The *recA*-deficient *Dictyostelium discoideum* Forms Large Fruiting Bodies

YASUNA HASEGAWA¹, YUKITO MASAMUNE² and HIRO YASUKAWA^{1*}

¹ Division of Bioengineering, Faculty of Engineering, Toyama University, Toyama 930–8555, Japan

² Department of Microbiology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920–0934, Japan

(Received July 12, 2004—Accepted August 27, 2004)

The cellular slime mold *Dictyostelium discoideum* expresses a RecA homolog that is distributed in mitochondria. A gene disruption mutant is hypersensitive to DNA-damaging agents, indicating that RecA is required for the repair of mitochondrial DNA. We analyzed the process by which a multicellular structure of *recA*[−] cells is formed. Four hours after starvation, the internal glucose level of *recA*[−] cells (11.8 ± 0.9 nmol/mg protein) was higher than that of wild-type cells (6.7 ± 0.1 nmol/mg protein). The degree of cell-cell adhesion of *recA*[−] cells was greater than that of wild-type cells, while the migration speed of *recA*[−] cells (3.5 ± 0.2 $\mu\text{m}/\text{min}$) was less than that of wild-type cells (4.3 ± 0.3 $\mu\text{m}/\text{min}$). The increase in cell-cell adhesion and decrease in motility of *recA*[−] cells gave rise to the formation of large multicellular structures. The diameters of mounds formed by *recA*[−] and wild-type cells were 0.30 ± 0.01 mm and 0.17 ± 0.01 mm, respectively.

Key words: *Dictyostelium discoideum*, *recA*, tissue size

Organisms form bodies of appropriate sizes. Little is known, however, about the mechanisms by which the sizes of such structures are determined. The cellular slime mold *Dictyostelium discoideum* is a suitable model for studying the molecular basis of size control. *D. discoideum* is a predatory amoeba feeding on bacteria in soil. Individual amoebae grow as a unicellular free-living microorganism, but aggregate to form multicellular structures under conditions of starvation. During the aggregation, cells form a dendritic pattern of aggregation streams. If there are too many cells in a stream, the stream breaks up into groups. Each of the groups forms a cell mass on “mound”. A mound changes form into a “slug” and, then forms a fruiting body consisting of spores and a stalk³). Each mound consists of about 10^5 cells, indicating that *D. discoideum* has mechanisms that regulate the cell number in a multicellular structure. Factors that are thought to control the cell number in a group are Countin, Countin2 and Countin3 proteins encoded by *countin*, *countin2* and *countin3*, respectively. The multicellular structures formed by gene disruption mutants differ in size

from those formed by wild-type cells. *countin*[−] cells form large multicellular structures, whereas *countin2*[−] cells and *countin3*[−] cells form small multicellular structures^{2,7,9,11}). Results of analyses of these mutants have contributed to an understanding of the mechanisms controlling the sizes of multicellular structures. In addition to analyses of these mutants, it is important to characterize other mutants that exhibit differences in sizes of multicellular structures in order to determine the molecules and signal pathways responsible for size control.

We have identified a RecA homolog of *D. discoideum*⁵). Fluorescence of *D. discoideum* RecA fused with GFP was detected in mitochondria. A gene disruption mutant showed hypersensitivity to UV light, H₂O₂ and cisplatin. These findings indicate that *D. discoideum* RecA is required for the repair of mitochondrial DNA⁵). Upon starvation, *recA*[−] cells formed larger multicellular structures than did wild-type cells.

We present here results obtained from assays for cell-cell adhesion, cell motility and glucose levels of *recA*[−] cells.

* Corresponding author; E-mail: hiro@eng.toyama-u.ac.jp, Tel: +81-76-445-6875, Fax: +81-76-445-6863

Materials and Methods

D. discoideum strains and culture conditions

D. discoideum Ax2 was used as a wild type in the experiments. Ax2 was cultured in HL5 medium (0.04% KH_2PO_4 , 0.05% Na_2HPO_4 , 0.7% yeast extract, 1.4% bacteriological peptone and 1% glucose, pH 6.5). The *recA*⁻ strain was established as described in a previous report⁵. The null cells were maintained in HL5 containing 5 mg/l of blasticidin S (Funakoshi, Tokyo, Japan).

Adhesion assay

Cells were grown in HL5 medium to a density of 5×10^6 cells/ml. Then the cells were harvested, washed with a phosphate buffer (1.66 g of NaH_2PO_4 in 1 L of H_2O , pH adjusted to 6.1 with KOH), suspended in the buffer at a density of 5×10^6 cells/ml and shaken slowly (60 rpm) for 8 h. The cells which did not aggregate were counted under a microscope.

