Growth Inhibition by Phospholipase C Inhibitor Peptides of Colorectal Carcinoma Cells Derived from Familial Adenomatous Polyposis

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Abstract
We reported previously the enhanced phosphoinositide metabolism and constitutive activation of phosphoinositide-specific phospholipase C (PLC) in two colorectal carcinoma cell lines, KMS-4 and KMS-8, derived from familial adenomatous polyposis patients. To study the physiological role of enhanced PLC activity in these cells, we analyzed the effect of PLC inhibitor (PCI) peptides on their growth and cell cycle. N-Myristoylated PCI peptide, myr-PCI(Y), originally developed based on the PCI sequence of PLC-γ2, inhibited activity of purified PLC isoforms in vitro. When myr-PCI(Y) was added to KMS-4 and KMS-8 cultures, it suppressed the production of inositol triphosphate, DNA synthesis, and cell growth, all of which were induced by serum in both KMS-4 and KMS-8 cells. The number of colonies grown in soft agar was also reduced significantly by treating KMS-8 cells with myr-PCI(Y) peptide. Flow cytometry analysis with propidium iodide labeling revealed marked decreases in the percentage of KMS-8 cells in S phase and increases in G0-G1, by the addition of myr-PCI(Y). On the other hand, myr-PCI(F), in which two of the tyrosine residues in myr-PCI(Y) are replaced by phenylalanine and which does not inhibit phosphatidylinositol 4,5-bisphosphate-hydrolyzing activity in vitro, did not significantly inhibit either inositol triphosphate production or cell growth. These results indicate that the activation of PLC is essential for growth and the transformed properties of these colorectal carcinoma cells.

Introduction
External stimuli enhance PIP2 metabolism through the activation of inositol phospholipid-specific PLC, followed by the generation of two second messenger molecules, IP3 and 1,2-diacylglycerol, which regulate intracellular levels of Ca2+ and PKC activity, respectively (1,2). The purification and molecular cloning of PLC isozymes reveal three families of isozymes, PLC-β, -γ, and -δ, with different regional and cellular expression in most mammalian tissues (3). Since each isozyme appears to couple to different receptors through distinct mechanisms, divergent effects can be observed in different tissues and cell types in response to a variety of external signals (4).

The importance of the PLC-mediated pathway in cell growth has been reported: (a) microinjection of a monoclonal antibody specific for IP3 into NIH3T3 cells results in the blocking of DNA synthesis induced by platelet-derived growth factor and bombesin (5); (b) microinjection of PLC-β or PLC-γ initiates DNA synthesis in quiescent NIH3T3 fibroblasts (6), and this effect is neutralized by the microinjection of a mixture of monoclonal antibodies against PLC-β and PLC-γ (7); and (c) histochemical analysis shows the enhanced expression of mRNA and PLC-γ protein levels in breast and colorectal carcinomas (6,9). Our previous study also demonstrated the enhanced expression of PLC-γ and PLC-δ and the constitutive elevation of intracellular IP3 levels in human colorectal carcinoma cell lines, KMS-4 and KMS-8, derived from FAP patients (10). Thus, it is worthwhile to examine whether the PLC signaling pathway is involved in the growth of these cells.

Recent findings demonstrate that PLC-γ possesses a PCI region adjacent to its SH2 and SH3 motifs that strongly suppresses the IP3 hydrolyzing activity of purified PLC isozymes in vitro (11). Synthetic peptides identical to the amino acid sequence of the PCI region (PCI peptides), including the octamer Tyr-Arg-Lys-Met-Arg-Leu-Arg-Tyr, inhibit IP3 hydrolysis induced by each PLC isozyme in vitro (12). On the other hand, it has been reported that the N-acetylation (e.g., myristoylation) of various synthetic peptides enhances their permeability to the plasma membrane, rendering the peptides potentially specific effectors or inhibitors inside intact cells (13,14). Based on the above information, we synthesized two myr-PCI peptides, myr-PCI(Y) and myr-PCI(F), in the latter of which two tyrosines in myr-PCI(Y) are replaced by phenylalanine, and examined their effects on serum-stimulated PLC activity, cell growth, and growth in soft agar using KMS-4 and KMS-8 cells. The results suggest the applicability of myr-PCI peptides to living cells and the

