phosphatase in TN-TX-BSA for 30 min. The membranes were washed three times with TN-TX, then transferred to a solution containing color development substrates (0.33 mg/ml of nitro blue tetrazolium and 0.17 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate) and incubated for 30 min at room temperature.

Cell growth in liquid medium and soft agar To assess the growth of cells in liquid medium, 2×10^4 cells were suspended in 3 ml of DMEM supplemented with 10% FCS and kanamycin. On days 1, 4, 7, 10, and 12 after starting the culture, the numbers of cells in triplicate wells per group were counted by using a Coulter Counter (Coulter Electrics, Ltd., UK). Viability of the cells was confirmed by measuring the ratio of the cells which exclude 0.1% trypan blue to that of the cells which include trypan blue by using a hemocytometer.

To assess the anchorage-independent growth of cells in soft agar medium, 5×10^4 cells were suspended in 1 ml of 0.3% Bacto-agar (Difco Laboratories, Detroit, MI) in DMEM containing 10% FCS and overlaid above a layer of 2 ml of 0.5% agar in the same medium, on 30-mm petri dishes. The cells were then overlaid with 1 ml of 0.3% agar in the medium once a week until the end of the culture. On the 28th day after starting the culture, colonies (with a diameter of more than 100 μm) were counted microscopically.

Tumorigenicity of cells in nude mice Actively growing cells were trypsinized, washed twice with PBS, and resuspended at a concentration of 10^7 cells/ml in PBS. Approximately 10^6 cells were injected subcutaneously into 4–5-week-old female Balb/c *nu/nu* athymic nude mice (Shizuoka Laboratory Animals Corporation, Shizuoka). Mice were dissected under anesthesia with diethylether on day 23 after the injection for measurement of the tumor weight.

Adhesion assay Cell adhesion assay was carried out according to the method of Benchimol *et al*.[32] Briefly, cell cultures in the late exponential phase of growth were changed into single cell suspensions by 3 min incubation at 37°C with 0.12% Bacto trypsin in PBS lacking Mg^{2+} and Ca^{2+} and containing 15 m*M* sodium citrate. After centrifugation, the cells were suspended by two or three passes through a 30-gauge needle in DMEM supplemented with 0.8% FCS and 10 μg/ml DNAase. Triplicate cell suspensions in 3 ml of the above medium at 10^6 cells/ml under an atmosphere of 5% CO_2 in polystyrene tubes were magnetically stirred at 70–80 rpm at 37°C. Samples were taken over a 2 h period to determine the total number of cells and the number of single cells by using a

A

B

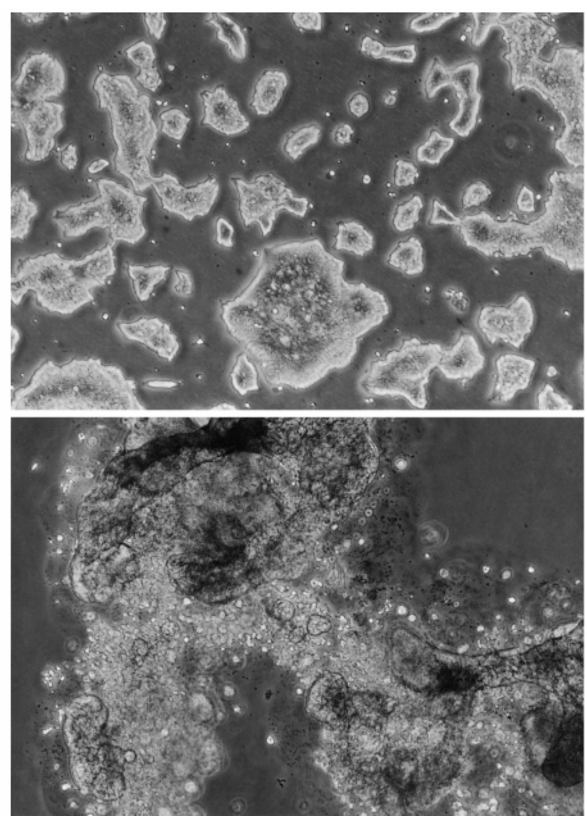

Fig. 1. Morphological changes induced in LS174T human colon adenocarcinoma cells by overexpression of PI-PLC β1 in liquid medium. pMV7-transduced control clone #3 (A) and PLC β1-transduced clone A1 (B) were incubated at 37°C in an atmosphere of 5% CO_2 for 3 days.

hemocytometer. The percentage of total cells remaining as single cells was used as a measure of the proportion of nonadherent cells.

