Original

Difference in sensitivity to amino acid starvation between the wild type and *sir2A*-null mutant of *Dictyostelium discoideum*

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SUMMARY

Dictyostelium discoideum transits from growth phase to developmental phase when nutrients are depleted. *D. discoideum* under a starved condition aggregates and forms a cell mass called mound within 12 h and then forms a fruiting body. The nutrients critical for the transition are amino acids. Thus, *D. discoideum* is thought to have a mechanism for sensing amino acid starvation. We examined in the current study the function of Sir2A in the initiation of development. The wild type initiated development and formed mounds within 12 h even in the presence of 1 ~ 3 mg/ml amino acids, while the null mutant did not form mounds within 12 h in the presence of 2 mg/ml amino acid. These results suggest that the null mutant is less sensitive to starvation than is the wild type and that Sir2A plays an important role in the recognition of amino acid starvation.

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INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* is a soil-dwelling microorganism. *D. discoideum* cells grow as unicellular free-living amoebae in the presence of nutrients. Upon starvation, amoebae aggregate and form a cell mass consisting of approximately 10^5 cells. The cell mass, called a 'mound', changes its form to a slug and migrates as a multicellular organism. Then the slug changes its form to a fruiting body (Firtel, 1995, 1996; Kessin, 2001). The nutrients critical for the transition from growth phase to developmental phase are amino acids. Amino acid starvation is the specific stimulus that initiates the developmental phase of the life cycle of *D. discoideum* (Marin, 1976). *D. discoideum* is therefore thought to have a mechanism for sensing amino acid starvation. However, proteins responsible for the mechanism are not well understood.

We have analyzed silent information regulator 2 (Sir2) proteins in *D. discoideum*. Sir2 proteins are an evolutionarily conserved family of NAD-dependent protein deacetylases (Brachmann et al., 1995; Imai et al., 2000). By deacetylating histone and other proteins, Sir2 proteins play important roles in various biological processes such as gene expression, apoptosis, metabolism and aging (Blander and Guarente, 2004).

Analysis of genomic databases has revealed that *D. discoideum* encodes at least four Sir2 homologs named Sir2A, Sir2B, Sir2C and Sir2D. Reverse transcription-PCR (RT-PCR) and whole-mount *in situ* hybridization (WISH) analyses showed that the genes for Sir2A, Sir2C and Sir2D were expressed at high levels in growing cells but at decreased levels in developing cells, whereas the gene encoding Sir2B was expressed in the prestalk -cell region in the developmental phase (Katayama and Yasukawa, 2008). We have tried to establish gene disruption mutants and overexpressing mutants to determine the importance of Sir2 proteins in *D. discoideum*.

In this report, we show the results obtained from analysis of a *sir2A*-disruption mutant. This mutant exhibits different sensitivity to amino acid starvation from that of the wild type. It is thought that Sir2A plays an important role in sensing amino acid starvation.

MATERIALS AND METHODS

Culture conditions

For dish culture, Ax2, an axenic strain of *D. discoideum*, was cultured in HL5 liquid medium (Watts and Ashworth, 1970), and the gene disruption mutant was maintained in HL5 supplemented with 5 mg/l blasticidin S (BS). For shaking culture, cells were grown in HL5 or HL5 containing the drug with shaking at 150 r.p.m. Cell culture was carried out at 20°C.

Fruiting body formation

For differentiation on bacterial lawns, cells grown in medium were collected, washed with a buffer (1.66 g/l NaH_2PO_4, pH adjusted to 6.1 with KOH), diluted, and spread on SM plates (10 g/l Bactopeptone, 1 g/l Yeast extract, 1.9 g/l KH_2PO_4, 1.0 g/l K_2HPO_4, 0.5 g/l $MgSO_4$, 10 g/l Glucose, 17 g/l agar) with *Escherichia coli* WA802 (*metB1 lac3 lacY1 galK2 galT22 supE hsdR2*). For differentiation on agar plates, cells grown in medium were collected, washed with the buffer, and plated on SM plates or non-nutrient agar plates at a density of 1 x 10^6 cells/cm^2. Amino acid mixture was added to the agar plates if necessary. The mixture of amino acids contained 18 of the 20 naturally occurring amino acids. Cysteine and tyrosine were omitted from routine experiments because of low stability and solubility, respectively. Cells were incubated at 20°C.

