Research Article

PKC-Mediated ZYG1 Phosphorylation Induces Fusion of Myoblasts as well as of Dictyostelium Cells

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We have previously demonstrated that a novel protein ZYG1 induces sexual cell fusion (zygote formation) of Dictyostelium cells. In the process of cell fusion, involvements of signal transduction pathways via Ca2+ and PKC (protein kinase C) have been suggested because zygote formation is greatly enhanced by PKC activators. In fact, there are several deduced sites phosphorylated by PKC in ZYG1 protein. Thereupon, we designed the present work to examine whether or not ZYG1 is actually phosphorylated by PKC and localized at the regions of cell-cell contacts where cell fusion occurs. These were ascertained, suggesting that ZYG1 might be the target protein for PKC. A humanized version of zyg1 cDNA (mzyg1) was introduced into myoblasts to know if ZYG1 is also effective in cell fusion of myoblasts. Quite interestingly, enforced expression of ZYG1 in myoblasts was found to induce markedly their cell fusion, thus strongly suggesting the existence of a common signaling pathway for cell fusion beyond the difference of species.

1. Introduction

Some species of Dictyostelium exhibit dimorphism in development: sorocarp formation as an asexual development and macrocyst formation as a sexual development. In the asexual development, amoeboid cells grow and multiply feeding on bacteria during the vegetative growth phase. Upon exhaustion of external nutrients, starving cells stop growing and enter the differentiation phase. They gather together to form cell aggregates. A tip is formed on the top of each cell aggregate, which then migrates as a slug-shaped mass. After migration, the slug changes its shape dramatically to form a sorocarp consisting of a stalk with an apical mass of spores.

In the sexual development, Dictyostelium mucoroides 7 (Dm7), one species of cellular slime molds, forms macrocysts homothallically without its opposite mating type. Macrocyst formation in Dm7 is characterized by the formation of large aggregates after starvation, which are then subdivided into smaller masses (precysts), each of which is surrounded by a fibrillar sheath. At the center of each precyst, there arises a cytophagic cell (a giant cell), which in turn engulfs all the other cells in the precyst. The engulfed cells (endocytes) are eventually broken down into granular remnants. The enlarged cytophagic cell finally becomes surrounded by a thick wall to form a mature macrocyst [1]. After a resting period, the macrocyst germinates to release several amoeboid cells and initiates a new life cycle [2]. Other species of cellular slime molds, such as Dictyostelium discoideum (D. discoideum), form macrocysts heterothallically with their opposite mating types [3, 4].

The giant cell which appears during macrocyst formation is clarified as a zygote which is formed by cell fusion and subsequent nuclear fusion [5]. Several molecules have been identified as regulators of zygote formation. Ethylene has been known as a potent inducer, while cAMP as an inhibitor [5, 6]. Ca2+ is also able to induce zygote formation because the efficiency of zygote formation is significantly elevated by the presence of extracellular Ca2+ [6–8]. Ca2+ is supposed to activate PKC (protein kinase C) in the signal transduction pathways involved in zygote formation. In this connection,
phorbol esters such as TPA (12-O-tetradecanoyl phorbol-13-acetate), potent activators of PKC, have been shown to enhance zygote formation [9, 10]. In contrast, staurosporine, an inhibitor of kinases including PKC, inhibits zygote formation markedly [9, 10] and therefore macrocyst formation [11]. It is quite possible that zygotes in heterothallic strains may be formed by essentially the same mechanism as that in homothallic strains [10, 12].

In the process of myoblast fusion, cells cease dividing and change their morphology from fibroblast-like to spindle (bipolar) shape. End-to-end alignment of cells is formed along with lateral-to-lateral alignment in a process termed recognition-alignment. Alignment is followed by adhesion, defined as the stage immediately prior to membrane union. The highly elongated cells of typical myoblast fusion, in which nuclei were arranged in a single file, were finally formed. Many molecules which regulate myoblast fusion have been reported [13, 14]. Among them, it has been well documented that the signaling pathway mediated by Ca²⁺ and PKC is closely involved in cell fusion of myoblasts during myogenesis [15–19]. Since the signaling pathway via Ca²⁺ and PKC is involved in myoblast fusion as well as in Dictyostelium cell fusion, we have interested to know if there is a functionally ZYG1-like protein capable of inducing cell fusion in myoblasts.