Migration assay of the cells

D. discoideum cells grown in HL5 were collected, washed and suspended in the phosphate buffer. A portion of the cell suspension was placed on a slide glass. Four hours after starvation, the cells were photographed at 2-min intervals (for 20 min) and traced.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA samples were prepared from cells starved for 2, 4 and 6 h and used in RT-PCR analyses. RT-PCR was performed using a One-Step RNA PCR Kit (Takara Shuzo, Kyoto, Japan). The primers and the reaction conditions were described in previous reports^{5,9}. Reaction products were electrophoresed to analyze the signal intensity.

Glucose assay

Cells were collected, suspended in the phosphate buffer and centrifuged. The pellets were lysed by freezing and thawing. The supernatants of the lysates were prepared by centrifugation and used in glucose assays. For the assays, the Glucose CII Kit (Wako, Tokyo, Japan) was used. Amounts of protein in the samples were assayed by the use of the Coomassie Protein Assay Kit (Pierce, IL, USA). These assays were performed according to the manufacturer's instructions.

Results

Size of the multicellular structures formed by the *recA*⁻ cells

The developmental process of *D. discoideum* plated on agar is shown in Fig. 1. *D. discoideum* cells aggregate and form a multicellular structure consisting of about 10^5 cells. To study the mechanism by which the size of the structure is determined, we have established and characterized mutants that show differences in the sizes of multicellular structures.

The *recA*-deficient mutant is one of those mutants. To analyze the mound size, equal numbers of Ax2 and *recA*⁻ cells were separately plated on non-nutrient agar, and incubated at 20 degrees C. Cells entered the multicellular stage of development under these conditions. The mounds formed on the plates were photographed to measure the diameters. The diameter of the mound formed by the wild type was 0.17 ± 0.01 mm, while that of the formed by the null was 0.30 ± 0.01 mm (Fig. 2A). The *p* value obtained from a *t* test was less than 0.01. An illustration of typical fruiting bodies observed under a microscope is shown in Fig. 2B. *recA*⁻ cells formed larger fruiting bodies than wild-type cells.

We assayed two null clones that had been established independently. These clones showed the same phenotype, indicating that disruption of the *recA* gene is responsible for the formation of a large multicellular structure.

Aggregate formation in a slow shaking buffer

Computer simulations and experimental results have demonstrated that the sizes of the multicellular structures of *D. discoideum* are determined by cell-cell adhesion¹⁰. We carried out an experiment to determine whether cell-cell ad-

Fig. 1. Developmental program of *D. discoideum*.

Diagrams illustrate successive stages in development. *D. discoideum* cells aggregate and form a mound consisting of about 10^5 cells. A mound changes its form to a slug. The slug migrates on agar until it initiates terminal differentiation. The slug changes its form to a mature fruiting body consisting of oval spores supported by a stalk made up of vacuolated stalk cells.

Fig. 2. Morphological phenotype of *recA*⁻.

(A) Size of the mounds formed on a plain agar plate. Equal numbers of Ax2 and *recA*⁻ cells were plated separately on non-nutrient agar (1.6×10^6 cells/cm²). The diameters of mounds were compared between the strains. The values are the means $\pm$ S.E. from 20 mounds (Ax2) and 20 mounds (*recA*⁻). (B) Illustration of fruiting bodies formed by the wild-type and *recA*⁻ cells. Fruiting bodies on agar plates were observed under a microscope and illustrated.

hesion is increased in *recA*⁻ cells. Cells cultured in HL5 medium were harvested, washed, suspended in phosphate buffer, and shaken slowly. Cells were starved, expressed cell-cell adhesion proteins, and formed aggregates under the experimental conditions. The aggregates in the buffer were photographed at 8 h after starvation. The aggregates of *recA*⁻ cells were larger than those of wild-type cells (Fig. 3A). Single cells, the cells that did not participate in the aggregation, were observed in the samples. But the number of single cells of *recA*⁻ was less than that of the wild type. The number of single cells in 8-h samples was counted under a microscope. The population of the single Ax2 cells decreased to $21.0 \pm 1.8\%$, while that of the single *recA*⁻ cells decreased to $12.0 \pm 3.0\%$ (Fig. 3B). The p value obtained from a t test was less than 0.05. These results indicate that cell-cell adhesion was increased in the mutant.

