Overexpression of Phospholipase C-γ1 in Familial Adenomatous Polyposis

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ABSTRACT

Phosphoinositide-specific phospholipase C (PLC) isozymes occupy a central role in the signal transduction system by regulating various cellular processes including proliferation and differentiation. In the present study, we examined the contents of PLCs in colorectal adenomas, carcinomas, and normal mucosa obtained from 4 familial adenomatous polyposis patients to find out whether this enzyme plays any role in the pathogenesis of adenomas and/or carcinomas in familial adenomatous polyposis. Radioimmunoassay and immunoblot analysis revealed that, in contrast to little difference in PLC-β1 and PLC-δ1 content, a considerably higher level of PLC-γ1 was detected in 3 of 4 cases for adenoma and in all cases for carcinoma as compared to normal mucosa. The level of PLC-γ1 expression increased from normal mucosa to adenoma, and finally to carcinoma progressively. Immunohistochemical findings also confirmed this observation. Likewise, activity of PLC-γ1 was considerably higher in adenomas than in normal mucosa. These results suggest that PLC-γ1-mediated signal transduction may play a significant role in the progression of colorectal tumors in patients with familial adenomatous polyposis.

INTRODUCTION

Cells respond to extracellular signals such as hormones, growth factors, neurotransmitters, and other agonists that bind to specific receptors on the external surface of a cell. Receptor occupancy by these substances initiates the production of active second messengers including cyclic AMP and cyclic GMP molecules as well as diacylglycerol and inositol trisphosphates (1).

Phosphoinositide-specific PLC (2) has attracted great attention in the studies on the signal transduction mechanism since it hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate 2 second messenger phosphates, PI,phosphatidylinositol. Much evidence indicates that inositol 1,4,5-trisphosphate releases Ca2+ from intracellular stores resulting in the activation of Ca2+/calmodulin-dependent kinases, and diacylglycerol activates protein kinase C. Both processes are known to be important in many cellular processes including cell differentiation and proliferation (2-4). Purification and molecular cloning of PLC isozymes have revealed that they constitute several families of isozymes (PLC-β1, -γ1, -δ1) which have different regional and cellular expression in most mammalian tissues (5). Furthermore, each isozyme appears to be activated by different receptors through distinct mechanisms. This may account in part for the diversity of responses observed in different tissues and individual cell types to various external stimuli (5, 6). Among many types of PLC isozymes, PLC-γ1 has received the most attention since it was found that the phosphorylation of PLC-γ1 by growth factors, such as platelet-derived growth factor (7), epidermal growth factor (8, 9), fibroblast growth factor (10), and nerve growth factor (11), increased the activity of PLC-γ1 in several cultured cells. These findings suggest that the activation of PLC-γ1 by tyrosine phosphorylation may be one of the important pathways involved in mitogenic signals generated by some growth factors. Also, the findings that overexpression of PLC-γ1 caused a specific enhancement of the inositol phospholipid-dependent second messenger system stimulated by growth factors (12) and induced a growth and oncogenic potential (13) suggest a possible role of this enzyme during carcinogenesis. It has been proposed that PLC enzyme may be present in abnormally high content in cancer cells (14). Furthermore, a recent observation that PLC-γ1 is highly expressed in hyperproliferative epidermal diseases (15) and breast carcinoma (16) obtained from human tissues supports the view that PLC-γ1 may be involved in proliferation and carcinogenesis.

FAP is an autosomal dominantly inherited disease characterized by development of hundreds to thousands of adenomatous polyps. One or more of those polyps inevitably progress to carcinoma if left untreated. FAP is regarded as the best supportive model for the hypothesis of adenomacarcinoma formation sequence that has become widely accepted in the carcinogenesis of a majority of cancers arising in the colon and rectum. Although cytogenetic study of a FAP patient (17) and linkage studies based on large FAP kindreds (18, 19) have localized the gene responsible for FAP to chromosome 5q21-22, a region which is also deleted frequently in sporadic colorectal adenomas and carcinomas (20), and the gene responsible for FAP (APC gene) has been cloned (21), its exact biological function still remains to be clarified.

In the present study, we examined the expression of 3 distinct PLC isozymes (PLC-β1, -γ1, and -δ1) in normal, adenoma, and carcinoma tissues obtained from the same FAP patient to determine whether any one of the PLC isozymes may be involved in the signal transduction system leading to the progression of adenoma and/or carcinoma in FAP.