As a gene involved in sexual fusion (zygote formation) during Dictyostelium development, zyg1 cDNA was isolated from Dm7 by differential screening [20]. The zyg1 gene encodes a novel protein (ZYG1; deduced Mr 29.4 × Dm7 from that ZYG1 protein also induces cell fusion in myoblasts. PKC is localized at the region where cell fusion occurs and is phosphorylated by PKC? (3) Can ZYG1 protein induce cell fusion in myoblasts as well as in Dictyostelium cells? The results obtained have showed that ZYG1 protein phosphorylated by PKC is localized at the region where cell fusion occurs and that ZYG1 protein also induces cell fusion in myoblasts.

### 2. Materials and Methods

#### 2.1. Cell Culture and Developmental Conditions. Dictyostelium discoideum Ax-2, its transformants (GFPCONT and GFP/ZYG1²E), and mouse myoblasts (C2C12) were used in this work. Vegetative cells of Ax-2 were grown axenically in PS-medium (1% Special Peptone (Oxoid: Lot no. 33356412), 0.7% Yeast extract (Oxoid), 1.5% D-glucose, 0.11% KH₂PO₄, 0.05% Na₂HPO₄·12H₂O, 40 ng/mL vitamin B₁₂, and 80 ng/mL folic acid) containing 200 μg/mL of streptomycin and 10 μg/mL tetracycline. Transformants derived from Ax-2 cells were grown axenically by the shaking culture in PS-medium containing 200 μg/mL of streptomycin, 10 μg/mL of tetracycline, and 50 μg/mL of G418. To allow cells to differentiate, cells were harvested during the exponential growth phase, washed once in BSS (Bonner’s salt solution; 10 mM NaCl, 10 mM KCl, and 2.7 mM CaCl₂) [23] as starvation medium, and developed under the submerged conditions in BSS at 5 × 10⁵ cells/cm². For the immunocytochemical observations, starved cells were settled on coverslips which were placed in glass dishes (3.6 cm diameter). This was followed by incubation at 22°C with or without 5 nM of TPA (Sigma).

Mouse C2C12 cells (myoblasts) were cultured at 37°C in 5% CO₂ at 95% humidity in McCoy’s 5A (GIBCO) supplemented with 10% of FBS (fetal bovine serum; Invitrogen, CA), 0.03% of L-glutamine, and 80 μg/mL of kanamycin.

#### 2.2. Vector Constructs

##### 2.2.1. The Vector Construct for Expression of gfp/zyg1 Fusion Gene. The v18-l-s65tgfp (8181 bps) vector containing v18 promoter and s65tgfp (green fluorescent protein with fast oxidizing mutation S65T) gene was used as the starting material. Ubi and lacI genes inserted between v18 promoter and s65tgfp gene (984 bps) were deleted from this vector. To produce the gfp/zyg1 fusion gene, the vector was treated by Xhol. zyg1 CDNA without initiation codon (ATG) was also treated by XhoI. A Xhol-treated zyg1 (-ATG) gene was inserted into a Xhol-treated v18-l-s65tgfp vector. Vectors in which zyg1 gene was inserted in the downstream of s65tgfp gene at the sense direction were clonally selected (gfp/zyg1 fusion gene).

##### 2.2.2. The Vector Construct for Expression of ha/mzyg1 Fusion Gene. A humanized version (mzyg1) of zyg1 cDNA (AB006956) was synthesized so that each amino acid codon was replaced by that most commonly found in mammalian cells (DNA 2.0 Inc). mzyg1 treated by BamHI and EcoRI was inserted into pUCD2 SRA [24] treated by BamHI and EcoRI to create the ha/mzyg1 fusion gene, in which the mzyg1 gene was inserted in the downstream of ha (3 × haemagglutinin) at the sense direction (ha/mzyg1 fusion gene). The pUCD2 SRA vector was kindly gifted from Dr. K. Ohashi (Tohoku University).