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3 The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PCI, phospholipase C inhibitor; IP3, inositol triphosphate; FAP, familial adenomatous polyposis; myr-PCI, myristoylated-PCI; FBS, fetal bovine serum; BrdUrd, bromodeoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PKC, protein kinase C; PKA, protein kinase A; APC, adenomatous polyposis coli.
important role of the PLC signaling system in the cell growth and the transformed phenotype, such as anchorage-independent growth, of KMS-4 and KMS-8 cells.

Results
PLC Inhibition in Vitro by myr-PCI Peptides. We have demonstrated previously that an octamer, Tyr-Arg-Lys-Met-Arg(Or Lys)-Leu-Arg-Tyr[YRKMR(Or K)LRY] is the minimum sequence required for PLC inhibition (Fig. 1A) and that the two tyrosine residues at the NH₂- and COOH-termini of the sequence are important for inhibition (12). To apply inhibitory peptides to living cells and to examine their inhibitory effect in vivo, we synthesized two peptides, myr-GLYRMRMLRY and myr-GLFMRLRF, designated myr-PCI(Y) and myr-PCI(F), respectively (Fig. 1B). The other myristoylated synthetic peptides used in this study are also listed in Fig. 1B.

We first examined the effect of myristoylation of the NH₂-terminal of PCI peptides on their PLC inhibitory activity in vitro. As shown in Fig. 2A, myr-PCI(Y) strongly suppressed PLC-γ₁-dependent PIP₂ hydrolysis in a concentration-dependent manner. myr-PCI(F) also inhibited PIP₂ hydrolysis, but with about 10 times less potency than myr-PCI(Y). The Kₛ of myr-PCI(Y) and myr-PCI(F) were about 4 and 40 μM, respectively. On the other hand, the Kₛ of nonmyristoylated PCI(Y) and PCI(F) were about 120 μM and more than 1000 μM, respectively (12). These results suggest that the myristoylation of the NH₂-terminal of PCI peptides potentiates their inhibition of PLC activity.

myr-PCI(Y) inhibited not only the PIP₂ hydrolysis induced by PLC-γ₁ but also that induced by other PLC isoforms, with the Kₛ of 15 μM for PLC-β₁ and 18 μM for PLC-δ₁ (Fig. 2B). In addition, myr-PCI(Y) inhibited the hydrolysis of PIP and PI induced by PLC isoforms (data not shown). On the other hand, other myristoylated peptides, such as myr-ΨPKA, myr-ΨPKC, which were already identified as exogenous PKC and PKA inhibitor peptides, respectively, and myristoyl glycerine showed no inhibitory activity on PIP₂ hydrolysis (data not shown). These results are consistent with the previous observations on nonmyristoylated PCI peptides (12).

Inhibition of FBS-induced IP₃ Formation by myr-PCI Peptides. We next examined the inhibitory effect of myr-PCI peptides on FBS-stimulated IP₃ production in vivo. KMS-8 cells precultured in serum-free medium for 2 days in the presence or absence of myristoylated peptides for the last 1 h were stimulated with FBS (5%, v/v) for 30 s, and IP₃ contents were measured. As shown in Fig. 3A, 5% FBS produced a stimulation in the production of IP₃ in KMS-8 cells from the basal level of 6 pmol/well to a maximum of 24 pmol/well at 30–40 s; the level then declined to the basal level after 3 min. myr-PCI(Y) at 10 μM strongly inhibited the production at all time points examined, while myr-PCI(F) at 10 μM had a small inhibitory effect on the production of IP₃ (Fig. 3A). The inhibition by myr-PCI(Y) was dose dependent (Fig. 3B), with an IC₅₀ for inhibition of about 40 nM. myr-PCI(F) also inhibited FBS-induced IP₃ production in a dose-dependent manner, but its inhibitory effect was about 500 times weaker than that of myr-PCI(Y). The other myristoylated pseudosubstrate peptides, myr-ΨPKC and myr-ΨPKA, did not inhibit the FBS-dependent production of IP₃ at any concentration used (Fig. 3B).

