J. Biochem. Mol. Biol. Vol. 30, No. 3, pp. 194~199 May 31, 1997

Overexpression of Protein Kinase Cβ_1 Restores Mitogenic Responses of Enterocytic Differentiated Colon Carcinoma Cells to Diacylglycerol and Basic FGF

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(Received February 28, 1997)

Abstract : Previous studies have shown that the HD3 human enterocytic differentiated colon carcinoma cell lines having low PKCβ activity did not respond to diacylglycerol and basic FGF by growth and by activation of pp57 MAP kinase, but undifferentiated cell lines exhibiting high PKCβ activity did. To confirm a role of PKCβ in colonocyte mitogenesis, derivatives of HD3 cell line that stably overexpress a full-length of cDNA encoding the β_1 isoform of human PKC were generated. The abundance and activity of PKCβ in two of the these cell lines, PKC3 and PKC8 were much higher than those in the C1 control cell line that carries the vector lacking the PKCβ_1 cDNA insert. Following exposure to diacylglycerol or basic FGF, proliferation of PKC3 and PKC8 cells increased about 50%; but this effect was not seen with the control C1 cells. Also, in contrast to the control cells, the PKCβ_1-overproducing cells displayed activation of pp57 MAP kinase when treated with diacylglycerol and basic FGF as undifferentiated cell lines did. These results provide direct evidence that PKCβ_1 which plays a key role in mitogenic responses of colon carcinoma cells to diacylglycerol and basic FGF is down-regulated in enterocytic differentiation of colon cells.
Key words : PKCβ, colon carcinoma, differentiation, mitogenesis, MAP kinase

Protein kinase C (PKC) is a calcium- and phospholipid-dependent serine-threonine protein kinase of fundamental importance in signal transduction and growth regulation. A variety of extracellular signals have been shown to activate phospholipid turnover and the formation of diacylglycerol (DAG), which then activates PKC by binding to the regulatory domain of this enzyme (Bell and Burns, 1991). The tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is also a highly potent activator of the enzyme (Castagna *et al.*, 1982). Once activated, PKC can modulate diverse cellular processes, presumably via phosphorylation of specific target proteins. Molecular cloning studies indicate that PKC belongs to a multigene family consisting of at least nine distinct genes (Nishizuka, 1989; Osada *et al.*, 1990; Bacher *et al.*, 1991). Since these isoforms are differentially expressed in different tissues, it seems likely that they perform somewhat different physiological functions.

Sublines of the HT29 colon carcinoma line have been obtained which are permanently committed to begin differentiation when grown to confluency (Hafez *et al.*, 1990). The sublines were isolated by cloning of HT 29 cells in the differentiation inducer hexamethylene bisacetamide (HMBA) for 20 days, then maintaining the lines in log phase growth without HMBA. Differentiation occurred either along the fluid-transporting enterocytic pathway or along the mucin-granule producing goblet cell pathway (Hafez *et al.*, 1990). The cell lines differentiated along enterocytic maturation pathway exhibited columnar polarized morphology with apical surface brush borders, while the undifferentiated cell lines retained multilayered morphology and disorganized, immature brush borders. These defined human colon cell lines could have been used like cell mutants to study the mitogenic signal transduction pathway, which might be altered during cell differentiation.

In previous studies, we have shown that enterocytic differentiated cell lines did not respond to the DAG diolein and basic FGF (bFGF) by growth and activation of pp57 mitogen activated protein (MAP) kinase, while undifferentiated colon carcinoma cell lines exhibited, when treated with DAG or bFGF, increased growth and increased tyrosine phosphorylation and activation of pp57 (Lee *et al.*, 1993a; 1993b). Interestingly, the enterocytic lines exhibited 30% of the total PKC activity and 10-20% of the abundance and activity of PKC β isoform

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that was expressed in the undifferentiated cell lines. Since bFGF rapidly increased the cellular concentration of long-chain DAGs (Pessin *et al.*, 1990; Lee *et al.*, 1993b), the results suggested that loss of mitogenic reponses to DAG and bFGF in the enterocytic differentiated cell lines is due to low cellular levels of PKC β activity.

To confirm this possibility, we generated derivatives of the HD3 enterocytic differentiated colon carcinoma cell line that stably overexpress large amounts of PKCβ1 and analyzed their mitogenic responses to DAG and bFGF. This report shows that overexpression of PKCβ1 in the enterocytic differentiated cells led the cells to respond to DAG and bFGF by growth and by activation of pp57 MAP kinase as the undifferentiated colon carcinoma cells having high PKCβ levels responded.

