Induction of macrocyst wall formation by ZYG1 in Dictyostelium discoideum

Aiko Amagai1*, Fumio Takahashi1,2, Toshinori Usui4,5, Tomoaki Abe4 and Yasuo Maeda1

Abstract

Background: Macrocyt is known as the sexual developmental form in cellular slime molds. ZYG1 is known to be involved in zygote formation by cell fusion during macrocyst formation in Dictyostelium mucoroides, one of the species of cellular slime molds.

Aim and methods: To know the effects of ZYG1 on the asexual and sexual development in Dictyostelium discoideum, another species of cellular slime molds, we observed the developmental process and forms of a transformant (GFP-ZYG1OE) derived from Dictyostelium discoideum, in which GFP-ZYG1 fusion protein is overproduced.

Results: GFP-ZYG1OE cells were developed without or with an opposite mating type, V12M2 cells. When GFP-ZYG1OE cells were developed without V12M2 cells, they formed macrocyst-like structures consisting of a central cell mass and a thick transparent macrocyst wall surrounding it. It was suggested that transparent zones in the macrocyst wall were formed by cell fusion. When GFP-ZYG1OE cells were developed with V12M2 cells, globular bodies which are likely the macrocysts surrounded by thin cellulosic walls were found besides macrocysts. They were formed directly from zygotic giant cells without the formation of cell aggregates. The formation of the globular body is not caused by ZYG1 overproduction, because GFPCONT cells, a transformant overproducing GFP protein as controls, also formed globular bodies.

Conclusion: By the use of GFP-ZYG1OE cells overproducing GFP-ZYG1 fusion protein, it is revealed that ZYG1 is closely involved in macrocyst wall formation. This is the new finding concerning the function of ZYG1. In addition, the formation of a globular body, which is likely the macrocyst surrounded by a thin cellulosic wall, is demonstrated as a novel process of sexual development.

Keywords: Dictyostelium, cellulose, cell fusion, macrocyst wall, ZYG1

Introduction

Macrocysts are formed as a sexual developmental process in cellular slime molds. Removal of any growing medium (starvation) causes cells to stop growing and form cell aggregates. Macrocyst formation in Dm7, a wild-type strain of Dictyostelium mucoroides 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. A giant cytophagic cell arises at the center of each precyst, which engulfs all the other cells in the precyst. The engulfed cells (endocytes) become oval and are eventually broken down into granular remnants. The enlarged giant cell is eventually surrounded by a thick macrocyst wall to form a mature macrocyst. The macrocyst wall has been shown electron-microscopically to consist of three layers: a thin inner layer of the least electron-density, a thick middle layer of medium electron-density, and a thin electron-dense outer layer [1]. The macrocyst wall is considered to be a product synthesized by a giant cell between the cell membrane...
and the outer-most fibrillar sheath [1]. After a resting period, the macrocyst germinates to release several amoeboid cells and initiates a new life cycle [2].

There are mainly two kinds of mating systems involved in macrocyst formation: homothallic and heterothallic [3-6]. Dm7 is a homothallic strain, which forms macroysts without mating types. Ax2, an anexic strain of *Dictyostelium discoideum* (*D. discoideum*) is a heterothallic strain, which mates with an opposite mating type strain, V12M2. In the developmental process of macrocyst formation in Ax2 cells co-cultured with V12M2 cells, zygotic giant cells attract local amoebae, resulting in the formation of precysts [7,8]. Thus giant cells appear within aggregates during macrocyst formation in Dm7 cells [1], while the giant cells are formed before distinct cell aggregation in the case of Ax2 cells co-cultured with V12M2 cells. The process of macrocyst formation, however, is believed to be basically the same between the two mating systems [9,10].

The cytophagia giant cells formed during macrocyst formation are zygotes produced by cell fusion and subsequent nuclear fusion [11]. As regulators of zygote formation, ethylene (a potent plant hormone) and 3',5'-cyclic adenosine monophosphate (cAMP) have been identified. Ethylene induces zygote formation [11], while cAMP inhibits zygote formation [12,13].

Concerning the signal transductions involved in zygote formation, the signaling pathway via Ca²⁺ and protein kinase C (PKC) is suggested in both homothallic Dm7 and heterothallic Ax2 cells [13-14,18]. As a gene involved in zygote formation, *zyg1* has been isolated from a cDNA library of Dm7 by time dependent differential screening (DDBJ/EMBL/GenBank, accession number AB006956) and from a genomic DNA of Dm7 by PCR (DDBJ/EMBL/GenBank, accession number AB479506). *Zyg1* encodes a protein (ZYG1; deduced Mr 29.4x10³) consisting of 268 amino acids. According to BLAST and FASTA searches, ZYG1 is translocated at regions of cell-to-cell contact where cell fusion occurs [23]. These results indicate that ZYG1 moves from the cytoplasm to the cell membrane, is phosphorylated by PKC and causes cell fusion through PKC mediated signal transduction pathways. Thus ZYG1 plays an important role in zygotic cell fusion.