##### 2.2.3. The Vector Construct for Expression of gfp and mzyg1 Genes. For statistical analyses, a pIRE2-AcGFP vector (Clontech) was used. mzyg1 treated by Xhol and EcoRI was inserted into the pIRE2-AcGFP vector treated by Nhel and EcoRI to create a vector-expressing gfp and mzyg1 genes (gfp and mzyg1).
2.3. Transformation of Ax-2 Cells. Ax-2 cells were grown by shake culture, harvested, and washed once with BSS. Starved cells were then washed twice with EB (electroporation buffer; 10 mM phosphate buffer (pH 6.2) containing 50 mM sucrose) and suspended in EB at 3 × 10^7 cells/mL. Introduction of the vector constructs into Ax-2 cells was performed by electroporation, as described by Howard et al. [25], using 10 μg of the vector containing gfp insert and 18 μg of the vector containing gfp/zyg1 insert, to gain the transformants GFP^CONT and GFP/ZYG1^OE, respectively. The original transformant pool was first selected by incubation in PS medium containing 10 μg/mL of G418 and finally cloned by selection in 50 μg/mL of G418.

2.4. Western Blot Analysis. GFP^CONT and GFP/ZYG1^OE cells (1 × 10^7) were boiled for 5 min in SDS-sample buffer (90 μL) (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 42 mM dithiothreitol, and 0.005% bromophenol blue) and then cooled on ice. The samples (3 μL) were separated by 10% SDS-PAGE and transferred on Immunoblot PVDF membranes (Bio-Rad). The membranes were blocked overnight with TBS-T (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) containing 0.5% BSA (bovine serum albumin, Sigma) at RT (room temperature). Subsequently, the membranes were probed by rabbit Phospho-(Ser) PKC Substrate antibody (Cell Signaling) diluted 1,000 times with enhancer A solution (Wako) for 1 h at RT. After two washings in TBS-T (10 min for each), the membranes were stained with goat HRP- (horseradish-peroxidase-) conjugated anti-rabbit secondary antibody (Amersham Biotech) diluted 500 times with TBS-T containing 5% skim milk for 1 h at RT. After two washings in TBS-T (10 min for each), the membranes were stained with goat Phospho-(Ser) PKC Substrate antibody (Cell Signaling) diluted 300 times with PBS containing 2% FBS, for 1 h at RT. The samples were washed by three changes of PBS and then stained with the goat rhodamine-conjugated secondary anti-rabbit IgG (H + L) (Thermo) for 1 h at RT. After washing in PBS, samples were mounted in PBS containing 20% glycerol.

2.6. Introduction of Vectors into C2C12 Cells (Myoblasts). C2C12 cells (myoblasts) were plated on coverslips and transfected with the vector containing a ha gene, a ha/mzyg1 fusion gene, a gfp gene, or gfp and mzyg1 genes using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. After 6 h of transfection in Opti-MEM (Invitrogen) without FBS, samples were washed twice with PBS and incubated with DMEM (Dulbecco’s Modified Eagle Medium, Invitrogen) containing 10% FBS and 80 μg/mL of kanamycin for 24 h at 37°C in 5% CO2 at 95% humidity.

2.7. Immunocytochemical Staining of C2C12 Cells Expressing HA or HA/ZYG1 Protein. After transfection, the samples were fixed with PBS containing 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 in PBS containing 2% FBS for 30 min at RT, followed by blocking with PBS containing 2% FBS for 30 min at RT. After three washings in PBS, the samples were stained with the mouse anti-HA antibody (Roche Applied Science) diluted 400 times with PBS containing 2% FBS, for 1 h at RT. Then they were washed with three changes of PBS and stained with the goat FITC-conjugated secondary anti-mouse IgG (H+L) (Alexa Fluor 488, Invitrogen) in PBS containing 2% FBS for 1 h at RT. After washings in PBS, the samples were mounted in PBS containing 20% glycerol and DAPI (4′,6-diamidino-2-phenylindole) (final concentration: 2.5 μg/mL) (Wako, Japan).