Materials and Methods

Materials

[γ-^{32}P]ATP and [methyl-^{3}H]thymidine were obtained from Du Pont-New England Nuclear, and ^{125}I-labeled protein A from Amersham-Life Science (Arlington Heights, USA). Cell culture media, lipofectin, neomycin G418, and rabbit anti-peptide antisera specific for PKCβ were purchased from Gibco-BRL (Grand Island, USA). Restriction enzymes and T_4 DNA ligase were obtained from Amersham-Life Science, Goat anti-mouse IgG and protein A-Sepharose from Bio-Rad (Hercules, USA), anti-phosphotyrosine monoclonal antibody PY69 from ICN biochemicals (Costa Mesa, USA), and PVDF transfer membrane from Millipore (Bedford, USA). All other chemicals including phosphatidylserine (PS), basic FGF (bFGF), phorbol 12-myristate 13-acetate (TPA), myelin basic protein (MBP), and histone H1 were purchased from Sigma (St. Louis, USA).

Cell culture

The HD3 colon carcinoma cell line was cloned from the HT29 cell line and maintained in DMEM containing 7% fetal calf serum as described (Hafez *et al.*, 1990).

Transfection experiments

A PKCβ1 expression plasmid, pcDL-SRα-PKCβ1 (Ohno *et al.*, 1987; 1988), was provided by Dr. Shigeo Ohno (School of Medicine, Yokohama City University). An *Eco*RI digested DNA fragment of 2.7 kb size, containing the translated sequence of PKCβ1, was subcloned into the *Eco*R1 site of pcDNA3 (Invitrogen), a selectable mammalian expression vector, down stream of a CMV promoter. A clone having correctly inserted PKCβ1 cDNA into pcDNA3 was confirmed with *Eco*R1/*Pvu*II digested DNA fragment of 0.8 kb size.

The linearlized pcDNA3-PKCβ1 vector (2 μg) with *Pvu*I digestion was introduced into HD3 cells by using lipofectin (Gibco-BRL) as described (Felgner *et al.*, 1987). Forty-eight hours later, the cells were trypsinized and replated in DMEM medium plus 10% FBS with 500 μg of the neomycin derivative G418 per ml. Resistant clones were cloned by ring isolation after 4 weeks of selection.

Selection of PKCβ overexpressers

Western blots were used to identify high PKCβ1 expressing clones, using rabbit anti-PKC peptide antibody as described previously (Lee *et al.*, 1993a). For immunoprecipitations, log phase transfectant cells 2 days after seeding were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 μM leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, and 0.1 μM aprotinin) for 20 min, centrifugated at 35,000×*g* for 30 min, and the supernatants (approximately 0.5 mg of cellular protein) were incubated with 5 μg of rabbit anti-PKCβ peptide antibody for 6 h at 4°C with rocking, then bound to protein A-Sepharose for 2 h, washed 3 times with RIPA buffer, and then once with 10 mM Hepes buffer, pH 7.2. Immune complex *in vitro* kinase reactions were performed as described (Lee *et al.*, 1993a), and reaction products were analyzed by SDS-PAGE and autoradiography. The total PKC activity (membrane and cytosolic) present in cultured transfectant cells was determined after partial purification of cell extract as described (Lee *et al.*, 1993a).

Growth stimulation assays

Transfectant cells were seeded in 24-well multiwell plate at 4×10^4 cells/well in serum-containing DMEM. One day postplating, the medium was aspirated and replaced with 2 ml serum-free DMEM containing *sn*-1,2-diolein micelles freshly prepared by sonication (Lee *et al.*, 1993a) at concentrations of 40 μM, bFGF at 5 ng/ml, or no additives for the controls. After 3 days of exposure to diolein or bFGF, 5 μCi/ml of [^{3}H]-thymidine were added to each well for 3 h of labeling and then aspirated. The cells were washed twice with 2 ml of PBS, then solublized with 1 ml of 1 N NaOH containing 1% SDS, and added directly to 10 ml of scintillation fluid for β-counting.