Similar results were obtained in experiments using KMS-4 and CCD841CoN cells (Table 1). IP₃ production after FBS stimulation was suppressed significantly in these cells by
pretreatment with 10 μM PCI(Y), but not in such an evident way by myr-PCI(F) (Table 1).

Inhibition of FBS-stimulated DNA Synthesis by myr-PCI Peptides. We next examined the effect of myr-PCI peptides on DNA synthesis in KMS-4 and KMS-8 cells. Logarithmically growing cells were maintained in serum-free medium in the presence of either myr-PCI(Y) or myr-PCI(F) for 2 days. The cells were then exposed to FBS, and DNA synthesis stimulation was assessed by measuring the incorporation of [3H]thymidine into the nuclear fraction. As shown in Fig. 4, the incorporation of [3H]thymidine to both KMS-4 and KMS-8 DNA was strongly inhibited in a dose-dependent manner by myr-PCI(Y), with an IC₅₀ of 0.2 μM. On the other hand, myr-PCI(F), up to 10 μM, had no significant effect on DNA synthesis, and about 20% inhibition was observed at higher concentrations (Fig. 4). When another cell proliferation assay, the incorporation of BrdUrd into the nuclear fraction, was used to assess growth, similar results were obtained (data not shown).

On the other hand, DNA synthesis in CCD841CoN cells derived from normal colon mucosa was slightly inhibited by high concentrations of myr-PCI(Y) (Fig. 4); 50 μM myr-PCI(Y) produced about 30% inhibition, and 50 μM myr-PCI(F) produced about 15% inhibition.

Inhibitory Effect of myr-PCI Peptides on Cell Growth. Since the inhibition of DNA synthesis suggests a loss of growth response to the stimuli, the effect of myr-PCI peptides on FBS-stimulated growth was examined by a colori-
Growth were as follows: addition (0, absence with cause CCD841CON FBS-stimulated) Results FBS. shown cells. InhIbItIon. lCscs Inhibitory effect of myr-PCI peptides on cell growth. Cells growing logarithmically in 96-well plates were maintained for 2 days in serum-free DMEM in the presence or absence of different concentrations of myr-PCI(Y) (●, ○) or myr-PCI(F) (□, △) peptides. Cells were stimulated with DMEM containing 5% FBS for 24 h, and the MTT assay was performed to determine cell growth. Results are expressed as percentages of control, which were determined as follows: ([(the average absorbance at 570 nm in myr-PCI-treated and 5% FBS-stimulated) – (the average absorbance in serum-free medium)] / ((the average absorbance in serum-free medium)]) × 100. Cells used were KMS-8 (●, ○), KMS-4 (■, □), and CCD841CoN (▲, △). Bars, SD (n = 5).

Fig. 6. Effect of preincubation time with myr-PCI peptides. Cells growing logarithmically were maintained for various times as indicated in serum-free DMEM medium containing 10 µM myr-PCI(Y) (●, ○) or myr-PCI(F) (□). Cells were stimulated with DMEM supplemented with 5% FBS at time 0. After 24 h, cell growth was assessed by MTT assay. Results are expressed as percentages of control as described in the legend to Fig. 5. Cells treated with myr-PCI(Y) for 7 days with serum-free medium changed every day are indicated (■). Bars, SD (n = 3).