2.8. Photographs. Photographs were taken under fluorescence microscope (Axiostkop, Zeiss) or confocal microscope (Fluoview FV 1000, Olympus).

3. Results

3.1. Localization of GFP/ZYG1 Fusion Protein. From our previous studies [10, 20], ZYG1 was expected to translocate to the cell membrane where cell fusion occurs and probably coupled with its phosphorylation by PKC. To examine this possibility, the gfp/zyg1 fusion gene was introduced into D. discoideum Ax-2 cells. In the present work, we used Ax-2 cells, instead of D. mucoroides (Dm7) that had been used in our previous works, because Ax-2 cells with 10-11 μm of diameter are bigger than Dm7 cells with 6-7 μm of diameter and more convenient for cytological studies. In addition, it is suggested that the mechanism of zygote formation might be fundamentally common [10, 12]. The vector containing only a gfp gene was also introduced into Ax-2 cells as a control. Cells overexpressing GFP/ZYG1 (GFP/ZYG1^OE) and overexpressing GFP (GFP^CONT) were selected by 50 μg/mL of G418, and the localization of GFP/ZYG1 fusion protein or GFP protein was monitored under fluorescence and confocal microscopes.
Figure 1: Localization of GFP and GFP/ZYG1 fusion protein. GFP<sup>CONT</sup> and GFP/ZYG1<sup>OE</sup> cells are starved for 2 h, fixed with 4% paraformaldehyde for 20 min, and observed under a fluorescence microscope. GFP protein in GFP<sup>CONT</sup> cells and GFP/ZYG1 fusion protein in GFP/ZYG1<sup>OE</sup> cells are shown as the green color of GFP. GFP/ZYG1 fusion protein is preferentially localized at the periphery of vesicles (c, d; arrow) and at the phagocytic cups which are attached to form cell-cell contacts (f; arrows). On the other hand, in GFP<sup>CONT</sup> cells, GFP protein is uniformly distributed in the cytoplasm (a, b; arrow), and its localization at the region of cell-cell contacts is not observed (e; arrow). This indicates that its localization is due to the presence of ZYG1, but not to GFP itself. Photographs of (b) and (c) are the magnified (a) and (d), respectively, and adjusted to get brighter images. Bars: 20 μm.

GFP protein in GFP<sup>CONT</sup> cells and GFP/ZYG1 fusion protein in GFP/ZYG1<sup>OE</sup> cells were observed as the green color of GFP. When GFP<sup>CONT</sup> cells were developed for 2 h after starvation, GFP protein was uniformly distributed in the cytoplasm (Figures 1(a) and 1(b)). On the other hand, in GFP/ZYG1<sup>OE</sup> cells, most GFP/ZYG1 fusion protein was localized at the periphery of vesicles (Figures 1(c) and 1(d); arrow), particularly around the phagocytic cups. The phagocytic cups became larger and attached to each other when cell-cell contacts were formed (Figure 1(f); arrows). Since the localization of GFP at the periphery of vesicles and the region of cell-cell contacts was never observed in GFP<sup>CONT</sup> cells (Figures 1(a) and 1(e); arrows), it is most likely that its localization is due to the presence of ZYG1, but not to GFP itself. Successive observation of intact cells observed under a fluorescence microscope has showed that GFP/ZYG1 fusion protein translocates to the cell cortex and then detaches from the cell cortex within 10 sec (Figure S1 see in supplementary material available online at doi:10.1155/2012/657423). Thus, the ZYG1 localization seemed to change temporarily.

When optical sections of GFP/ZYG1<sup>OE</sup> cells under a confocal microscope were observed, a part of two contacted cells seemed to fuse to each other because demarcation was lost in sections 2 and 3 of Figure 2 (DIC; yellow arrows). Considering the merged images of DIC (Differential Interference Contrast) and green fluorescence emitted from GFP, it is quite possible that GFP/ZYG1 fusion protein is preferentially localized at the region of cell fusion (Figure 2). It was also observed that GFP protein was localized in nuclei (shown as red in Figure 2). These results showed that ZYG1 was predominantly localized at the regions of cell-cell contacts and cell fusion.