MAP kinase assay of ERK-like pp57

Antiphosphotyrosine immunoprecipitates with monoclonal antibody PY69 were prepared as described previously (Lee *et al.*, 1993a) with cytosolic fractions of untreated control transfectant cells or PKCβ1 transfectant cells treated for 2 min with 40 μM diolein or 5 ng/ml bFGF and then boiled with Laemmli sample buffer

(Laemmli, 1970) and electrophoresed on an SDS-7.5% polyacrylamide gel containing 0.5 mg/ml MBP. After removal of SDS, denaturation with guanidine-HCl, and renaturation, phosphorylation of MBP within the gel was carried out exactly as described (Lee *et al.*, 1993a).

Results

Generation of HD3 cell lines that stably overproduce PKCβ$_1$

To construct human colon carcinoma cell lines that stably overproduce PKCβ$_1$, a mammalian expression vector pcDNA3 containing the full-length cDNA coding sequence for human PKCβ$_1$ and the selectable marker gene *neo* (Fig. 1) was used to transfect a colon carcinoma enterocytic line HD3, which has been shown to contain very low level of PKCβ when compared with other HT29 colon carcinoma cell sublines (Lee *et al.*, 1993a), by using a cationic liposome, lipofectin. After 48 h, the cells were replaced and grown in medium containing 400 μg of G418 per ml. Individual clones resistant to G418 were isolated and expanded. In a similar fashion, the plasmid pcDNA3 lacking the PKCβ$_1$ cDNA insert was used to generate control cell lines resistant to G418.

The clonal cell lines thus derived were then analyzed for their PKCβ protein amount by immunoblotting using anti-PKCβ antibody as described in Materials and Methods. Several cell lines obtained with the PKCβ$_1$ expression vector displayed increased levels of PKCβ protein when compared with the parental HD3 cells. Two of these clones, PKC3 and PKC8, which exhibited much higher levels of PKCβ protein than clone C1, a control cell line generated with the pcDNA3 vector lacking the PKCβ$_1$ cDNA insert, were selected for further analysis (Fig. 2-A).

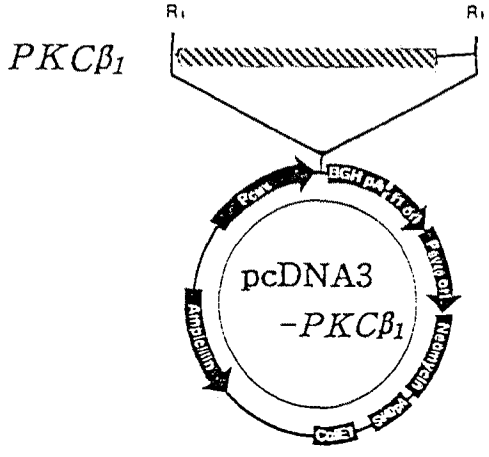

Fig. 1. Structure of the PKCβ$_1$ cDNA expression vector.

PKC activity of PKCβ$_1$-overproducing HD3 cells

Immune complex kinase assays for PKCβ were performed using histone H1 as the substrate to confirm the immunoblotting data. No incorporation of [γ-^{32}P]ATP into histone H1 was observed when the reaction was carried out in the absence of calcium and PS. Little calcium/PS-dependent protein kinase activity was detected in an immune complex of PKCβ from a control transfectant line C1 (Fig. 2-B). However, the histone phosphorylation was increased to a great extent when immune complexes of PKCβ from the PKC3 and PKC8 cells were assayed in the presence of calcium and PS. Therefore, both assays shown in Fig. 2 demonstrated that PKCβ was considerably more abundant in the PKC3 and PKC8 cells than in the control C1.

To compare total PKC activity, PKC was partially purified from the PKC3, PKC8, and C1 cells and assayed for calcium/PS-dependent and -independent protein kinase activity (Table 1). The PKC3 and PKC8 cells exhibited about 2.5- and 3.2-fold increases in the total