Determination of carcinoembryonic antigen (CEA) A nonionic detergent, Triton X-114 (Sigma), was purified as described by Bordier[33] to eliminate the most hydrophilic molecules from the commercial preparation. Confluent cells (10^7–10^8) were washed twice with PBS, and after centrifugation, the pellet was homogenized in 1 ml of 10 m*M* Tris-HCl, pH 7.4, 100 m*M* NaCl, 1% Triton X-114, 5 m*M* iodacetamide, 1 m*M* PMSF, and 5 m*M* EDTA. The homogenate was incubated for 1 h in a 1.5-ml Eppendorf microfuge tube at 4°C under agitation. CEA levels were determined by an immunoradiometric method using the CEA Kit "Daiichi" II (Daiichi Radioisotope, Tokyo). The sensitivity of the assay was 1 ng/ml.

RESULTS

Cloning of PI-PLC β1 overproducers Individual G418-resistant clones were isolated from the dishes which had been transduced with pMV7-PLC β1 or pMV7 vector plasmid only when the clones made small colonies. The cell lines (pMV7-PLC β1) were designated A1, A2, and C. The control clones were designated #3, #4, #5, and #6. All of three PI-PLC β1 clones grew as cell clusters floating in the medium and had weak activity to adhere to the bottom of dishes (Fig. 1B). The viability of the cultured cells was confirmed to be more than 90% by means of

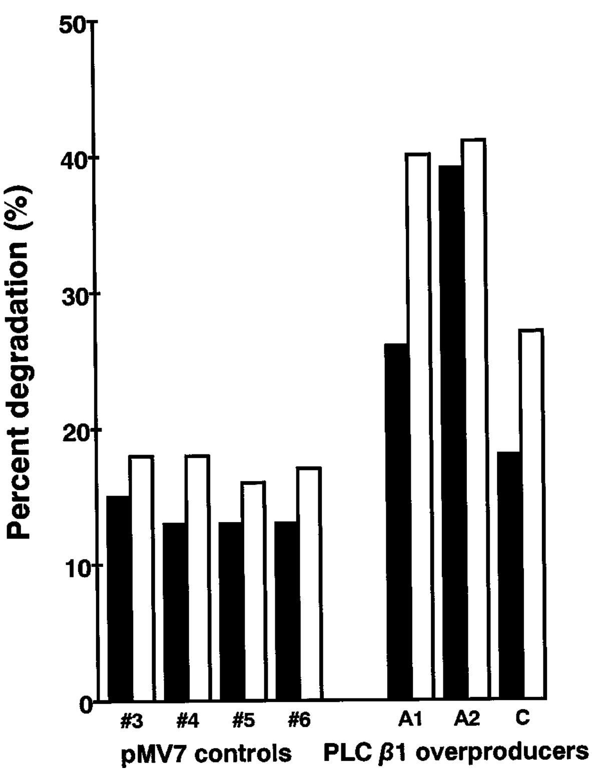

Fig. 2. Enhancement of PI- and PIP_2-PLC activities of extracts of LS174T cells by overexpression of PLC β1. Extracts from control clones and PLC β1 overproducers were incubated with labeled PI or PIP_2 in the presence of 1 mg/ml sodium deoxycholate at 37°C for 60 min (PI, black column) or 15 min (PIP_2, white column). Results were expressed as percent degradation of the labeled substrate.