Expression of gp24 and gp80 in the cells

Cell-cell adhesion is mediated by gp24 and gp80 proteins during the process of aggregation¹⁾. We compared the expression levels of the genes for these proteins in the strains. Cells shaken slowly in buffer were recovered at 2, 4 and 6 h to extract total RNA. Expression levels of the genes for gp24 and gp80 were analyzed by RT-PCR. RT-PCR products amplified by various cycles after reverse transcription were resolved by gel electrophoresis, and their signal inten-

sities were analyzed. Ax2 expressed gp24 in the early stages of starvation and then the expression level of gp80 increased. The expression levels of the genes were higher in *recA*⁻ cells than wild-type cells (Fig. 4). The reaction products derived from *actin15* mRNA⁸⁾ are shown as a control.

Migration of the cells

Motility is also thought to be important for the size control of *D. discoideum*. If the ratio of random cell motility force to cell-cell adhesion force is low, the aggregation stream of the cells will stay intact. But if this ratio is high, the random motility will overcome the adhesion and the aggregation stream will dissipate¹⁰⁾. We carried out an experiment to determine whether the motility is altered in *recA*⁻ cells. Cells were placed on a slide glass with buffer and starved for 4 h. They were then photographed at 2-min intervals under a microscope. Wild-type cells migrated at 4.3 ± 0.3 μ m/min, while *recA*⁻ cells migrated at 3.5 ± 0.2 μ m/min. The p value obtained from a t test was less than 0.05. The motility of *recA*⁻ cells was lower than that of wild-type cells.

Internal glucose levels of the cells

The Countin protein has been identified as a factor that down-regulates the size of the multicellular structure²⁾. *countin*⁻ cells have increased cell-cell adhesion and de-

Fig. 3. Cell-cell adhesion of Ax2 and *recA*⁻.

(A) Aggregates formed in the phosphate buffer. Cells were suspended in the buffer and shaken slowly. Cells photographed at 0 h and 8 h are shown. Bars, 0.2 mm. (B) Population of the single cells in the buffer at 8 h. The single cells in the samples were counted. The values are the means $\pm$ S.E. from 4 experiments (Ax2) and 3 experiments (*recA*⁻).

Fig. 4. Expression of *gp24* and *gp80*.

Expression kinetics of *gp24* and *gp80* in the strains were compared. Cells shaken slowly in the phosphate buffer were recovered at 2, 4 and 6 h to extract total RNA. The RT-PCR was conducted for 14–24 cycles with the use of specific primers. The reaction products of 16 cycles are presented.

creased motility, resulting in the formation of large multicellular structures^{6,10,12}. The phenotype of *countin*⁻ cells is similar to that of *recA*⁻ cells. The internal glucose level of *countin*⁻ cells is higher than that of wild-type cells¹⁰. These observations suggest that the internal glucose level is increased in *recA*⁻ cells. We therefore compared the levels in *recA*⁻ and wild-type cells. Vegetative cells in a medium and aggregating cells in a buffer were collected to measure glucose levels. Internal glucose levels of the growing cells

were 11.0 ± 0.8 nmol/mg protein (wild type) and 18.3 ± 1.1 nmol/mg protein (*recA*⁻). The glucose levels at 4 h after the starvation were 6.7 ± 0.1 nmol/mg protein (wild type) and 11.8 ± 0.9 nmol/mg protein (*recA*⁻) (Fig. 5). The *p* values are indicated in the figure. The glucose level was higher in *recA*⁻ cells than wild-type cells.

Expression of countin in the cells

We compared the expression levels of the *countin* gene in the strains. Cells cultured in HL5 medium were recovered to extract total RNA for RT-PCR. RT-PCR products amplified by various cycles after reverse transcription were resolved by gel electrophoresis, and their signal intensities were analyzed. The experimental results indicated that *recA*⁻ cells expressed *countin* at almost the same level as did the wild-type cells (Fig. 6). The reaction products derived from *recA* mRNA and *actin15* mRNA are also shown.

Discussion

Garrod and Ashworth⁴) showed that wild-type cells grown with a high concentration of glucose formed larger fruiting bodies than did control cells. Jang *et al.* reported that exogenous glucose increased the cellular glucose

Fig. 5. Glucose levels in the cells.

Glucose levels in the cells cultured in a medium (V) and starved for 4 h in a buffer (4 h) were analyzed. The values are the means $\pm$ S.E. obtained from three independent assays.

Fig. 6. Expression of *countin*.

Expression levels of *countin* in the strains were compared. Cells grown in HL5 medium were recovered to extract total RNA. The RT-PCR was conducted for 16–24 cycles with the use of specific primers. The reaction products of 18 cycles (*countin* and *recA*) and 16 cycles (*actin15*) are presented.

level⁶). Cells that had a high concentration of glucose showed increased cell-cell adhesion and decreased cell motility⁶). Thus, the intercellular glucose level seems to determine the size of the multicellular structure by controlling cell-cell adhesion and cell motility. We found in the present study that *recA*⁻ cells have higher cell-cell adhesion and lower cell motility than do wild-type cells and that they formed larger multicellular structures than wild-type cells. The phenotype of the cells would be the result of an increased glucose level.

It is presently not clear why the internal glucose level is increased in *recA*⁻ cells. The amount of *countin* mRNA in *recA*⁻ cells was almost the same as that in wild-type cells. This finding suggests that Countin protein is not responsible for the increase in glucose levels in *recA*⁻ cells. However, the possibility that the translation of Countin protein is down-regulated in *recA*⁻ cells cannot be ruled out. A study to determine the mechanisms by which the glucose level is increased in *recA*⁻ cells is in progress.

Acknowledgment

We thank H. Asashima (Toyama University) for technical support.

References

- 1) Beug, H., F.E. Katz, A. Stein and G. Gerisch. 1973. Quantitation of membrane sites in aggregating *Dictyostelium* cells by use of tritiated univalent antibody. *Proc. Natl. Acad. Sci. USA* **70**: 3150–3154.
- 2) Brock, D.A. and R.H. Gomer. 1999. A cell-counting factor regulating structure size in *Dictyostelium*. *Genes Dev.* **13**: 1960–1969.
- 3) Escalante, R. and J.J. Vicente. 2000. *Dictyostelium discoideum*: a model system for differentiation and patterning. *Int. J. Dev. Biol.* **44**: 819–835.
- 4) Garrod, D.R. and I.M. Ashworth. 1972. Effect of growth conditions on development of the cellular slime mould, *Dictyostelium discoideum*. *J. Embryol. Exp. Morphol.* **28**: 463–479.
- 5) Hasegawa, Y., M. Wakabayashi, S. Nakamura, K. Kodaira, H. Shinohara and H. Yasukawa. 2004. A homolog of *Escherichia coli* RecA in mitochondria of the cellular slime mold *Dictyostelium discoideum*. *DNA Repair* **3**: 515–525.
- 6) Jang, W., B. Chiem and R.H. Gomer. 2002. A secreted cell number counting factor represses intracellular glucose levels to regulate group size in *Dictyostelium*. *J. Biol. Chem.* **277**: 39202–39208.
- 7) Katayama, T., A. Takano, T. Okuwa, M. Issaeva, Y. Masamune, K. Kodaira and H. Yasukawa. 2003. *Dictyostelium discoideum* (*countin3*⁻) forms small fruiting bodies. *Microbes Environ.* **18**: 223–226.
- 8) Knecht, D.A., S.M. Cohen, W.F. Loomis and H.F. Lodish. 1986. Developmental regulation of *Dictyostelium discoideum* *actin* gene fusions carried on low-copy and high-copy transformation vectors. *Mol. Cell. Biol.* **6**: 3973–3983.
- 9) Okuwa, T., T. Katayama, A. Takano, K. Kodaira and H. Yasukawa. 2001. Two cell-counting factors regulate the aggregate size of the cellular slime mold *Dictyostelium discoideum*. *Dev. Growth Differ.* **43**: 735–744.
- 10) Roisin-Bouffay, C., W. Jang, D.R. Caprette and R.H. Gomer. 2000. A precise group size in *Dictyostelium* is generated by a cell-counting factor modulating cell-cell adhesion. *Mol. Cell* **6**: 953–959.
- 11) Takano, A., Y. Akaza, Y. Hasegawa, M. Issaeva, Y. Masamune, K. Kodaira, H. Shinohara and H. Yasukawa. 2004. Expression of genes coding for cell-cell adhesion proteins in *Dictyostelium discoideum* mutants that form small fruiting bodies. *Microbes Environ.* **19**: 76–78.
- 12) Tang, L., T. Gao, C. McCollum, W. Jang, M.G. Vicker, R.R. Ammann and R.H. Gomer. 2002. A cell number-counting factor regulates the cytoskeleton and cell motility in *Dictyostelium*. *Proc. Natl. Acad. Sci. USA* **99**: 1371–1376.