The developmental process and forms of GFP-ZYG1OE cells co-cultured without or with an opposite mating type, V12M2 cells, were also examined during the course of the study. We report here a new function of ZYG1 and the unique process for macrocyst formation obtained consequently by use of GFP-ZYG1OE derived from Ax2 cells, which overproduces GFP-ZYG1 fusion protein.

**Materials and methods**

**Cell cultures and developmental conditions**

GFPcont and GFP-ZYG1OE, transformants derived from Ax2 (an anexic strain of *D. discoideum*) (Amagai et al, 2012) and V12M2 (an opposite mating type strain of Ax2) were used in this work. Vegetative GFPcont and GFP-ZYG1OE cells were grown axenically by shake cultures in PS-medium (1% Special Peptone [Oxoid: Lot No. 333 56412], 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 streptomycin, 10 µg/ml tetracycline and 50 µg/ml Geneticin (G418) (GIBCO BRL). To allow the cells to differentiate, they were harvested during the exponential growth phase, washed once in BSS (Bonner’s salt solution: 10 mM NaCl, 10 mM KCl, 2.7 mM CaCl₂) [25] as a starvation medium. V12M2 cells were grown by simultaneous culture with Escherichia coli B/r. Vegetative cells at the exponential growth phase were harvested and washed three times with BSS for starvation. Cell culture and development were carried out in dark conditions at 22°C. For observation and time-lapse imaging under an inverted microscope (Nikon), the cells were settled on glass based dishes (Iwaki, 3910-035, 35 mm diameter) and developed under submerged conditions in BSS at 1 ~ 6x10⁶ cells/cm².

**Calcofluor staining**

Immediately after one drop of Calcofluor (10 µg/ml; Sigma F-3397) was added into the 2 ml cell suspension, one drop of cell suspension was placed on cleaned coverslips for observation.

**Imaging of living cells**

Glass dishes with adhered living cells were placed on an inverted microscope for observation (Nikon, Japan). Time-lapsed images were acquired at one frame per 20 seconds using a CCD camera (Leica Dc250, Germany).

**Observations and photographs**

Developing cells and forms were observed and photographed under an inverted microscope, a phase-contrast microscope (Nikon, Tokyo) and a fluorescence microscope using UV excitation...
As described above, the structure consisting of a central cell mass and peripheral cells surrounding it. Since the peripheral cells did not become transparent synchronously, three types of developmental forms eventually coexisted. As described above, the structure consisting of a central cell mass and peripheral cells surrounding it. The peripheral cells surrounding the central masses were eventually lost, thus resulting in the formation of a macrocyst-like structure consisting of a central cell mass and a thick transparent wall (a macrocyst wall) surrounding it. The peripheral cells did not become transparent synchronously, three types of developmental forms eventually coexisted.

Figure 1. Development of GFP-ZYG1OE cells. Starved GFP-ZYG1OE cells were developed without their mating cells at a density of 6x10^6 cells/dish. (a) A phase-contrast photomicrograph at 1 day of development. Most of the cells finished aggregation and form loose aggregates. (b) A phase-contrast photomicrograph at 3 days of development. Loose aggregates change to tight aggregates, each of which is surrounded by a sheath (arrows). The cells located at the center of an aggregate seem to be optically denser than the peripheral cells. (c) A phase-contrast photomicrograph at 7 days of development. The optically denser part of an aggregate becomes a tight aggregate (a central cell mass, arrow). This structure looks like a macrocyst (a macrocyst-like structure). (d) A bright-field photomicrograph at 18 days of development. This shows a macrocyst-like structure consisting of a central cell mass and peripheral cells surrounding it. (f) A bright-field photomicrograph of a macrocyst-like structure at 17 days of development. This shows a complete macrocyst-like structure consisting of a central cell mass and a transparent macrocyst wall surrounding it.

Although macrocyst-like structures are morphologically similar to normally formed macrocysts, it is presently unknown whether or not the central cell mass is a zygotic giant cell containing endocytes.

When the macrocyst-like structure was stained with Calcofluor before the completion of a transparent wall, the whole of the central cell mass was stained. As the outer surface of the peripheral cells surrounding the central cell mass was also stained, each peripheral cell was easily recognized. Comparing the size (diameter) of the cells, the cell shown by an arrow was about 4 times larger than that shown by an arrowhead. Large-sized cells seem to fuse with each other to increase their size.