metric assay using MTT. Logarithmically growing cells were pretreated with either myr-PCI(Y) or myr-PCI(F) in serum-free medium for 2 days and then stimulated for growth with 5% (v/v) FBS. As shown in Fig. 5, pretreatment of KMS-8 cells with myr-PCI(Y) inhibited cell growth in a dose-dependent manner, with an IC₅₀ of about 0.2 µM. The inhibitory effect of myr-PCI(Y) was not derived from its cytotoxic effect, because the number of living cells did not change significantly, when cells were maintained for 7 days in serum-free medium containing 10 µM myr-PCI(Y) with a medium change everyday (Fig. 6). On the other hand, myr-PCI(F) did not inhibit the FBS-stimulated growth of KMS-8 cells as myr-PCI(Y) did; only 10% inhibition was observed at 20 µM myr-PCI(F). Similar results were obtained in experiments using KMS-4 cells.

We also tested the effect of PCI peptides on FBS-dependent growth of various colorectal carcinoma cell lines as well as CCD841CoN cells derived from normal mucosa. Myr-PCI(Y) inhibited the growth of DLD-1 and Colo205 cells with IC₅₀ of 20 and 30 µM, respectively (data not shown), an effect less potent than that observed in KMS-4 and KMS-8 cells. A significant effect was not observed in CCD841CoN cells (Fig. 5), consistent with the result from DNA synthesis.

Requirement of Long-Term Preincubation for Growth Inhibition. The effect of the length of pretreatment with myr-PCI(Y) on the inhibition of cell growth was examined. As shown in Fig. 6, an inhibitory effect of myr-PCI(Y) was apparent, when cells were pretreated for between 12 h and 3 days. When cells were cultured in fresh medium containing 10 µM myr-PCI(Y) and 5% FBS for 7 days with a medium change every day, the growth-inhibitory effect was most evident. On the other hand, the growth of KMS-8 cells was not inhibited by pretreatment with myr-PCI(F) at any concentration or at any pretreatment time tested.

Inhibitory Effect of myr-PCI Peptides on Anchorage-Independent Growth. Anchorage-independent growth to form colonies in soft agar is considered to be a typical phenotype of cultured malignant cells. We examined the effect of myr-PCI peptides on this malignant property by
characterizing the ability to grow in soft agar, which is also considered to be representative of tumor-forming ability. Results from the soft agar assay are shown in Table 2. KMS-8 cells were found to form colonies in soft agar. The ability to form colonies after the treatment with myr-PCI(Y) was reduced to 25% at 2 μM and 3.5% at 20 μM. On the contrary, when KMS-8 cells were treated with myr-PCI(F) at 20 μM, colony-forming ability was not suppressed significantly. The inhibition of KMS-8 cells by the treatment with myr-PCI(Y) to represent anchorage-independent growth suggested that their malignant property had been suppressed.

Inhibitory Effect of myr-PCI on the Progression of the Cell Cycle from G2/M to S Phase. The cell cycle progression of KMS-8 cells exposed to myr-PCI(Y) peptide was analyzed by flow cytometric analysis with propidium iodide staining of DNA. DNA histogram analysis indicated that three distinct cell populations, corresponding to G0/G1, S, and G2/M phases, were observed in untreated cells at 16 h after stimulation by 5% FBS (Fig. 7A). On the contrary, pretreatment of KMS-8 cells with 10 μM myr-PCI(Y) for 48 h induced marked and reproducible decreases in the percentage of cells in the S phase and increases in the G0/G1 phase of the cell cycle (Fig. 7B). The time dependence of the myr-PCI(Y) effect on cell cycle progression was studied by changing the length of pretreatment with myr-PCI(Y) for 6, 24, 48, and 72 h. The inhibition of cell cycle progression was observed at 24 h, and the peak effect of this inhibition was observed by pretreating KMS-8 cells for 48 h, with the percentage of cells in the G0/G1 phase increasing and the percentage of cells in the S phase decreasing (data not shown). In contrast to myr-PCI(Y)-treated KMS-8 cells, G0/G1, S, and G2/M populations displayed no significant difference among control CDB41CoN cells or 10 μM myr-PCI(F)-treated cells.