Gene disruption

The construct for disruption of the gene encoding Sir2A was generated as follows. The coding region was amplified from the Ax2 genome by PCR using the primers 5'-ATAAAGCTTATGTACGCAGTGAATCCAATTGAA and 5'-AATGAATTCGAACTATTTTGGATATTTACAATATG. A gene cassette for blasticidin S resistance (a *bsr* cassette; 1.35-kb in size) (Sutoh, 1993) was inserted into the *Spe*I site of the Sir2A coding region. The gene disruption construct was introduced into Ax2 cells by electroporation. Cells in which the gene was disrupted were selected in HL5 supplemented with blasticidin S. Gene disruption was confirmed by PCR and RT-PCR using the primers 5'-GTGCTGGTATCAGTGTTGCTGCTGGTATTC and 5'-CCTCACGTGCACAATCATTGAATCTTGATGGC. RT-PCR for *rnlA* (Ogawa et al., 2000) was carried out as a control using the primers 5'-TTACATTTATTAGACCCGAAACCAAGCG and 5'-TTCCCTTTAGACCTATGGACCTTAGCG. RT-PCR was performed with a One Step RT-PCR Kit (Takara Bio, Shiga, Japan) using total RNA

isolated from cells.

RESULTS AND DISCUSSION

Establishment of a *sir2A*-null mutant

We investigated the importance of Sir2A in *D. discoideum* by transforming Ax2 with a knock-out construct of the gene that was disrupted by a *bsr* cassette (Fig. 1A). The cells were cultured in HL5 supplemented with BS, and genomic DNA extracted from a selected clone was used as a template for PCR. Comparison of the sizes of the PCR products indicated that the gene in the BS-resistant strain was disrupted by insertion of the 1.35-kb fragment (Fig. 1B). RT-PCR using total RNA as a template also indicated that *sir2A* was expressed in the wild-type cells but not in the BS-resistant strain (Fig. 1C). These results indicate that the *sir2A* gene in the strain tested was disrupted by insertion of the *bsr* cassette.

We established two independent clones. Since they exhibited the same phenotype, we show in this report the experimental results obtained from one of these clones. The *sir2A*-null mutant was maintained stably in liquid medium. The growth rate of the mutant was almost the same as that of the wild type (data not shown), indicating that Sir2A is not essential for growth in *D. discoideum*.

Development of null cells

D. discoideum amoebae were spread on SM plates with bacteria, incubated, and photographed (Fig. 2A). The wild-type amoebae formed plaques on the bacterial lawns. Inside the plaques, where the bacteria were depleted, *D. discoideum* formed

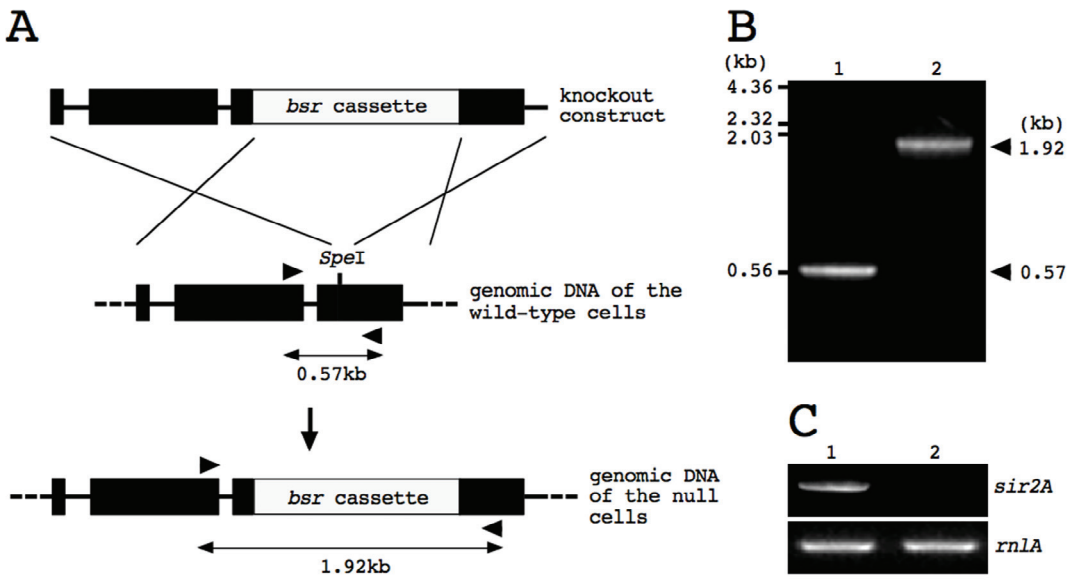

Fig. 1. Establishment of the *sir2A*-null mutant.
(A) Construct used for gene disruption. Black boxes indicate exons. The *bsr* cassette (1.35 kb) was inserted into the *Spe*I site in the *sir2A* coding region. Arrowheads indicate the primers used in PCR and RT-PCR, which were carried out to confirm gene disruption. (B) PCR analysis. (C) RT-PCR analysis. Amplification of *rnlA* was performed as a control. In (B) and (C), lane 1 contains products from Ax2, and lane 2 contains products from the *sir2A*-null mutant.

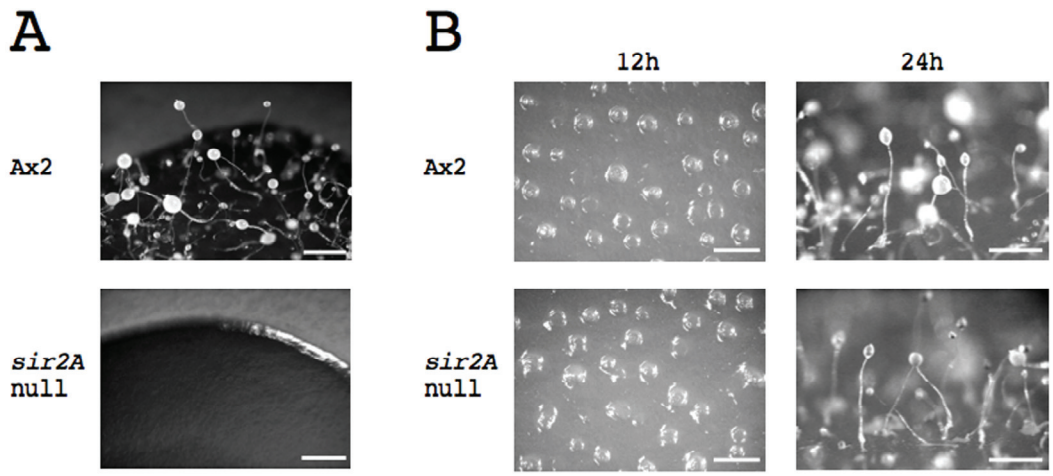

Fig. 2. Morphological phenotype of Ax2 and the *sir2A*-null mutant. (A) *D. discoideum* cells were axenically grown in HL5, harvested, diluted, and spread on SM plates with *E. coli*. (B) Amoebae cultured in HL5 were collected, washed, and plated on plain agar plates. Mounds and fruiting bodies formed by the *D. discoideum* cells were photographed at the indicated times after incubation. The bars indicate 0.5 mm.

fruiting bodies. The *sir2A*-null mutant also formed plaques but did not aggregate. We confirmed that the null mutant did not aggregate on SM plates without bacteria (data not shown). This observation eliminates the possibility that bacterial load or a bacterial metabolite inhibits the development of the null mutant.

We then examined whether the null mutant could develop on a non-nutrient agar plate. Log-phase cells were harvested from a shaking culture and plated on plain agar plates. Both the wild-type cells and null-mutant cells initiated development and formed mounds within 12 h and completed fruiting body formation within 24 h (Fig. 2B). These results indicate that a factor(s) in the SM plate inhibited the initiation of development of the mutant.

Sensitivity to amino acid starvation

Amino acids are the only nutrients that inhibit the initiation of development. Under a starved condition, *D. discoideum* usually initiates the developmental process, aggregates and forms mounds within 12 h. We determined in the next experiments the difference in sensitivity to amino acid starvation between the wild type and the null mutant. *D. discoideum* cells were plated on agar containing various concentrations of amino acids, incubated, and photographed (Fig. 3). The wild-type cells aggregated and formed mounds normally on the agar plates even in the presence of 1 ~ 3 mg/ml amino acids. No aggregate was observed at 12 h on the agar containing more than 4 mg/ml amino acids.

The null cells formed mounds within 12 h on the agar plates containing 1 mg/ml amino acids. On the agar containing more than 2 mg/ml amino acids, no aggregate was observed 12 h after the starvation. We obtained almost the same results from three sets of experiments.

Our results suggest that the wild-type cells recognize amino acid starvation at 3 mg/ml, whereas the *sir2A*-null cells do so when the concentration of amino acids falls to 1 mg/ml. Thus, it

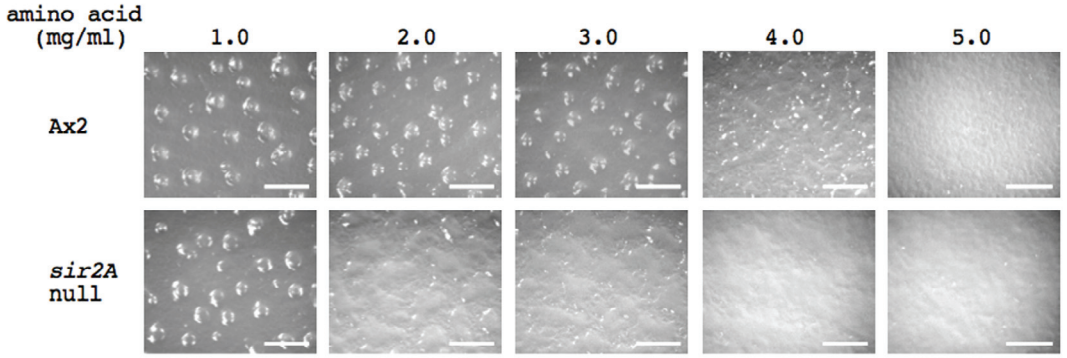

Fig. 3. Development of Ax2 and the *sir2A*-null mutant in the presence of amino acids. Amoebae grown in liquid medium were collected, washed, and plated on agar plates containing various concentrations of amino acids. Multicellular structures formed on the plates were photographed at 12 h after the plating. The bars indicate 0.5 mm.

appears that null cells are less sensitive to starvation than are wild-type cells.

In summary, the current results indicate that Sir2A is dispensable for growth but plays an important role in the sensing of amino acid starvation. Further analysis to determine the function of Sir2A in the transition from growth phase to developmental phase is in progress.

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REFERENCES

Blander, G. and Guarente, L. (2004) The Sir2 family of protein deacetylases. Annu. Rev. Biochem., 73, 417-435.

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L. and Boeke, J.D. (1995) The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev., 9, 2888-2902.

Firtel, R.A. (1995) Integration of signaling information in controlling cell-fate decisions in *Dictyostelium*. Genes Dev., 9, 1427-1444.

Firtel, R.A. (1996) Interacting signaling pathways controlling multicellular development in *Dictyostelium*. Curr. Opin. Genet. Dev., 6, 545-554.

Imai, S., Armstrong, C.M., Kaeberlein, M. and Guarente, L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature, 403, 795-800.

Katayama, T. and Yasukawa, H. (2008) Developmental and spatial expression of *sir2* genes in the cellular slime mold *Dictyostelium discoideum.* Microbes Environ., 23, 40-43.

Kessin, R.H. (2001) *Dictyostelium*-evolution, cell biology, and the development of multicellularity. Cambridge University Press. Cambridge.

Marin, F.T. (1976) Regulation of development in *Dictyostelium discoideum*: I. Initiation of the growth to development transition by amino

acid starvation. Dev. Biol., 48, 110-117.

Ogawa, S., Yoshino, R., Angata, K., Iwamoto, M., Pi, M., Kuroe, K., Matsuo, K., Morio, T., Urushihara, H., Yanagisawa, K. and Tanaka, Y. (2000) The mitochondrial DNA of *Dictyostelium discoideum*: complete sequence, gene content and genome organization. Mol. Gen. Genet., 263, 514-519.

Sutoh, K. (1993) A transformation vector for *Dictyostelium discoideum* with a new selectable marker *bsr*. Plasmid, 30, 150-154.

Watts, D.J. and Ashworth, J.M. (1970) Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J, 119, 171-174.