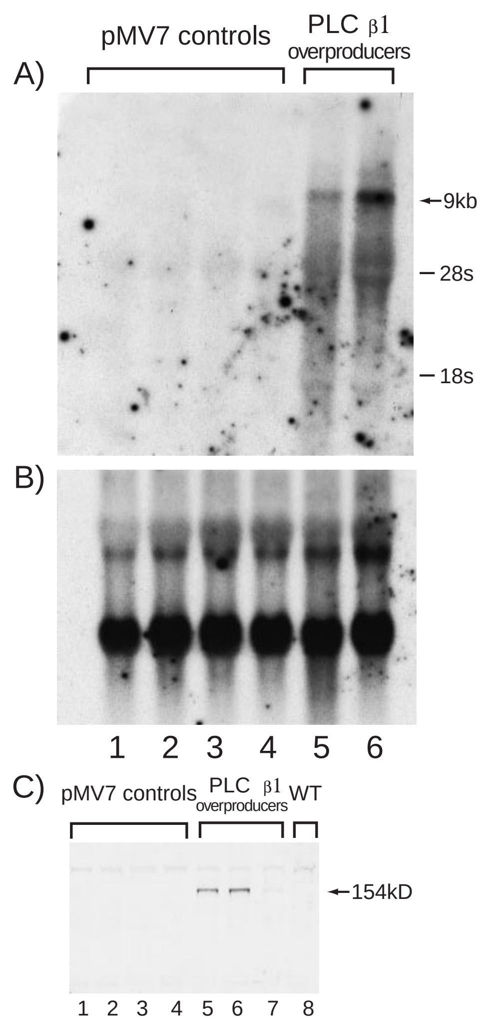

Fig. 3. Overexpression of PI-PLC β1 in LS174T. Total RNAs (20 μg/lane) from the LS174T wild type (WT), pMV7-transduced control clones (#3, #4, #5, #6) and PLC β1 overproducers (A1 and A2) were analyzed with PLC β1 (A) and γ actin (B) probes as described in "Materials and Methods." Partially purified protein extracts from the control clones (#3, #4, #5, and #6) and PLC β1 overproducers (clones A1, A2, and C) were subjected to electrophoresis (50 μg of protein/lane), blotted and hybridized to a PLC β1 antibody, as described in "Materials and Methods" (C). Lanes 1–4, pMV 7 control #3, #4, #5, #6; lanes 5–7, PLC β1 A1, A2, C; lane 8, LS174T wild type.

the 0.2% trypan blue dye exclusion test. Even after trypsinization, the single cell suspensions of PI-PLC β1 clones formed clusters of cells in a short period when they were incubated again in the liquid medium and they maintained the acquired phenotypic change after more than 50 passages. Mucous substances surrounding the aggregates were clearly apparent (Fig. 1B). None of the four control clones showed any morphological change as compared to wild type LS174T (Fig. 1A).

Overexpression of PI-PLC β1 in LS174T To determine the levels of PI-PLC and PIP_2-PLC activities, each of the cell lines was assayed for the enzyme activity *in vitro* by using crude extracts of the cell lines. As shown in Fig. 2, the derivatives displayed significant increases in total enzyme activity towards both substrates when compared with the control clones. The level of the enzyme activity in clone C was less than those of clones A2 and A1.

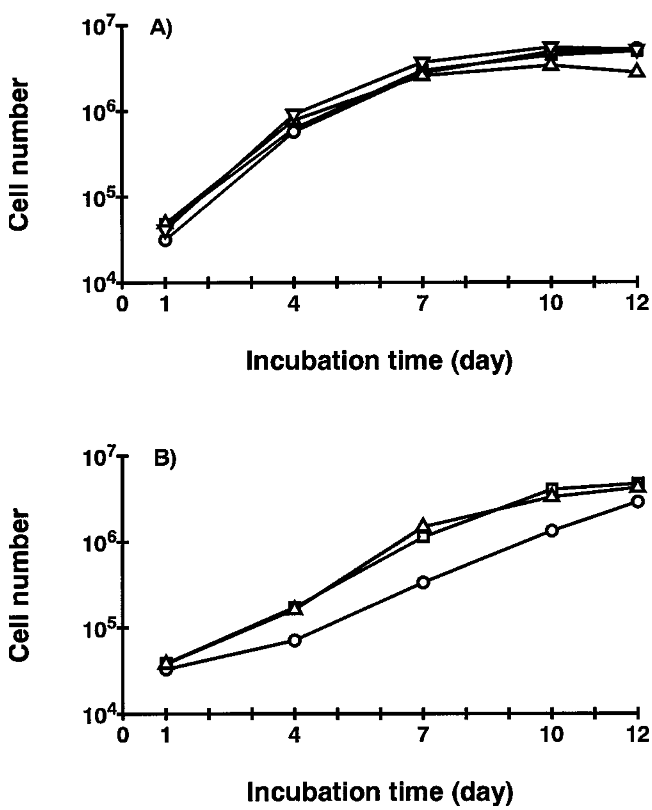

Fig. 4. Inhibition of growth of LS174T in liquid medium by overexpression of PI-PLC β1. Cells (5×10^4) of control clones (#3, #4, #5, #6) and PLC β1-overproducing cells were cultured in 6-well flat-bottomed dishes at 37°C in an atmosphere of 5% CO_2 and air. Cell number in each well was counted on days 1, 4, 7, 10, and 12 after starting the culture, and the results are indicated as the mean of duplicate wells. A) pMV7 control clones. □ #3, ○ #4, △ #5, ▽ #6. B) PLC β1 overproducers. □ A1, ○ A2, △ C.