MATERIALS AND METHODS

Tissue Specimens. Surgical total colectomy specimens of 4 FAP patients were obtained from the Department of Surgery at Seoul National University Hospital, Seoul, Korea. Fresh specimens of normal mucosa, adenomas, and carcinomas were immediately frozen in liquid nitrogen and stored at −80°C. For immunohistochemical investigation, a part of each specimen was formalin-fixed and paraffin-embedded. Parallel samples were processed for histological examination with hematoxylin and eosin staining. The pathological diagnosis was confirmed on respective paraffin-embedded material.

Immunoprecipitation and Immunoblot Analysis. For immunoprecipitation of PLC isozymes, tissues were homogenized in homogenizing buffer [20 mm N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid], pH 7.2, 10% glycerol, 150 mm NaCl, 1 mm Na2VO4, 50 mm NaF, 1% Triton X-100, 10 μg/ml leupeptin, 1 mm phenylmethanesulfonyl fluoride] and centrifuged at 20,000 x g for 30 min. Five mg of the supernatant were treated for 1 h with 30 μl of 20% Pansorbin (Calbiochem, San Diego, CA)-precocated anti-mouse IgG antibody developed in rabbit (Sigma, St. Louis, MO). Precooled homo
genates were incubated with 5 μg of K92-3 plus K32-3 (monoclonal anti-PLC-β1 antibodies), or F7-2 plus D7-3 (monoclonal anti-PLC-γ1 antibodies), or S11-2 plus Z78-5 (monoclonal anti-PLC-δ1 antibodies). After 2 h of incubation in an ice bath, 30 μl of 20% Pansorbin-precocated anti-mouse IgG antibody were added. Immunocomplexes were recovered by centrifugation at 20,000 x g for 30 min. Five mg of the supernatant were treated for 1 h with 30 μl of 20% Pansorbin (Calbiochem, San Diego, CA)-precocated anti-mouse IgG antibody developed in rabbit (Sigma, St. Louis, MO). Precooled homo
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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PLC, phospholipase C; FAP, familial adenomatous polyposis; PI, phosphatidylinositol.
2,000 × g for 5 min, and then washed 5 times with a washing buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate). Immunoprecipitated proteins were released by heating at 95°C for 5 min with Laemmli cooking buffer, and separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane and incubated with their respective monoclonal antibodies for 4 h. Immunoreactive bands were visualized using alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

Double-determinant Tandem Radioimmunoassay. In order to determine the protein level of PLC isozymes (PLC-β1, γ1, and δ1), a double-determinant tandem radioimmunoassay was performed as previously described by Suh et al. (22). All samples were assayed in triplicate.

PLC Activity Assay. For the enzyme activity assay of PLC-γ1, immunoprecipitates were prepared as described above and were washed 3 times with a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylene glycol-bis(β-aminobutyrylamino)-N,N'-tetraacetic acid, and resuspended in 100 μl of the same buffer. The enzyme activity of PLC-γ1 was determined in the 200-μl assay mixture containing 50 μM P1 (20,000 cpm, [3H]P1), 1 mM ethylene glycol-bis(β-aminobutyrylamino)-N,N'-tetraacetic acid, 10 mM CaCl2, 0.1% sodium deoxycholate, and 50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid], pH 6.8. The reaction was performed by incubating with 10 μl of resuspended immunoprecipitate at 37°C for 10 min and terminated as described previously (23).

Immunohistochemistry. The immunohistochemical staining of paraffin sections was carried out using monoclonal antibodies against PLC-γ1. Serial 5-μm sections were cut from the formalin-fixed and paraffin-embedded tissue. After deparaffinization and hydration, sections were incubated for 20 min in 10% normal goat serum to block nonspecific binding protein. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 15 min. Sections were then incubated with monoclonal antibodies (1 μg/ml, mixture of F7-2, D7-3, and B16-5 as described by Sub et al. (22)). After overnight incubation, sections were washed 3 times in phosphate-buffered saline and incubated with avidin-biotin reagents from the Vecta Stain avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). For control experiments, phosphate-buffered saline or anti-PLC-γ1 antibodies preadsorbed with PLC-γ1 protein were substituted for incubation with monoclonal anti-PLC-γ1 antibodies. The immunohistochemical procedures were repeated using the most representative sections to assess the relative intensity of immunoreactivity in normal, adenomas, and cancers and also to ensure reproducibility of the staining distribution. All sections were treated with 3,3-diaminobenzidine as the chromogen and photographed under an Olympus BH 12 light microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

RESULTS

Clinicopathological characteristics of 4 FAP patients are summarized in Table 1. All patients had multiple carcinomas developed from their adenomatous polyps at the time of colectomy. There are 2 patients with well-differentiated and 2 with moderately differentiated carcinomas. Three patients were found to have metastasis in regional lymph nodes of their carcinoma.