Discussion
In this study, we used myristoylated PCI peptides to examine the role of the PLC signaling system in the cell growth of colorectal carcinoma cells including two cell lines, KMS-4 and KMS-8, derived from FAP patients. When KMS-4 and KMS-8 cells were treated with myr-PCI(Y) at concentrations up to 10 μM, a remarkable inhibition of FBS-stimulated IP3 production was observed, whereas myr-PCI(F) caused little inhibition. The IC50s of myr-PCI(Y) and myr-PCI(F) for FBS-induced IP3 production in KMS-8 cells were 40 nM and 20 μM, respectively, consistent with their Ks, measured in vitro against purified PLC-γ1, of 2 and 80 μM, respectively. The inhibitory effect of myr-PCI(Y) does not seem to be specific for the FAP-derived cell lines. Similar results were obtained using other cells, including Swiss 3T3 fibroblasts, in which myr-PCI(Y), but not myr-PCI(F), strongly inhibited IP3 production induced by platelet-derived growth factor, epidermal growth factor, bombesin, and FBS.4 PCI peptides lacking myristate had no inhibitory effect in nonpermeabilized cells in vivo. These results indicate that NH2-terminal myristoylation allows peptides to inhibit endogenous PLC in intact cells and that myr-PCI(Y) can be used as a specific inhibitor of PLC.

myr-PCI(Y) did not inhibit the incorporation of [3H]thymidine into the nuclear fraction or cell growth in CDB41CoN cells. This was not due to a difference between CDB41CoN and FAP-derived cells in their permeability to myr-PCI(Y), since myr-PCI(Y) inhibited FBS-induced IP3 production in CDB41CoN cells in the same manner as observed in KMS-8 cells. The steady-state level of IP3 may explain the difference. There was 30–34 pmol IP3/106 KMS-8 cells (i.e., constitutive activation of PLC), significantly higher than in CDB41CoN cells, where the level was less than 15 pmol/106 cells (10). The results presented here suggest an important contribution of the PLC signaling systems to the growth of KMS-4 and KMS-8 cells. Analysis of PLC signaling systems in other cell lines whose growth is sensitive to myr-PCI(Y) may provide more clues to understanding the mechanism of myr-PCI(Y)-sensitive cell growth.

The effect of myr-PCI(Y) peptides was dependent on pretreatment time. It is of interest that cells should be pretreated with myr-PCI(Y) at least for 12 h prior to FBS stimulation to observe an inhibition of cell growth. The growth of cells pretreated with myr-PCI(Y) for 2 h was not inhibited significantly (data not shown). However, pretreatment with the peptide for only 10 min was enough to suppress the IP3 production induced by FBS (data not shown). This discrepancy can be explained by a mechanism that requires persistent PLC inhibition for the suppression of growth in KMS-8 cells and may correlate with the degradation of myr-PCI peptides by cellular proteases.

Pretreatment with myr-PCI(Y), but not with myr-PCI(F), inhibited the DNA synthesis, growth, and anchorage-independent growth of KMS-4 and KMS-8 cells, in which the constitutive activation of endogenous PLC isoforms could be observed (10). To test the possibility that this effect of myr-PCI(Y) on cell proliferation derives from its cytotoxic effect, cells were cultured in the presence of myr-PCI(Y) for an extended time. However, even when cells were pretreated with myr-PCI(Y) for 7 days, no significant differences in cell numbers between control and myr-PCI(Y)-treated cells could be detected. In addition, neither morphological changes under microscopy at any dose of myr-PCI(Y) tested nor changes in the mitogenic response to FBS stimulation could be observed (data not shown). These results suggest that the effect of myr-PCI(Y) peptides derives from the inhibition of endogenous PLC activity, not from cytotoxicity. The inhibitory effect did not seem to be derived from cellular apoptosis.