3.2. Phosphorylation of ZYG1 by PKC. To know if ZYG1 is phosphorylated by PKC (protein kinase C), ZYG1 phosphorylation in GFP/ZYG1<sup>OE</sup> cells was examined by Western blotting using the antibody that specifically recognized the serine residues phosphorylated by PKC. Results showed that a small amount of GFP/ZYG1 fusion protein (67.4 kDa) was...
Figure 2: Behavior of ZYG1 during cell fusion. GFP/ZYG1OE cells were starved for 2 h and fixed with methanol for 10 min. Pictures of their serial sections (1–3; 4 μm thick) were taken under a confocal microscope. Pictures on the upper side show DIC images and those on the lower side the merged images of DIC, GFP, and DAPI staining. The color of DAPI staining is shown as red. In the DIC images (upper panels), the boundaries shown by white arrows are clear in all sections. On the other hand, the boundary shown by a yellow arrow is clearly observed in section 1, while the demarcation becomes unclear in sections 2 and 3. This indicates that the two cells are fusing around here (2, 3). In merged images (lower panels), GFP/ZYG1 fusion protein is localized at the region of cell fusion (2, 3; yellow arrows). Nuclei become yellow by merging GFP with DAPI staining. Bar: 20 μm.

Figure 3: Phosphorylation of GFP/ZYG1 fusion protein by cPKC. GFPCONT and GFP/ZYG1OE cells were collected at 2-hour intervals after starvation. Each sample was separated by 10% SDS-PAGE, followed by Western blottings using the rabbit Phospho-(Ser) PKC Substrate antibody and the rabbit anti-GFP antibody. Hours after starvation are designated at the bottom. The GFP/ZYG1 fusion protein (67.4 kDa) is phosphorylated by cPKC (a; arrow), while no bands around 67.4 KD are detected in the samples obtained from GFPCONT cells. GFP/ZYG1OE cells also express GFP protein, as detected by the anti-GFP antibody (b; arrow).

The phosphorylation of ZYG1 protein was examined also by immunocytochemical staining. As TPA, a potent activator of cPKC, is known to enhance the formation of zygotes [9], experiments were carried out with or without TPA (5 nM). In GFP/ZYG1OE cells, GFP/ZYG1 fusion protein shown as the green color of GFP was localized at the region of cell-cell contacts (Figure 4(d); arrow). On the other hand, the localization of GFP protein at the region of cell-cell contacts was not observed in GFPCONT cells (Figure 4(c); arrow). This indicates that ZYG1 protein, but not GFP, localizes at the regions of cell-cell contacts in GFP/ZYG1OE cells, as shown already in Figure 1. The protein phosphorylated by PKC shown as the red color of Rhodamine was also localized at the region of cell-cell contacts in GFP/ZYG1OE cells (Figure 4(f); arrow). This localization was not observed in GFPCONT cells (Figure 4(e); arrow). When GFP was merged with Rhodamine, the image exhibited yellow color in GFP/ZYG1OE cells (Figure 4(h); arrow). This means that ZYG1 protein was colocalized with the proteins phosphorylated by cPKC at the regions of cell-cell contacts (Figure 4, arrows). Naturally enough, the colocalization of GFP and Rhodamine was not observed in GFPCONT cells (Figure 4(g); arrow).