The quantitation of PLC isozymes (PLC-β1, γ1, and δ1) in crude homogenates of normal, adenoma, and carcinoma tissues was performed by using double-determinant tandem radioimmunoassay (Fig. 1). Whereas the amounts of PLC-β1 and PLC-δ1 isozymes in adenoma and carcinoma tissues were nearly the same as that in their normal mucosal tissue, the PLC-γ1 showed different levels of expression. As shown in Fig. 1, the amount of PLC-γ1 in adenomas was significantly increased as compared with normal mucosa, with the exception of patient 156, in whom only a slight increase was observed.

Table 1 Summary of the clinicopathological findings of FAP patients with immunohistochemical data

<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>No. of polyps</th>
<th>No. of cancers</th>
<th>Size of cancer (cm)</th>
<th>Node</th>
<th>Differentiation*</th>
<th>IHC</th>
<th>N</th>
<th>A</th>
<th>C</th>
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<td>34</td>
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<td>8000</td>
<td>2</td>
<td>3</td>
<td>1/45</td>
<td>MD</td>
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<td>–</td>
<td>+</td>
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<td>M</td>
<td>1350</td>
<td>2</td>
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<tr>
<td>156</td>
<td>48</td>
<td>M</td>
<td>700</td>
<td>4</td>
<td>15</td>
<td>0/32</td>
<td>WD</td>
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<td>–</td>
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* WD, well differentiated; MD, moderately differentiated; IHC, result of immunohistochemistry; N, normal; A, adenoma; C, carcinoma.

Fig. 1. Quantitation of PLC isozymes by using double determinant tandem radioimmunoassay. The crude homogenates (100 μl, 5 mg/ml) of normal, adenoma, or carcinoma tissues were added to the 96-well microtiter plates coated with anti-PLC-β1, γ1, or δ1 antibody and incubated for 4 h at room temperature. The antibody-PLC complex was purified from HeLa cell extract overexpressing each PLC isozyme by using vaccinia virus expression system. All samples were assayed in duplicate.
When serial sections of adenoma and carcinoma tissues were examined, immunoreactive PLC-γ1 in adenomas showed either a focal and weak staining pattern (Fig. 5A) or a diffuse but weak staining pattern (Fig. 5B). On the other hand, carcinoma tissues consistently demonstrated strong positivity compared to normal mucosa (Fig. 5C). From the multimodality analyses to PLC assessment, PLC-γ1 was elevated in both adenoma and carcinoma of FAP, whereas β1 and β3 were not changed as compared with normal mucosa.

DISCUSSION

The PLC-γ1 is activated when specific tyrosine residues on PLC-γ1 become phosphorylated, and this phosphorylation is achieved by receptor tyrosine kinase for growth factors such as platelet-derived growth factor, epidermal growth factor, and nerve growth factor (7–11, 24). Also similar activation of PLC-γ1 by nonreceptor tyrosine kinase has been described in ligation of membrane IgM in B-lymphocytes (25) and the multicomponent T-cell receptor-CD3 complex in T-cells (26). All of these facts suggest that the phosphorylation of PLC-γ1 is a biologically important event in phosphoinositide metabolism stimulated by growth factors, and perhaps plays an important role in the signal transduction during cell proliferation.

Structural analysis of PLC-γ1 has revealed domains that are related in sequence to noncatalytic regions of the nonreceptor tyrosine kinase of the src family, termed SH2 and SH3, which are not found in other types of PLC isozymes (27, 28). While SH3 domain has been implicated in the interaction with the cytoskeleton, the SH2 domain, which is also found in ras GTPase activator protein, crk oncogene product, and in products of various tyrosine kinase-related oncogenes (6, 28), functions as a recognition site that links components of the mitogenic pathway to activated growth factor receptors in a process which is regulated by phosphorylation/dephosphorylation of specific tyrosine residues within the receptors. Hence, it has been suggested that since the normal physiological role of SH2 domain is probably to target signaling molecules for binding to activated growth factor receptors and other tyrosine phosphorylated proteins, aberrant expression or overexpression of substances containing SH2 domains such as PLC-γ1 may lead to a pathological status such as cancer (29).