The overexpression of PI-PLC β1 at the messenger RNA level was confirmed by northern blot analysis (Fig. 3, panel A). PI-PLC β1 clones contained elevated levels of a prominent 9-kilobase species, which corresponds to the predicted size for an mRNA transcript that initiates in the 5′ long terminal repeat (LTR) and terminates in the 3′ LTR of the pMV7-PLC β1 construct. Only weak bands of an endogenous transcript homologous to the PI-PLC β1 probe were detected on the lines of control RNAs. Thus, in these cells, there is negligible expression of the endogenous gene encoding PI-PLC β1. Expression levels of mRNA for γ actin were similar for all of the clones tested (Fig. 3, panel B). The level of the enhanced expression of the isoform in clone C was less than those in clones A1 and A2 (data not shown).

Western blot analysis by using cell extracts and monoclonal antibody to the isoenzyme also showed elevated expression of the 154 kD PI-PLC β1 in PI-PLC β1-overproducing cells, but no detectable expression of the species in control cells or LS174T wild type cells (Fig. 3, panel C). The level of overexpression in clone C was apparently less than that of A1 or A2 clone: the relative expression levels of PI-PLC β1 protein were 2603:3392:100 for A1:A2:C.

Growth characteristics of PI-PLC β1-overproducing cells To characterize further the phenotypic changes of PLC β1 overproducers, the clones were examined in detail with respect to their growth rates in liquid medium as compared to those of control cell lines. As shown in Fig. 4, PLC β1-overproducing cells showed significant lower growth rate when compared with control cell lines. The mean doubling times of the individual clones A1, A2, and C were 30.2, 31.1, and 23.1 (h), respectively. The doubling times of control clones #3, #4, #5, and #6 were

Table I. Inhibition of Growth of LS174T by Overexpression of PI-PLC β1

Clone	Colony formation in soft agar medium[a] (mean±SD)	Growth in nude mice[b] (g, mean±SD)
Control #3	223±19	0.79±0.70
Control #4	578±22	0.67±0.30
Control #5	313±31	0.43±0.31
Control #6	797±139	0.72±0.35
PI-PLC β1 A1	99±3	0.14±0.04
PI-PLC β1 A2	25±10	0.00±0.01
PI-PLC β1 C	77±10	Not tested

a) 2×10^4 cells of each clone were seeded into 6-well dishes, and the numbers of colonies were counted after incubation for 4 weeks.
b) 10^6 cells of each clone were injected into BALB/c *nu*/*nu* mice (6 mice/group), and the tumor nodules were weighed on day 23 after the transplantation.

16.2, 13.5, 18.2, and 11.8 (h), respectively. There was no significant difference in saturation density between the control clones and PLC β1-overproducing clones.

We also assayed these cell lines for the ability to form colonies in soft agar. As shown in Table I, the control cells formed many more large colonies as compared with PLC β1-overproducing clones. The effects of constitutive PLC β1-overexpression on tumorigenicity in nude mice were examined by subcutaneously injecting 1×10^6 cells per mouse. The control clones formed palpable tumors in nude mice (Table I). In contrast, growth of PI-PLC β1-overproducing cells in nude mice was significantly slower than that of the control cells.

Intercellular adhesion function of cultured cells To test for possible changes in the intercellular adhesion function caused by overexpression of PLC β1, a widely used assay for adhesion was applied, which measures the ability of single cells to form aggregates in suspension culture. The results, shown in Fig. 5 and expressed as the percentage of cells remaining as single cells as a function of time, indicate that the PLC-overproducing cell lines aggregate more readily than the pMV7-transduced cell lines. The size of the aggregates varied from doublets up to large aggregates of more than 50 cells (data not shown).