Multinucleate cells were formed more frequently in GFP/ZYG1OE cells that had been incubated with TPA, as compared...
Figure 4: Immunocytochemical detection of the PKC-mediated ZYG1 phosphorylation. Starved GFP\textsuperscript{CONT} and GFP/ZYG1\textsuperscript{OE} cells were developed for 2 h, fixed with methanol, and stained by the Phospho-(Ser) PKC Substrate antibody. This was followed by the rhodamine-conjugated anti-rabbit secondary antibody to detect the proteins phosphorylated by cPKC, as described in Section 2. Photographs were taken under DIC (a and b) and fluorescence microscope (GFP, (c) and (d); Rhodamine, (e) and (f); GFP and Rhodamine merged, (g) and (h)). Photographs represent the same fields of GFP\textsuperscript{CONT} cells (a, c, e, g) and of GFP/ZYG1\textsuperscript{OE} cells (b, d, f, h). GFP protein in GFP\textsuperscript{CONT} cells and GFP/ZYG1 fusion protein in GFP/ZYG1\textsuperscript{OE} cells are shown as the green color of GFP (c and d). In GFP/ZYG1\textsuperscript{OE} cells, GFP/ZYG1 fusion protein is localized at the region of cell-cell contacts (d; arrow). On the other hand, the localization of GFP protein at the region of cell-cell contacts is not observed in GFP\textsuperscript{CONT} cells (c; arrow). This indicates that its localization is due to the presence of ZYG1, but not to GFP itself. The protein phosphorylated by PKC is shown as the red color of Rhodamine in both cells ((e) and (f)). The protein phosphorylated by PKC is also localized at the region of cell-cell contacts in GFP/ZYG1\textsuperscript{OE} cells (f; arrow). This localization shown by Rhodamine is not observed in GFP\textsuperscript{CONT} cells (e; arrow). Since the merged color of GFP and Rhodamine shows yellow in GFP/ZYG1\textsuperscript{OE} cells (h; arrow), it is evident that ZYG1 protein is phosphorylated by cPKC at the regions of cell-cell contacts. Bar: 25 μm.
with those incubated without TPA, thus suggesting that cell fusion in GFP/ZYG1OE cells might be accelerated by TPA. Since there is no boundary between the two cells in GFP/ZYG1OE cells incubated with TPA (Figure 5(a); black arrow), it is quite likely that cell fusion actually occurs between them. GFP/ZYG1 fusion protein was co-localized with the proteins phosphorylated by cPKC at the region of cell fusion, because the image exhibited yellow color around the region of cell fusion occurred (Figure 5; arrows). These results suggest that ZYG1 is phosphorylated by PKC at the region of cell-cell contacts and cell fusion.

Interestingly, the region where cell-cell contacts and cell fusion occurred was found to be composed of a cluster of small vesicles (Figure 2).

3.3. Enforced Expression of ZYG1 Protein in Mouse Myoblasts Induces Their Fusion. Ca^{2+} and PKC are known to be involved in myoblast fusion during myogenesis [15–17] and in zygotic cell fusion of Dictyostelium cells [6–9]. Therefore, it is possible that there may be a common signal transduction pathway for cell fusion in both of the cell lines. To know if ZYG1 is able to induce myoblasts, a ha/zyg1 fusion gene was transfected into C2C12 cells (myoblasts). In spite of many trials, however, ZYG1 protein was never expressed in C2C12 cells. For the purpose of ZYG1 expression in mammalian cells, a humanized version (mzyg1) of the zyg1 gene was synthesized in which each amino acid codon was replaced by that most commonly found in mammalian cells (DNA 2.0 Inc.). When a ha/mzyg1 fusion gene (ha/mzyg1) was transfected into C2C12 cells, the expression of HA/ZYG1 fusion protein was revealed by the anti-HA antibody (mouse) and then the FITC-conjugated secondary anti-mouse antibody. As a control, C2C12 cells transfected with a vector containing only a ha gene were prepared. At 24 h of incubation in

Figure 5: The phosphorylation of GFP/ZYG1 fusion protein by cPKC occurs at the region of cell fusion. Starved GFP/ZYG1OE cells were developed for 2 h in the presence of TPA (5 nM). The sample of GFP/ZYG1OE cells for the immunocytochemical detection was prepared as described in Figure 4. The same fields are shown as images of DIC (a), Rhodamine (b), GFP (c), and GFP and Rhodamine merged (d). Since it is evident by the DIC image that there is no boundary between the two cells (a; black arrow), cell fusion is occurring between two cells. Since the merged image shows yellow color (d; a white arrow), it is evident that GFP/ZYG1 fusion protein and proteins phosphorylated by cPKC are colocalized particularly at the region where cell fusion occurred. Bar: 25 μm.
Figure 6: Behavior of C2C12 cells (myoblasts) transfected with the ha/mzyg1 fusion gene. C2C12 cells were transfected with the ha gene (control; a, c) or ha/mzyg1 fusion gene (HA/ZYG1; b, d), fixed with 4% paraformaldehyde and stained by antibodies and DAPI according to the methods described in Section 2. Phase-contrast micrographs on the upper sides represent the same field as the merged images of FITC and DAPI on the lower sides ((a) and (c), (b) and (d)). C2C12 cells expressing the HA/ZYG1 fusion protein stained with the anti-HA antibody and then FITC-conjugated anti-mouse secondary antibody are detected as the green-colored cells under a fluorescence microscope though a considerable number of cells are intermingled as untransfected ones in the population (d). Nuclei stained with DAPI are shown as purple color by UV-excitation (c and d). The transfection rate with the ha/mzyg1 gene was 36.8 ± 8%. The expression of HA protein in control cells was scarcely detected (c). Bar: 100 μm.