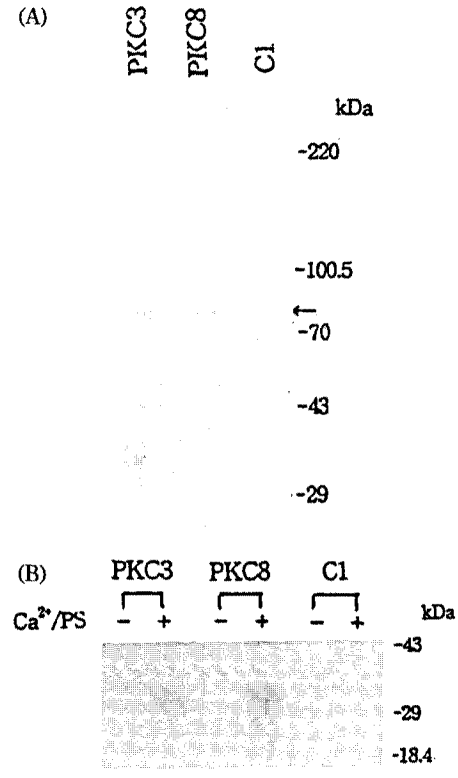

Fig. 2. PKC expression of transfected HD3 colon carcinoma clones. (A) Cell lysates prepared from the indicated PKCβ$_1$ transfectant cell lines were analyzed by immunoblotting using anti-PKCβ antibody as described in *Materials and Methods*. Molecular weight size markers are indicated by *dashes*, and *arrows* indicate the position of PKCβ$_1$. (B) Immune complex kinase reactions for PKCβ were performed using immunoprecipitates from cell lysates of the indicated PKCβ$_1$ transfectant cell lines as described in *Materials and Methods*.

Table 1. PKC activities of derivatives of HD3[a]

Cell line	Ca^{2+}/PS-dependent	Ca^{2+}/PS-independent
	cpm×10^{-5}/*mg protein*/*min*[b]	
C	30.1±0.4	2.2±0.5
PKC3	74.7±1.3	1.8±0.7
PKC8	95.3±0.9	2.4±0.3

[a]Cell extracts were applied to DEAE-cellulose columns, eluted with 0.1 M NaCl, and assayed for calcium- and phosphatidylserine-dependent and independent protein kinase activity on histone 1.
[b]Values are a mean of three determinations.

activity of cellular PKC, respectively when compared with the C1 cells. No differences in calcium/PS-independent protein kinase activities were detected in the cell extracts of any of the lines. This result clearly shows that the overproduction of PKCβ1 in the PKC3 and PKC8 cells contributed to the increase in total PKC activities of those cells.

Mitogenic responses of PKCβ1-overproducing HD3 cells to DAG and bFGF

The growth characteristics of the PKCβ1-overproducing cells were analyzed in the absence and presence of DAG and bFGF. It has been previously shown that DAG and bFGF both induced the proliferation of undifferentiated colon carcinoma cell lines, HP1 and U9H, but not that of HD3 enterocytic differentiated colon carcinoma cell line which has been used in this study for the PKCβ1 transfection (Lee *et al.*, 1993a; 1993b). The previous reports also revealed that the abundance and activity of PKCβ in the HP1 and U9H lines were 5-10 fold higher than those in the HD3. Since bFGF rapidly elevated intracellular DAG levels in the HP1 cells (Lee *et al.*, 1993b), our previous findings raised a possibility that the increase of PKCβ level in the HD3 cells lead the cells to respond to DAG and bFGF by growth. [^{3}H]-Thymidine incorporation assay indicated that the two PKCβ1-overproducing cell lines, PKC3 and PKC8, grew at about the same rate as the C1 control line when grown in the absence of DAG and bFGF (Fig. 3). However, treatment of these HD3 transfectant cells with DAG (40 μM) or bFGF (5 ng/ml) resulted in a marked growth stimulation of the two PKCβ1-overproducing cell lines but had no appreciable effect on the growth of the C1 control cells (Fig. 3). This result demonstrated that the low responses of the enterocytic differentiated colon carcinoma cell lines to DAG and bFGF were in part due to low endogenous PKCβ1 levels in those cells, compared to the undifferentiated colon carcinoma cell variants.