In our present study, we examined the amount of 3 distinct PLC isozymes (PLC-β1, -γ1, and -δ1) in adenoma and carcinoma tissues served. In carcinomas, the PLC-γ1 showed an even greater increase than that in adenomas. Fig. 2 revealed that the PLC-γ1 level in carcinomas was substantially higher than that in adenoma and normal tissues (normal, 119 ± 20; adenoma, 186 ± 15; carcinoma, 226 ± 30 ng/mg of protein), suggesting that the level of PLC-γ1 increases as the normal mucosa undergoes progression to adenoma, and finally to carcinoma.

Fig. 3 showed the expression of PLC-γ1 by immunoblot analysis. Whereas PLC-γ1 is not readily detected in normal mucosa, 3 of 4 adenomas and all of the carcinomas show apparent overexpression. This finding is consistent with the observation with radioimmunoassay.

[3H]PI-hydrolyzing enzymatic activity of PLC-γ1 was measured in immunocomplexes precipitated with anti-PLC-γ1 antibody. The activity of PLC-γ1 revealed a stepwise increase from normal to adenoma to carcinoma (Fig. 4), although the difference between adenoma and carcinoma was barely detectable in one case (patient 135).

Immunohistochemical analysis exhibited weak staining in 3 of 4 adenomas, but all carcinomas showed stronger positivity (Table 1). Fig. 3. Detection of PLC-γ1 on immunoprecipitation and immunoblot analysis. Normal, adenoma, or carcinoma tissue samples were homogenized and immunoprecipitated with F7–2. Immune complexes were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. The proteins were identified by immunoblot using F7–2 plus D7–3 as described in “Materials and Methods.” Lanes: N, normal tissue; A, adenoma; C, carcinoma; Arrows, prestained molecular weight standard marker.
there were either no associated carcinomas or the size of the carcinoma was too small. Hence, only 4 patients were eligible for analysis. Although the number of samples assessed is relatively small because of collection limitation, from the multimodality analysis consisting of immunoblotting, radioimmunoassay, immunocytochemistry, and activity assay, it appeared that PLC-γ1 is highly expressed in adenoma and carcinoma as compared with normal mucosa. Therefore, this finding suggests that the overexpression of PLC-γ1 may play an

obtained from FAP patients by using specific antibodies. Even though there were no apparent increases in levels of PLC-β1 and PLC-δ1, a remarkable increase in the PLC-γ1 level was detected in adenomas and carcinomas as compared with normal mucosal tissue. We found that the level of PLC-γ1 increases as the normal mucosa progress through adenoma to carcinoma sequence in FAP. PLC-γ1 activity was also found to be increased in adenomas and carcinomas. In adenomas, however, a noticeable difference of PLC-γ1 expression was detectable in only 3 of 4 cases as compared to normal mucosa. Also, a stepwise increase in PLC-γ1 activity from normal to adenoma to carcinoma tissue was clearly visible in all cases, while in only 2, slight differences exist between adenoma and carcinoma tissues.

Our observations indicate that PLC-γ1 overexpression may contribute to the amplification of PLC-γ1-mediated signaling during the development of adenomas and carcinomas in FAP. However, it is not clear whether overexpression of PLC-γ1 is the result or the cause of the progression to adenomas and carcinomas in FAP.

In a recent study, we reported that significantly higher levels of PLC-γ1 protein were expressed in 15 of 17 sporadic colorectal carcinomas as compared to adjacent normal mucosa (30). This result is consistent with the observation from our present study, indicating that the level of PLC-γ1 is higher in carcinoma than that in normal tissue. Although factors that may influence the level of PLC-γ1 expression still remain obscure, recent reports showed that the majority of human primary breast carcinomas contained considerably higher levels of PLC-γ1 protein as compared with normal breast tissues, and that all carcinomas in which tyrosine phosphorylated and overexpressed PLC-γ1 was present, also had epidermal growth factor receptor or erb B-2 highly expressed (16), suggesting that PLC-γ1 may play an important role in growth factor-stimulated hyperproliferative effect in breast cancer. Such a event may be a universal one in all tumor tissues.

As stated previously, FAP is regarded as the best model for the study of the adenoma-carcinoma sequence in colon and rectum. Our interest in FAP began in 1990 when we established a central registry and organized management program. Since then, 18 patients have undergone surgery at Seoul National University Hospital. The main purpose of this study was to compare the level of PLC-γ1 in normal mucosa, adenoma, and carcinoma tissues from the same FAP patients. However, we had difficulty in obtaining the appropriate samples. In some cases, adenomas were too profuse for us to obtain sufficient amounts of intervening normal mucosal tissues, while in other cases...
important role in the development of adenomas and carcinomas in FAP.

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