Table 2  Soft agar colony-forming assays for myr-PCI-treated KMS-8 cells

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Colony no.*</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55.3 ± 8.7</td>
<td>100</td>
</tr>
<tr>
<td>myr-PCI(Y), 2 μM</td>
<td>13.6 ± 3.5</td>
<td>25</td>
</tr>
<tr>
<td>myr-PCI(Y), 20 μM</td>
<td>2.7 ± 1.5</td>
<td>3.5</td>
</tr>
<tr>
<td>myr-PCI(F), 20 μM</td>
<td>47.7 ± 11.1</td>
<td>86</td>
</tr>
</tbody>
</table>

* Cells (5 x 104) were plated in 0.33% soft agar in growth medium in the presence or absence of myr-peptides as indicated. Data represent the means (±SD) of quadruplicate determinations.

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either, since laddering of nuclear DNA was not observed after the treatment with myr-PCI(y) or myr-PCI(f) (data not shown).

The cell cycle analysis demonstrated that myr-PCI(y) seemed to induce a specific cell cycle block at G0/G1 in KMS-8 cells. myr-PCI(y) was most effective in reducing the proliferative response when added 48 h before FBS stimulation. The reason for such a long pretreatment may be derived from the difficulty of KMS-8 cells to grow synchronously, even with serum deprivation. These results raise the possibility that myr-PCI(y) modulates the endogenous machinery regulating cell cycle progression. PLC might be an important candidate for this role, probably as an upstream signal transducer located in the cytoplasmic phase of the plasma membranes. Support for such a hypothesis will be provided by the ability of myr-PCI peptides to inhibit downstream signals, such as cyclins, cyclin-dependent kinases, or retinoblastoma gene product (15,16). Further examination may clarify this hypothesis.

FAP is a common autosomal-dominant malignant disease in humans. The APC gene responsible for FAP on chromosome 5q21 has been cloned by positional cloning (17,18). Although most mutations of APC, both germline and somatic, result in the truncation of gene products (19,20), its functional role in cellular malignancy has not yet been clarified. APC has been predicted to encode a coiled-coil protein that might interact in vivo with itself or with other cytoskeletal proteins such as catenins (21–23). The contribution of APC to tumorigenesis in FAP and to the development of sporadic colorectal cancers remains obscure. Genetic studies on a mutant mouse (Min) strain indicated that the secretory phospholipase A2 act as a novel modifier of intestinal polyp number (24). Park et al. (9) reported higher levels of PLC-y1 protein and activity in colorectal adenomas and carcinomas than in normal mucosa obtained from four FAP patients. Considered with our previous findings (10), these results suggest that lipid metabolism, including PLC-mediated signal transduction, may play a significant role in the development of adenomas and carcinomas in FAP patients.

In summary, we demonstrate that myr-PCI(y) can be applied to living cells to regulate endogenous PLC activity. Using these myr-PCI peptides, we show that the inhibition of DNA synthesis by pretreatment with myr-PCI(y) may be caused by the inhibition of PLC-mediated PIP2 breakdown and that the PLC signaling system may be important for the growth of KMS-4 and KMS-8 cells but not for the growth of normal CCD841CoN colon cells. The results suggest the applicability of specific PLC inhibitors as antitumor drugs. In this context, protease-resistant myr-PCI peptides, such as o-amino acid derivatives, should be tested for their inhibitory effect using other tumor cells with activated PLC and tumor-bearing animals.

Materials and Methods

Materials. PIP2 (bovine brain) and phosphatidyethanolamine (soybean) were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]PIP2, [3H]thymidine, and the IP3 assay system were obtained from DuPont-New England Nuclear (Wilmington, DE). The cell proliferation assay system (BroUrd assay) was from Amersham International (Buckinghamshire, England). MTT assay for cell proliferation was from Chemicon International, Inc. (Temecula, CA).