Expression and spontaneous release of carcinoembryonic antigen (CEA) from LS174T cell lines The total CEA content of LS174T cell lines was quantified after Triton-X114 extraction (Fig. 6). The mean amount of CEA expressed in the three PLC β1-overproducing cell lines was more than 3 times the levels in the pMV7-transduced control clones ($P<0.01$). These cell lines also released CEA into culture medium (Fig. 7). The spontaneous release of CEA was not due to cell death since more than 99% of the cells excluded trypan blue (data not shown). PLC β1-overproducing cells spontaneously released more than 100-fold more CEA when compared with that released by the vector-transduced control cell lines. The amounts of CEA released from the PLC β1

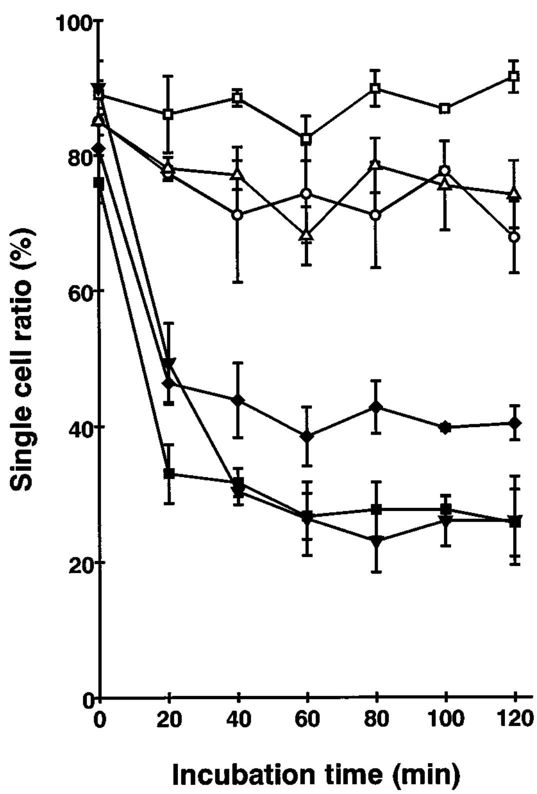

Fig. 5. Enhancement of intercellular homotypic adhesion by overexpression of PLC β1 in LS174T cells. Single cell suspensions of LS174T clones at a concentration of 10^6 cells/ml were incubated at 37°C in an atmosphere of 5% CO_2 and air. The number of single cells was counted at the time points indicated in the figure, and the percentage of total cells remaining as single cells is shown as the single cell ratio. Symbols: □ pMV7 control #3, ○ pMV7 control #4, △ pMV7 control #6, ◆ PLC β1 overexpresser A1, ■ PLC β1 overexpresser A2, ▼ PLC overexpresser C.

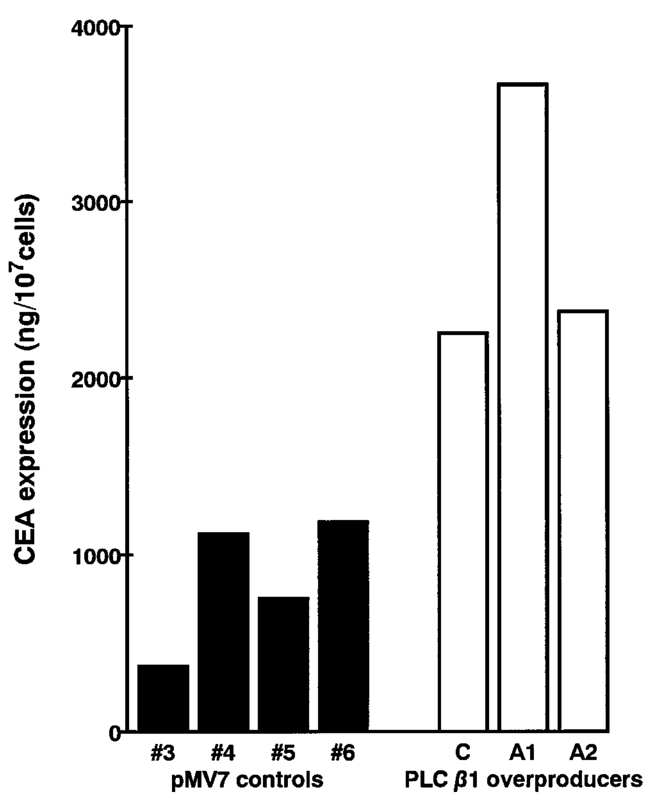

Fig. 6. Increased expression levels of CEA in PLC β1-overproducing cells. LS174T cells were treated with 1% Triton X-114 as described under "Materials and Methods." The assay of extracted CEA was performed by radioimmunoassay.