DMEM containing 10% FBS after transfection, cells were fixed with 4% formaldehyde, followed by immunostaining. As a result, cells expressing HA/ZYG1 fusion protein (HA/ZYG1) were recognized because of their green color of fluorescence (Figure 6(d)). The transfection rate was 36 ± 8%. The expression of HA protein in control cells was scarcely detected though the HA gene was present in the vector used for transfection (Figure 6(c)).

Some HA/ZYG1 cells showed a typical spindle shape as a sign of myoblast fusion (Figure S3(A) see in supplementary material available online at doi:10.1155/2012/657423) though a large number of cells exhibited fibroblast-like morphology. The spindle-shaped cells showed a characteristic alignment that was the parallel apposition of the long axes of myoblasts forming end-to-end alignment as a sign of acquisition of fusion competence (Figure S3(B) see in supplementary material available online at doi:10.1155/2012/657423) [18]. In addition, HA/ZYG1 cells formed giant multinucleate cells frequently (Figure 7) and exhibited fibroblast-like morphology. As binucleate cells were frequently observed to have larger sizes of cells and nuclei (Figure 7(a)), it is quite different from the cells at the mitosis (Figure S4 see in supplementary material available online at doi:10.1155/2012/657423),

The highly elongated cells known as a sign of typical myoblast fusion, in which nuclei were arranged in a single file, were not observed. In addition, nuclei tended to fuse and form a large size of a fused nucleus or nuclei (Figure 7(d)) though this event was never observed in the normal process of myoblast fusion.

Since the characteristics described above were also observed in control cells, the numbers of cells having multinuclei were counted and statistically analyzed using a pIRES2-AcGFP vector, which contains the gfp gene and expresses constitutively GFP protein independently from the gene inserted. The expression of GFP was weak, but significantly higher compared to GFP-uninfected cells (Figure S5 see in supplementary material available online at doi:10.1155/2012/657423). By transfection with the pIRES2-AcGFP vector or pIRES2-AcGFP vector containing an mzyg1 gene, almost all of the cells expressed GFP or GFP and ZYG1 protein, respectively (Figure S6 see in supplementary material available online at doi:10.1155/2012/657423). Thus, the rates of cell fusion, which were designated as fusion indexes, were found to be significantly higher in cells transfected with the vector containing an mzyg1 gene than the control cells (Figure 8). The difference of fusion index between GFP cells
Figure 7: Formation of multinucleate cells in C2C12 cells transfected with the ha/mzyg1 fusion gene (HA/ZYG1). C2C12 cells were transfected with the ha/mzyg1 fusion gene, fixed with 4% paraformaldehyde, and stained by antibodies and DAPI according to the methods described in Section 2. The FITC images merged with DAPI images of large-sized HA/ZYG1 cells containing 2 nuclei (a), 3 nuclei (b), and 7 nuclei (c) are shown. Considering some gulfs observed in a nucleus, it is evident that a large nucleus is formed by nuclear fusion (d; arrows). Bar: 100 μm.

and GFP and ZYG1 cells was statistically significant (P < 0.002), indicating that the myoblast fusion is markedly enhanced by the mzyg1 gene. These results indicate that ZYG1 is able to cause cell fusion in myoblasts.

4. Discussion

Previous studies have suggested that the signal transduction pathway via Ca^{2+} and PKC may participate in sexual fusion (zygote formation) of Dictyostelium cells [10, 12]. Since ZYG1 protein has several deduced sites phosphorylated by PKC, ZYG1 protein was expected to be a likely substrate of PKC. Provided that ZYG1 is phosphorylated by PKC prior to cell fusion, ZYG1 protein will be translocated to the cell membrane where cell fusion occurs.

As presented in this study, the ZYG1 protein was found to actually translocate to the cell cortex including the cell membrane, where cell-cell contacts and subsequent cell fusion occur. Importantly, ZYG1 localizes at the periphery of vesicles including phagocytic cups. Since cell-cell contacts are often observed between phagocytic cups of two facing cells, it is likely that cell fusion may occur through the fusion of those phagocytic cups. The phosphorylation by cPKC of ZYG1 at the regions of cell-cell contacts and cell fusion was verified by Western blot analysis and immunocytochemical study. PKC is a multigene family of serine/threonine (ser/thr) kinases that play critical roles in many signal transduction pathways [26, 27]. This kinase family is composed of at least 11 different isozymes classified into the following 3 groups: (1) cPKC (conventional) α, β, and γ which require Ca^{2+} and DAG (diacylglycerol) for maximal activity [28]; (2) nPKC (novel) δ, ε, η, μ, and θ isoforms, which are Ca^{2+} independent but require DAG [29–32]; (3) aPKC (atypical) ζ, λ, and τ, which are Ca^{2+} and DAG insensitive [33, 34]. Since Ca^{2+} is required for sexual fusion in Dictyostelium cells [2–4], we used the Phospho-(Ser) PKC Substrate antibody that specifically recognizes cPKC substrates containing phosphoserine.
As a result, we found that ZYG1 protein was phosphorylated by cPKC among the PKC family. After homology search, ZYG1 protein has seven sites phosphorylated by cPKC [20]. Among the seven deduced phosphorylation sites, five are serine residues though it is presently unknown if some or all of the serine residues are phosphorylated by cPKC for cell fusion. There are two threonine-phosphorylation sites, but their phosphorylation by cPKC was not detected in our preliminary experiment.

Considering the data presented here, a possible signal transduction pathway involved in sexual cell fusion has been proposed, as follows: (1) cPKC is activated by the increase of intracellular Ca\(^{2+}\). (2) ZYG1 is translocated to the cell membrane. (3) ZYG1 protein is phosphorylated by activated cPKC. (4) The sexual cell fusion is induced by the phosphorylated ZYG1 protein.

Since cPKC is known to be involved in myoblast fusion [15–17], the fusion processes in myoblasts and Dictyostelium cells might share a similar signal transduction pathway. When C2C12 cells (myoblasts) were transfected with the fusion gene of *ha* and humanized *zyg1* (*ha/mzyg1*) or pIRES2-AcGFP vector containing the *mzyg1* gene (gfp and *mzyg1*), the process of myoblast fusion was found to be accelerated. Since ZYG1 has not a mammalian ortholog, protein(s) other than ZYG1 may be phosphorylated by PKC during myogenesis. However, the result presented here has suggested that ZYG1 is able to take place of the protein phosphorylated by PKC in mammalian cells. Also, the protein phosphorylated by PKC during myoblast fusion may be functionally similar to ZYG1, though the target protein of PKC in myoblasts remains to be identified. The multinucleate cells formed by the transfection with *mzyg1*, however, exhibited fibroblast-like morphology, while highly elongated cells in which nuclei arrange in a single file as observed in the normal process of myoblast fusion were not formed. The following two explanations for this difference might be possible. During the transfection carried out without FBS for 6 h, myoblasts stop their growth and start differentiation. After a short period of differentiation, myoblasts restart growth in the medium containing FBS. As a result, the multinucleate cells formed by cell fusion assume fibroblast-like morphology. Another possibility is that HA/ZYG1 cells might be able to fuse by ZYG1 even under the growth conditions. In all events, it is of importance to note that a novel protein, ZYG1, has an inductive effect on myoblast fusion as well as on sexual fusion of Dictyostelium cells.

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