Peptides. Peptides were prepared on an automatic peptide synthesizer (Applied Biosystems; Perkin-Elmer, Foster, CA) using t-butoxycarbonyl-protected amino acids, including myristoyl glycine, and purified on a C18 reverse-phase column by high performance liquid chromatography.
The synthetic peptides used in this study were myr-PCI(Y), NH₂-terminal myr-PCI, myr-PCII(Y), two of the tyrosine residues in myr-PCI(Y) were replaced by phenylalanine, myr-γPKC, myristoylated pseudosubstrate peptide of PKCδ, and myr-γPKA, myristoylated pseudosubstrate peptide of PKA (Refs. 13 and 14). (Fig. 1).

**Cell Culture.** KMS-4 and KMS-8 were colorectal carcinoma cells derived from patients with FAP and were grown in RPMI 1640 containing 10% (v/v) FBS (25). CDD841CoN cells were established from normal human fetal colonic mucosa and show epithelial-like morphology (26). CDD841CoN cells were grown in DMEM containing 10% FBS. These cells were cultured in a humidified 5% CO₂ incubator at 37°C.

**PLC Assay.** A conventional in vitro PLC assay was carried out as described (10).

**IP₃ Assay.** Cells (1 x 10⁵) were plated onto 6-well culture plates, and logarithmically growing cells were starved for 48 h in serum-free medium consisting of DMEM containing 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 1 mg/ml bovine serum albumin. Prior to stimulation, cells were pretreated with or without synthetic peptides for 1 h in the presence of 10 μm LCI during the last 10 min. The cells were then stimulated for 30 s with 5% (v/v) FBS and stopped by the addition of ice-cold perchloric acid at a final concentration of 4%. IP₃ was extracted and assayed according to the manufacturer's directions.

**Cell Growth Assay.** Cells (1 x 10⁶) were seeded into 96-well plates, and the medium was replaced with serum-free medium after the cells reached semiconfluence (about 4 x 10⁵ cells/well). Cells were starved for 48 h in serum-free medium with or without myr-PCI peptides. The cells were then stimulated with 5% (v/v) FBS. To determine the incorporation of [³H]thymidine into DNA, 37 kBq of [³H]thymidine were added to each well 3 h after stimulation and incubated for an additional 18 h. [³H]Thymidine incorporated into DNA was recovered on glass filters as trichloroacetic acid-insoluble materials. The radioactivity was measured in a liquid scintillation counter.

For the incorporation of BrdUrd into nuclear DNA, an aliquot of BrdUrd solution was added to each well 16 h after stimulation with 5% (v/v) FBS, and the cells were incubated for an additional 2 h. The cells were washed with PBS and fixed in 60% (v/v) methanol. BrdUrd incorporated into the nucleus was detected according to the manufacturer's directions. As another cell proliferation assay, a colorimetric assay was also performed using MTT (27). An aliquot of MTT solution was added to each well 24 h after stimulation, and the cultures were incubated for an additional 2–4 h. The number of living cells was analyzed by determining the formazane product by measuring the absorbance at 570 nm.

**Scr Agar Colony-Forming Assay.** KMS-8 cells (5 x 10³) from a single-cell suspension by trypsinization were mixed in 3 ml of 0.33% Noble agar (Difco Laboratory, Detroit, MI), which was supplemented with complete growth medium in the presence of 10 μM of either myr-PCI(Y) or myr-PCII(Y). Cell suspensions were overlayed on 5 ml of a base medium containing 0.5% agar in 60-mm dishes. The plates were set at room temperature until the agar was solidified and were subsequently incubated at 37°C and 5% CO₂ for 18–21 days. Visible colonies were counted. Each assay was performed in duplicate and three times.

**Cell Cycle Analysis by Flow Cytometry.** KMS-8 cells (2 x 10⁵) on 100-mm dishes cultured in serum-free medium in the presence of 10 μM myr-PCI(Y) or myr-PCII(Y) cell suspensions were overlayed on 5 ml of a base medium containing 0.5% agar in 60-mm dishes. The plates were set at room temperature until the agar was solidified and were subsequently incubated at 37°C and 5% CO₂ for 18–21 days. Visible colonies were counted. Each assay was performed in duplicate and three times.

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**References**