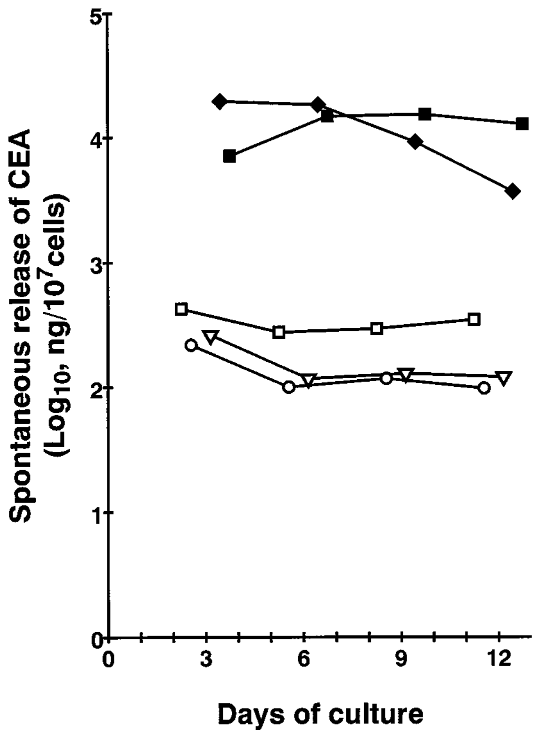

Fig. 7. Enhancement of spontaneous release of CEA by overexpression of PLC β1 in LS174T cells. Trypsinized cells (5×10^4) were incubated for 12 days at 37°C in an atmosphere of 5% CO_2 and air. The amounts of CEA in the culture supernatant were determined by radioimmunoassay. Symbols: □ pMV7 control #3, ○ pMV7 control #4, △ pMV7 control #6, ◆ PLC β1 overexpresser A1, ■ PLC β1 overexpresser A2.

overproducers were maintained at the increased levels during the culture period.

DISCUSSION

The present study provides the first description of the effects of overexpression of the PLC β isoform in a human cell system and demonstrates that overexpression of the β1 isoform of PLC in the LS174T human colon adenocarcinoma cell line tends to inhibit cell growth. Three derivatives of LS174T cells that overexpress PLC β1 displayed marked alterations in morphology, inhibition of growth in liquid culture, and a marked decrease in anchorage-independent growth in soft agar without any stimulation. On the other hand, the parental LS174T cells and four individual control cell lines which had been transduced with the vector plasmid did not display these effects.

The mechanism of the inhibition of cell growth by PLC β1 is not clear. Kalinec *et al*. reported that transfection of a mutated $G\alpha_q$ subunit into NIH 3T3 cells resulted in the transformation of the cells, but also in low colony-forming activity in soft agar medium, and they suggested that the toxicity of the mutated $G\alpha_q$ subunit is a consequence of high levels of intracellular Ca^{2+} induced by increased IP_3.[34] Moreover, recent studies have demonstrated that colon tumors generally display decreased levels of protein kinase enzyme activity[35–38] and diacylglycerol levels[39, 40] and that overexpression of the β1 isoform of protein kinase C in the HT 29 human colon carcinoma cell line results in dramatic inhibition of growth and loss of anchorage-independent growth in soft agar upon exposure to the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA).[41] Overall, it seems likely that enhancement of liberation of both IP_3 and DAG by PLC β1 increases intracellular Ca^{2+} and activates PKC, respectively, leading to inhibition of cancer cell growth.

