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TRANSCRIPTIONAL REGULATION OF PHOSPHOLIPASE C-γ1 GENE DURING MUSCLE DIFFERENTIATION

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SUMMARY : Phospholipase C (PLC) has been known to be a key effector protein in signal transduction pathway for cell proliferation and differentiation. Here, we report the transcriptional regulation of PLC-γ1 during myogenic differentiation. Both PLC-γ1 mRNA and protein levels were increased during myogenesis. The activity of PLC-γ1 was also comparable to the amount estimated by Western blot. Comparing promoter activities of various PLC-γ1 gene upstream regions in myoblast and myotube, the responsible region was mapped between -551 and -480. From these results, we conclude that the transcription of PLC-γ1 is increased by the control of GPE1 region of PLC-γ1 gene during myogenesis.

Many cellular responses are elicited by extracellular signals through activation of phospholipase C (PLC) which generates two second messengers, inositol-1,4,5trisphosphate and diacylglycerol (1, 2). Many PLCs have been cloned from various tissues and species. According to the primary structures, PLCs are divided into three types β, γ, and δ, each of

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The abbreviations used are: PLC-γ1, phospholipase C-γ1; GPE1, PLC-γ1 gene positive element1; SH, *src* homology; CAT, chloramphenicol acetyltransferase; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; GES, PLC-γ1 expression stimulator.

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which consists of several subtypes (3, 4). Among them, PLC-γ1 has so-called SH2 and SH3 domains through which PLC-γ1 interacts with various cellular proteins. PLC-γ1 is phosphorylated and activated by growth factor receptors, such as epidermal growth factor receptor (5), platelet-derived growth factor receptor (6), and fibroblast growth factor receptor (7). However, its role in mitogenic signal transduction is still obscure.

During the development of rat nervous system, PLC-γ1 expression is dramatically changed in several regions (8). The amount of PLC-γ1 mRNA and protein in rat whole brain is significantly decreased after birth[1]. Additionally, changes of PLC-γ1 expression were observed during development of rat aorta (9). These observations, that the expression of PLC-γ1 is regulated during animal developmant, imply the involvement of PLC-γ1 in cellular differentiation. However, the mechanism by which the expression of PLC-γ1 is regulated during differentiation is unknown.

Recently, we have cloned and characterized the promoter region of PLC-γ1 gene to understand the transcriptional control mechanism of PLC-γ1 gene during cellular transformation or differentiation (10). In this study, we examined whether the expression of PLC-γ1 is modulated by myogenic differentiation. PLC-γ1 is increased during multinucleated myotube formation through transcriptional activation. The responsible element is located in -551/-480 of PLC-γ1 gene.

MATERIALS AND METHODS

Cell culture conditions: C2C12 cells (mouse myoblast cells) were maintained at low density to avoid fusion in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 15% fetal bovine serum (HyClone Lab., Inc., Logan, UT). To promote differentiation of C2C12 cells to multinucleated myotubes, the medium was replaced with DMEM supplemented with 2% horse serum (HyClone Lab., Inc.) and after 3-4 days, differentiated cells were obtained (11). Cells were maintained in a humidified incubator at 37°C in 5% CO_2.

DNA transfection and CAT assay: Reporter CAT plasmids (10) were transfected into C2C12 cells by the calcium phosphate coprecipitation method (12). Ten μg of plasmid DNA was cotransfected with 3 μg of pCH110 (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden), a β-galactosidase expression vector which was used as an internal control to normalize for transfection efficiency. After exposure to the DNA precipitate for 12-16 hr, cells were washed and fresh media was then added. The cells were harvested 48 hr later and CAT activities were measured in the cell lysates (13). To measure transcriptional activities in differentiated myotubes, transfected C2C12 cells were maintained in differentiation media and harvested 3 days later. One tenth of cell lysate was used to

[1]Suh, P.-G. *unpublished result.*

determine β-galactosidase activity. For each CAT reaction, cell lysate was combined with 4 μl of [^{14}C]chloramphenicol (Amersham Corp., Buckinghamshire, United Kingdom, 55mCi/mmol, 25μCi/ml), 10 μl of 20 mg/ml acetyl-coenzyme A, and 250 mM Tris, pH 7.8, to final volume of 150 μl. The mixtures were incubated at 37°C for 2 hr, and the reactions were stopped by extraction with 1 ml of ethylacetate. Acetylated and nonacetylated chloramphenicol were separated by thin-layer chromatography.

RNA extraction and Northern blot analysis: Total RNAs were extracted from C2C12 cells and differentiated myotubes as described by Chomczynski and Sacchi (14). Ten μg of RNA was seperated on 1% agarose gel containing 1 M formaldehyde and transferred onto nylon membrane (Amersham, Buckinghamshire, UK). RNAs were crosslinked with nylon filter by UV illumination. Rat PLC-γ1 cDNA was labeled with ^{32}P by random primed labeling method (kit from Amersham) and used as a probe. Hybridization was performed in the solution containing 1% bovine serum albumin, 7% SDS, 0.5 M NaH_2PO_4, pH 7.0, 1 mM EDTA, and 2×10^6 cpm/ml ^{32}P-labeled probe at 65°C overnight as described by Church and Gilbert (15). The membrane was, then, washed in 0.1% SDS and 1 x SSC at 70°C for 1 hour, and visualized by autoradiography.