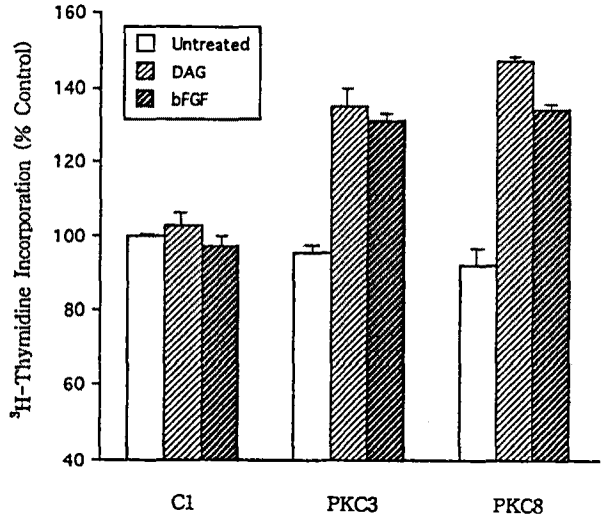

Fig. 3. Mitogenic effects of DAG and bFGF on PKCβ1 transfected clones. One day after seeding a series of plates, the cultures were treated with 40 μM sn-1,2-diolein, 5 ng/ml bFGF, or no additives for the controls. After 3 days of treatment incorporation of [^{3}H]-thymidine was determined as described in *Materials and Methods*. The data are the mean of two experiments. Each bar is the mean of values from 3 wells per experiment, and the standard error is represented by a line on the top of the bar.

MAP kinase activity of ERK-like pp57 in PKCβ1-overproducing HD3 cells

Our previous reports showed that the HD3 cells, when treated with DAG or bFGF, did not exhibit increased tyrosine phosphorylation and MAP kinase activity of ERK-like pp57 (Lee *et al.*, 1993a; 1993b). It seemed that the association between loss of PKCβ activity and blocked signaling through pp57 in HD3 cells results in loss of mitogenic responses to DAG and bFGF. To confirm this reasoning, antiphosphotyrosine immunoprecipitates of cytosolic fractions of PKCβ1-overproducing cells and the control C1 cells, which had been either untreated or treated with DAG or bFGF, were assayed for MAP kinase activity on MBP. As expected, little MAP kinase activity was detected in any of the lines, unless the cells were treated with DAG or bFGF (Fig. 4). MAP kinase activity of the C1 cells was not induced by treatment of cells with DAG or bFGF. However, the PKC3 and PKC8 cells treated with DAG or bFGF exhibited greatly enhanced MAP kinase activity at position of 57 kDa, along with 85 kDa (Fig. 4). The increase in activity of pp57 was due to either increased yield of pp57 in the anti-phosphotyrosine immunoprecipitates from the treated PKCβ1-overproducing cells because of their increased tyrosine phosphorylation or activation of the pp57 MAP kinase because of its increased tyrosine phosphorylation, as occurred with other ERKs (Ander-

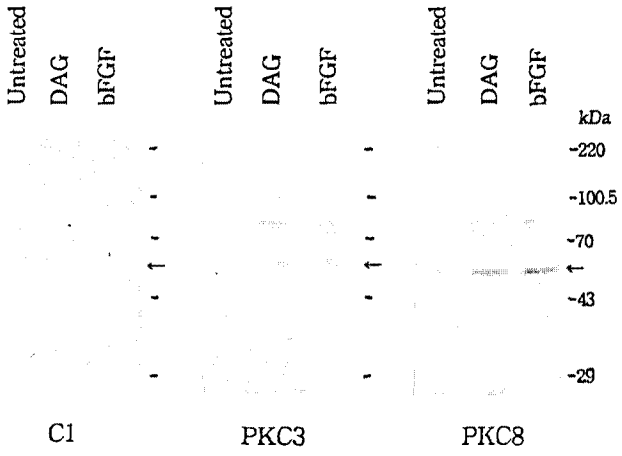

Fig. 4. Kinase activity of pp57 on MBP in the cell lines treated with DAG or bFGF. Antiphosphotyrosine immunoprecipitates of cytosolic fractions of the cells treated with 40 μM *sn*-1,2-diolein or 5 ng/ml bFGF and the untreated cells were electrophoresed on SDS-polyacrylamide gels containing MBP, after denaturation with SDS. Following renaturation, phosphorylation of MBP within the gel was carried out as described in *Materials and Methods*. Molecular weight size markers are indicated by *dashes*, and *arrows* indicate the position of pp57 MAP kinase.

son *et al.*, 1990), or a combination of both. This result clearly indicated that PKCβ$_1$ is part of a signal transduction system activating pp57 MAP kinase, which consequently induces mitogenic responses of colon carcinoma cells to DAG and bFGF.