Our recent study demonstrated that PI-PLC β1 is expressed only weakly at both the mRNA and protein levels in human colon epithelial tissues.[17] In normal colon epithelium or in some primary colon adenocarcinomas tested, no differences in the expression levels of the isoform were observed, and various kinds of human colon adenocarcinoma cell lines tested also expressed only low levels of β1 isoenzyme, suggesting that the isoform plays a minor role in the growth of human colon epithelial cells or the primary colon adenocarcinoma cells. Although we have not confirmed whether the results that we obtained by using LS174T in this study also hold for other human colon adenocarcinoma cell lines, the present study raises the possibility that overexpression or stimulation of PI-PLC β1 may result in the inhibition of the growth of malignant colon tumors. Since stimulation of PI-PLC β1 in LS174T by the exogenous addition of various ligands to G-protein-linked receptor molecules, such as M1 acetylcholine receptor, α1B adrenergic receptor or serotonin 1C receptor, may be possible, we are currently examining the effects of various receptor ligands on the growth of colon cancer cells.

The most important findings in the present study are that PLC β1-overproducing cells acquired a tendency to grow in clusters in liquid medium and showed enhanced ability to form aggregates in suspension culture. There has been a report that aggregation of *Dictyostelium* cells by starvation is due to activation of endogenous PLC-β.[42] On the other hand, Teixeira *et al*. have reported that eosinophils, when activated with inflammatory mediators, including platelet-activating factor (PAF), C5a and leukotriene B_4 (LTB_4), undergo homotypic aggregation and that the C5a and LTB_4 signaling pathways are negatively modulated by PKC, possibly at the level of PLC β.[43] These results suggest that the β isoform of PLC is related to the homotypic aggregation of eukaryote cells.

PLC β1-overproducing cells produce increased amounts of CEA and release a great deal of CEA during *in vitro* culture (Figs. 6 and 7). CEA is a member of a family of cell-surface glycoproteins that are produced in excess in essentially all human colon carcinomas and in a high proportion of many other cancers, such as pancreatic, breast, and non-small-cell lung carcinomas.[44] Malignant colon epithelial cells frequently express 10- to 100-fold higher levels of CEA than their normal counterparts; in addition, the cellular distribution of this molecule is altered and large amounts are secreted into the circulation.[45] Its release by colorectal cancer cells has made serum CEA measurements useful in the management of some patients, but the clinical value of CEA is limited due to the frequent failure of serum concentrations to become elevated until late in the course of colorectal cancer,[46] and it remains uncertain why some patients with colon cancer have normal serum CEA levels despite the presence of CEA in their tumors, as determined histochemically.[47, 48] Serum levels are influenced by several factors, including not only cellular CEA, but also the rate of CEA release. Because CEA is one of the glycoproteins anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) moiety which can be cleaved with PI-PLC,[49, 50] it has been suggested that the activity of endogenous PI-PLCs may represent one of the factors regulating the expression and spontaneous release of CEA. Our present findings provide the first evidence that endogenous activation of PLC results in a marked increase in both the spontaneous release and expression of CEA by cultured human colon cancer cells.

Kitsuki *et al*. have found that there was a significant correlation between the degree of cell aggregation and CEA expression by colorectal carcinoma cells by examining the smears of ascites fluid obtained from 27 patients with colorectal cancer.[51] It has been reported that overexpression of CEA cDNA in murine fibroblasts enhanced the intercellular homotypic aggregation, and that CEA mediates Ca^{2+}-independent, homotypic aggregation of cultured cells of LS180 human colon adenocarcinoma, which is the parent cell line of LS174T.[52] The LS174T cell line expresses CEA and releases a large amount of CEA in liquid culture upon exogenous addition of PI-PLC of bacterial origin.[26, 53] The thick mucous substance surrounding clusters of PLC β1 overproducing LS174T (Fig. 1B) cells could be seen clearly. A possible working hypothesis for the dramatic architectural alterations in the PI-PLC β1-overproducing LS174T cells is that overexpression of the β1 isoform of PI-PLC might cause changes in the CEA-mediated intercellular adhesion via modulation of the GPI anchoring of CEA on the cell membrane. Although our findings seem contrary to the current concept that an increase in CEA expression on tumor cells enhances the metastatic potential,[51, 54] the decrease in the growth rate caused by PI-PLC β1 overexpression may have overcome possible malignant changes induced by the increased expression of CEA molecules on the PI-PLC β1 overproducer clones, so that the net effect is a decrease in the tumorigenicity of the clones in nude mice. In any case, this is the first evidence that overexpression of a specific PI-PLC isoform changes the ability of the cells to undergo intercellular homotypic aggregation. Further studies are needed to examine possible changes in the expression levels of adhesion molecules in PI-PLC β1 overproducers.

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