Immunoprecipitation, Western blot analysis, and PLC activity assay: For immunoprecipitation, 5 μg of monoclonal antibody to PLC-γ1 (16) was added to 1 mg of cell lysates along with 30 μl of 20% solution of formalin-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem, San Diego, CA) precoated with rabbit anti-mouse Ig (Sigma, St. Louis, MO), and incubated for 2 hr on ice. The immunecomplex was washed extensively and used in Western blot analysis and PLC activity assay as described by Suh *et al.* (16) and Ryu *et al.* (17), respectively.

RESULTS

In order to examine whether PLC-γ1 expression is regulated during differentiation, RNAs were isolated from C2C12 myoblasts and differentiated myotubes and were analyzed by Northern blotting with rat PLC-γ1 cDNA as a probe. As shown in Fig 1, the expression of PLC-γ1 RNA in differentiated myotubes was 2.5 times the expression of PLC-γ1 RNA in myoblasts, while the amounts of loaded RNAs were the same. The increase was measured by densitometer. When PLC-γ1 was immunoprecipitated from undifferentiated and differentiated C2C12 cells and subjected to Western blotting with anti-PLC-γ1 monoclonal antibody, the differentiated cells have twice as much PLC-γ1 as the undifferentiated cells. Enzyme activity of PLC-γ1 was measured from the same immunoprecipitate used in Western blotting. The activity of PLC-γ1 was comparable to the amount estimated by Western blot analysis. Since the extents of the increases in RNA and protein levels were similar, it is likely that the upregulation of PLC-γ1 during myogenesis is controlled at transcription level.

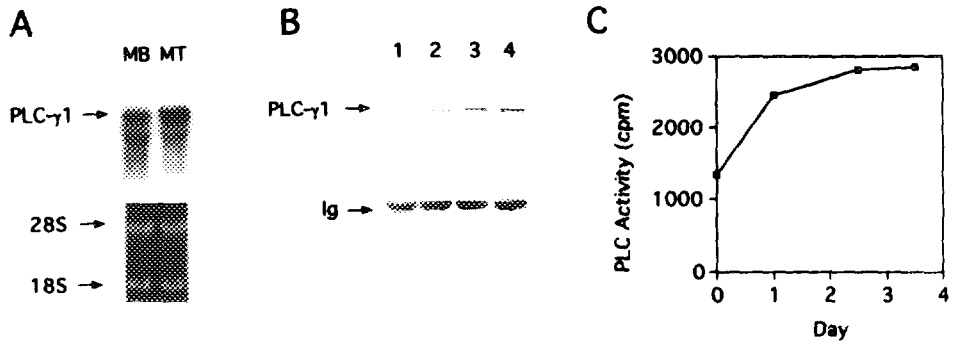

Fig. 1. **Change of PLC-γ1 expression during muscle differentiation of C2C12 cells.** (A) Northern blot analysis of PLC-γ1 in myoblast and myotube. MB and MT indicate myoblast and myotube, respectively. The total RNA of myotube was isolated from C2C12 cells which were differentiated in the medium containing 2% horse serum for 3 days. (B) Western blot analysis and (C) PLC-γ1 activity assay. After C2C12 cells were removed to differentiation medium, the cells were harvested at the indicated days. Extracted proteins were immunoprecipitated with anti-PLC-γ1 monoclonal antibody and immunoblotted with the same antibody (B). Ig indicates immunoglobulin. Each lane indicates the proteins from various stages (lane 1, undifferentiated myoblast; lane 2, 1 day; lane 3, 2.5 days; lane 4, 3.5 days). A part of the immunoprecipitated proteins were used in PLC activity assays (C). These results were representatives of several independent experiments.

In order to identify the transcriptional regulatory sequence which is responsible for the stimulation, transcription activities of deleted 5'-fragments were measured in C2C12 myoblasts and myotubes (Fig. 2). Since it is not easy to compare the activities of a reporter plasmid directly in two different cells, we compared the relative activity profiles revealed by serial deletion in myoblasts and myotubes. The CAT activities of pPLCγ1034CAT were defined as 100 and 250 in myoblasts and myotubes, respectively, since myotube has 2.5-times more PLC-γ1 mRNA than myoblast. The profiles of CAT activities were similar in both types of cells until the deletion reached -551. The removal of the sequence, -551/-480, caused greater loss of CAT activity in myotubes than in myoblasts. The relative activities of pPLCγ479CAT to pPLCγ1034CAT were 47.9% and 21.3% in myoblast and differentiated myotube, respectively. This result suggests that the region, -551/-480, which was identified as a transcriptional regulatory region and named GPE1 region in the previous study (10), might be responsible for the transcriptional activation during myogenesis.