Some macrocyst-like structures were in process of forming transparent walls by losing peripheral cells. Since some lines consequently became visible in the macrocyst-like structure, it was realized that the central cell mass was surrounded by several parts of transparent zones.

Apparently, there was no significant difference in the macrocyst walls in either macrocyst-like structures or macrocysts. To confirm this similarity, the localization of GFP-ZYG1 fusion protein and cellulose in the macrocyst-like structure and macrocyst was examined by staining with Calcofluor. In the macrocyst-like structure, GFP-ZYG1 fusion protein was localized at the whole central cell mass and the outer layer of the macrocyst wall, co-localizing with Calcofluor staining.

In the macrocyst formed in GFPCONT cells co-cultured with V12M2 cells, GFP protein was localized at the whole giant cell and the outer layer of the macrocyst wall, co-localizing with Calcofluor staining.

As a result, GFP-ZYG1 fusion protein and GFP protein were co-localized with cellulose in both macrocyst-like structures and macrocysts. This indicates the similarity of the macrocyst wall in both macrocyst-like structures and macrocysts. However, as GFPCONT protein also showed the same localization as GFP-ZYG1 fusion protein, co-localization of GFP-ZYG1 fusion protein with cellulose does not depend on ZYG1 overproduction.

When GFPCONT cells were developed without V12M2 cells, they formed loose aggregates, but never macrocyst-like structures. Therefore, the formation of macrocyst-like structures in GFP-ZYG1OE cells was concluded to be due to ZYG1 overproduction.
Macrocyst formation by GFP-ZYG1⁰⁷ and GFPCONT cells co-cultured with V12M2 cells

To elucidate the function of ZYG1 protein during macrocyst formation, GFP-ZYG1⁰⁷ cells were developed with V12M2 cells. As a result, mainly two types of developmental forms were found: naturally-occurring mature macrocysts surrounded with a thick macrocyst wall (Figure 4Aa) and globular bodies (Figure 4Ab). These forms were also found when GFPCONT cells were developed with V12M2 cells as controls (Figure 4Ac). In consequence, there seemed to be no essential differences in the developmental forms between GFP-ZYG1⁰⁷ and GFPCONT cells. In macrocysts, two types of macrocyst wall were observed. One was lined with peripheral cells (Figure 4Aa, arrowed), and another with few peripheral cells (Figure 4Aa, arrowhead). Macrocysts formed by GFPCONT cells co-cultured with V12M2 cells also showed two types of macrocyst wall. Although most of the macrocyst walls were clear zones in mature macrocysts developed for 21 days (Figure 4Ad), macrocyst walls in which peripheral cells were still retained were sometimes observed even after 21 days of development (Figure 4Ae).

With regard to globular bodies, there has been no precise description yet. The mature globular bodies were filled with endocytes (Figure 4B, upper panel, arrowhead). Apparently, a thick macrocyst wall was not formed in the globular body. However, as the periphery of the globular bodies was stained with Calcofluor (Figure 4B, lower panel, arrowed), it is evident that they have a thin wall consisting of cellulose.

To precisely examine how globular bodies are formed, the developmental process in a mixed culture of GFP-ZYG1⁰⁷ and V12M2 was observed under phase-contrast microscopy and also recorded using time-lapsed imaging. Cell aggregates (arrowhead) and giant cells (arrows) were formed at 1 day of development after starvation, although many cells remained as non-aggregated single cells (Figure 5a). After 4 days of development, giant cells full of granular endocytes appeared (Figure 5b, arrows). This was followed by formation of globular bodies after 6 days of development (Figure 5c, arrows). As the developmental process did not progress synchronously, giant cells and globular bodies coexisted for several days of development.

When GFPCONT cells were developed with V12M2 cells as controls, they showed a similar developmental process as
globular bodies returned to the giant cells spreading their lamellipodia. In this case, however, they finally became globular bodies.

Discussion

In the present study, the developmental process and forms of GFP-ZYG1OE cells overproducing GFP-ZYG1 fusion protein were examined. As a result, the interesting findings concerning the function of ZYG1 and the developmental process of macrocyst formation were obtained.

When GFP-ZYG1OE or GFPCONT cells were co-cultured with V12M2 cells, they formed macrocyts and many free-living giant cells. The free-living giant cells contained endocytes, retracted their lamellipodia and developed directly into globular bodies. Globular bodies have the characteristics of a macrocyst including the formation of giant cells containing endocytes and a wall stained with Calcofluor, though they do not form a thick macrocyst wall. Consequently, the globular body is likely the macrocyst formed directly by a giant cell without the process of cell aggregation.