Discussion

The results of this study confirm validity of our previous suggestion that loss of mitogenic response to DAG and bFGF in the enterocytic differentiated cell lines resulted from down-regulation of PKCβ, during the differentiation of colon carcinoma cells by the long-term treatment of the multipotent HT29 parental line cells with HMBA (Lee *et al.*, 1993a; 1993b). This suggestion was based on the finding that the colon carcinoma cell lines with undifferentiated characteristics contain 5-10 fold higher PKCβ levels than the cell lines exhibiting enterocytic differentiated phenotypes, such as apical surface brush borders. In this study, we demonstrated that overexpression of PKCβ$_1$ in the enterocytic differentiated colon carcinoma cells indeed led the cells to respond to DAG and bFGF (Fig. 3). Choi *et al.* (1990) also reported that PKCβ$_1$ overproducing HT 29 colon carcinoma cells blocked expression of the brush border differentiation marker alkaline phosphatase when treated with a cell differentiation agent, sodium butyrate. Therefore, it is evident that PKCβ$_1$ isoform is down-regulated in colon enterocytic differentiation. It explains the reason why exogenous DAGs did not induce the proliferation of normal colonic cells in primary culture, while benign and malignant colon tumor cells in primary culture replicated in response to exogenous DAGs (Friedman *et al.*, 1989). Thus, all these experiments raise a possibility that the cellular PKCβ$_1$ levels are increased during the transformation of normal colonocytes into colon tumor cells. If this is the case, exposure to fecal DAGs, with levels of 10-100 μM in normal humans' feces, could give a selective growth advantage to undifferentiated colon tumor cells which have up-regulated their PKCβ$_1$ levels. The idea that activation of PKC stimulates cell proliferation and perturbs growth control was also supported by several transfection studies of PKCβ$_1$ or PKCα on rodent fibroblasts (Housey *et al.*, 1988; Persons *et al.*, 1988; Krauss *et al.*, 1989).

The data in our previous reports have shown that acidic and basic FGFs and diolein induce proliferation of undifferentiated colon carcinoma cell lines having high PKCβ by a common mechanism, raising the intracellular levels of long-chain DAGs and then activating PKCβ (Lee *et al.*, 1993a; 1993b). It was also suggested that PKCβ, in turn, activates some member(s) of the protein kinase cascade, which activates a pp57 MAP kinase kinase, which activates pp57 by increasing its phosphorylation on tyrosine and threonine residues. The result of this study showing that pp57 MAP kinase activation by DAG and bFGF occurred only in the PKCβ$_1$-overproducing cells demonstrate that PKCβ$_1$ is a part of the signal transduction system activating pp57 MAP kinase in colon carcinoma mitogenesis (Fig. 4). This result is in good accordance with a finding that TPA did not cause rapid phosphorylation of pp42 ERK or the activation of MAP kinase in a 3T3 variant cell line mitogenically nonresponsive to TPA (L'Allemain *et al.*, 1991). Meanwhile, we have also reported that goblet cells with low levels of c-*src* kinase activity have normal PKC levels including β isoform, yet do not transduce signals from DAG (Lee *et al.*, 1993b). Therefore, it was suggested that *c-src* lies downstream of PKCβ in the MAP kinase signal transduction pathway. Signal transduction by FGF or nerve growth factor in the PC 12 pheochromocytoma cell line has been reported to be mediated by a sequence of *c-src*, then *ras* (Kremer *et al.*, 1991). Other members of the *src* family may also participate in the protein kinase cascades that activate MAP kinase. Purified p56^{lck} indeed tyrosine phosphorylated and activated a purified pp42 MAP kinase from sea star oocytes (Ettehadieh *et al.*, 1992). However, it is now believed that MAP kinase in the general signal transduction system in mammalian cells is activated by MAP kinase kinase (MEK), which is activated by either Raf1 or MAP kinase kinase kinase (MEK ki-

nase) (Lange-Carter *et al.*, 1993; Heystead *et al.*, 1994). It was also reported that PKCα activates Raf1 by direct phosphorylation in NIH3T3 cells (Kolch *et al.*, 1993). Thus, it is very likely that the Raf1 activation by PKCβ1 leads to MEK activation in the protein kinase network mediating the activation of pp57 MAP kinase during the early mitogenic responses of colon carcinoma cells to DAG and bFGF.

Acknowledgements

This study was supported by a research grant from the Korea Science and Engineering Foundation (931-0500-036-2).

The author thanks Dr. Friedman of Memorial Sloan-Kettering Cancer Center for providing HD3 enterocytic differentiated colon carcinoma cell line and Dr. Ohno of Yokohama City University for his generous gift of PKCβ1 expression plasmid, pcDL-SRα-PKCβ1.

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