DISCUSSION

Myogenesis proceeds with multifactorial events such as cell cycle arrest, cell migration, and muscle-specific gene expression (18). These

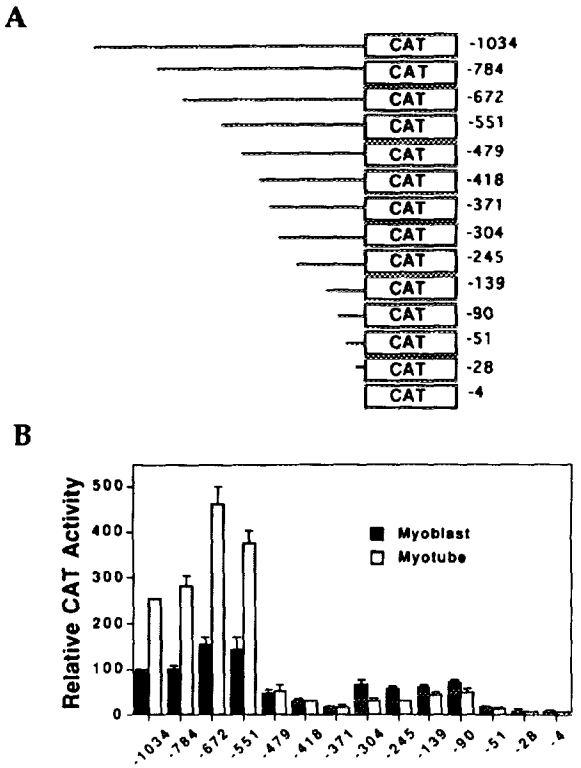

Fig. 2. **Comparison of the transcriptional activities of various fragments from PLC-γ1 promoter in myoblast and myotube.** (A) Schematic representation of the reporter plasmids which possess various lengths of PLC-γ1 gene upstream region. (B) Transcriptional activities of various reporter plasmids in myoblast and myotube. The CAT activities in C2C12 myoblasts (hatched bar) were measured 2 days after transfection. To measure the CAT activities in myotubes, cells were maintained in differentiation media after transfection for 3 days and then harvested.

events are controlled probably by extracellular signals which is transmitted to nucleus by cooperation of intracellular signal transducers. Therefore, differentiating cells might modulate the gene expressions of these signalling compounds according to its need. In this study, we examined the transcriptional regulation of PLC-γ1 gene during muscle differentiation. As differentiation proceeded, the expression of PLC-γ1 was increased by transcriptional activation. GPE1 region, -551 to -479 in the 5'-upstream region of PLC-γ1 gene, was identified as a responsible region for the transcriptional activation during myogenesis.

Myogenic differentiation progresses with drastic changes in cytoskeletal structures. Although there is no direct evidence of the involvement of PLC-γ1 in myogenesis, recent studies implicate that PLC-γ1

might regulate the cytoskeletal organization. In REF52 cells, PLC-γ1 is associated with actin microfilament through the SH3 domain (19). This association requires the growth factor-induced tyrosine phosphorylation, followed by the translocation to actin cytoskeleton (20, 21). A substrate of PLC-γ1, PIP_2, have been found to associate tightly and specifically with many actin-binding proteins, such as profilin (22), cofilin (23), gelsolin (24), gCap39 (25), and α-actinin (26). These proteins regulate polymerization and depolymerization of actin and the associations with PIP_2 seem to be important for its activity. For example, only PIP_2-bound α-actinin showed the maximum activity of F-actin bundling (26). From these results, we may speculate that the activation of PLC-γ1 leads its translocation to cytoskeleton and the hydrolysis of PIP_2 which is associated with the actin binding proteins, such as α-actinin. It is followed by the inactivation or release of the actin binding proteins and reorganization of the actin microfilaments. In addition, one of the products of PLC-γ1 activation, diacylglycerol, could stimulate actin nucleation (27), and the other one, IP_3-induced Ca^{2+}, may regulate the actin binding regulatory proteins.

In the early stage of muscle differentiation, myoblasts migrate and align to fuse among themselves. The increase of PLC-γ1 protein also occurred in the early stage of differentiation (Fig. 1). Recently, it was demonstrated that the phosphorylation of PLC-γ1 by PDGF receptor was necessary for PDGF-induced cell migration (28). From these results, it is possible that PLC-γ1 may be involved in the migration of myoblast in the initial stage of myogenic differentiation, although the activation signal of PLC-γ1 during myogenesis is not known,

GPE1 region is one of the transcriptional control regions in the 5'-upstream region of PLC-γ1 gene. In our recent study, we identified at least three binding proteins of this region, which were named GES1, GES2, and GES3[2]. Studies on the modulation of these GPE1 binding proteins may lead to the understanding of the nature of transcriptional regulation of PLC-γ1 gene during cellular differentiation.

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