Since globular bodies were also observed in a mixed culture of GFPCONT and V12M2 cells, the formation of the globular body...
ZYG1OE cells failed to form globular bodies without the help of another cell type. The macrocyst-like structure consisted of a central cell mass with a macrocyst wall surrounding it. According to the descriptions by Filosa and Dengler (1972), the central cell mass is a zygotic giant cell containing endocytes. Considering that ZYG1 is known to induce cell fusion (21,23), it is supposed that globular bodies formed only by cell fusion may be formed by ZYG1 overproduction. However, the fact that the GFP-ZYG1OE cells failed to form globular bodies without the help of mating type cells seems to indicate that the functional zygotic giant cells might not be formed only by ZYG1 overproduction. On the other hand, it is most likely that the wall formation in a macrocyst-like structure may be induced by ZYG1 overproduction.

In the macrocyst-like structure, GFP-ZYG1 fusion protein, as well as cellulose, was localized at the whole central cell mass and the outer layer of the macrocyst wall (Figure 3a). As macrocysts formed by co-cultures of GFPCONT with V12M2 cells (Figure 3b), this indicates the similarity in macrocyst walls of macrocyst-like structures and macrocysts. Consequently, according to the descriptions by Filosa and Dengler (1972), the thick transparent zone of the macrocyst-like structure corresponds to the thin inner layer and thick middle layer of the macrocyst wall in the macrocyst. The outer layer of the macrocyst-like structure, stained with Calcofluor, corresponds to the electron-dense, thin outer layer of the macrocyst wall in the macrocyst.

The formation process of transparent zones in macrocyst-like structures was also suggested in the present study; that is, peripheral cells in the macrocyst-like structure increase their size by cell fusion and eventually become transparent zones (Figure 2). Coupling with the enlarged cells, GFP-ZYG1 fusion protein may be pushed toward the cell membrane and co-localized finally with cellulose at the outer layer of the macrocyst wall (Figure 3). Since ZYG1 is known to induce cell fusion (21), it is quite possible that ZYG1 may induce cell fusion in the early stages of macrocyst wall formation.

In macrocysts formed by GFP-ZYG1OE or GFPCONT cells co-cultured with V12M2 cells, two types of macrocyst wall were observed; a clear zone (Figure 4Aa, arrowhead and 4Ad) and a zone filled with cells (Figure 4Aa, arrowed and 4Ae). From this, it is supposed that the cell fusion process could be involved also in the wall formation of the macrocyst. The macrocyst wall is considered to be a product synthesized by a giant cell between the cell membrane and the outermost fibrillar sheath (1). Both giant and periphery cells are possibly involved in the wall formation. However, at present, the accumulation of evidence is insufficient to clarify the precise mechanism of wall formation.

In the absence of a zyg1 gene in GFPCONT cells, how is the macrocyst wall formed in the macrocysts? Since the existence of a zyg1 homologue in V12M2 genome has been suggested by the genomic Southern analysis (21), ZYG1 could be supplied by V12M2 cells.

The idea of macrocyst wall formation by cell fusion of peripheral cells suggests that peripheral cells surrounding a giant cell might be necessary for the formation of transparent zones in a macrocyst wall. This may explain the reason why a globular body formed from a free-living giant cell has no thick transparent zones in a macrocyst wall. From the observations that the globular body is not surrounded by peripheral cells, cell fusion and subsequent vacuolization may not occur outside of a giant cell, thus resulting in lack of transparent zones of a macrocyst wall.

Recently, by BLAST search (26,27), uncharacterized ZYG1-like protein has been reported from prokaryotes such as Enterobacter cloacae (DDBJ/EMBL/GenBank, accession number AYIG01000002, E value: 3e-93) and Escherichia coli (DDBJ/EMBL/GenBank, accession number AWCH01000020, E value: 3e-91). As cellular slime molds except axenic strains such as Ax2, feed on bacteria phagocytotically as a food supply, their cells contain a lot of bacteria. It might be that a long time ago a bacterial zyg1 gene incorporated into the genome of cellular slime mold. Considering the functions of ZYG1, the information about cyst formation also might originate from bacteria. We hope that these interesting ideas will be further examined in the future.

**Competing interests**
The authors declare that they have no competing interests.
Authors’ contributions

<table>
<thead>
<tr>
<th>Authors’ contributions</th>
<th>AA</th>
<th>FT</th>
<th>TU</th>
<th>TA</th>
<th>YM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research concept and design</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Collection and/or assembly of data</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Data analysis and interpretation</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Writing the article</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Final approval of article</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Acknowledgement

We thank Dr. Ian G. Gleadall, Tohoku University, and Dr. Richard Halberstadt for their critical reading and helpful comments regarding this manuscript.

Publication history

Senior Editor: Tzi Bun Ng, The Chinese University of Hong Kong, China.
Received: 18-Aug-2014 Final Revised: 17-Sep-2014
Accepted: 11-Oct-2014 Published: 17-Oct-2014

References


Citation: