In Situ Analysis of the Membrane-binding Region of Multiple Mitochondrial DNA Molecules in the Mitochondrial Nucleus of *Physarum polycephalum*

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A single mitochondrion contains multiple mitochondrial DNA (mtDNA) molecules in vertebrates (review for Clayton 1991), yeast (Stevens 1977), tobacco (Satoh *et al.* 1993) and slime mold (Kawano *et al.* 1983, Sasaki *et al.* 1994). These multiple mtDNA molecules are organized with proteins to form mitochondrial nuclei (mt-nuclei, review for Kuroiwa 1982) in which replication and segregation of multiple mtDNA molecules are orchestrated. By analogy with the bacterial chromosome (Jacob *et al.* 1963), it has been assumed that the association of mtDNA with the mitochondrial membrane plays an important role in the mtDNA replication and the segregation of daughter mtDNA molecules (Kuroiwa *et al.* 1994). The association of mtDNA with the mitochondrial membrane in intact cells has been observed in thin sections with electron microscopy (Nass *et al.* 1965, Kuroiwa *et al.* 1977). Thin sections, however, fail to clearly show the association of multiple mtDNA molecules in the mt-nucleus with the membrane in a whole mitochondrion.

The mitochondrion in the slime mold *Physarum polycephalum* has a rod-like mt-nucleus which contains 18–32 copies of mtDNA (Kawano *et al.* 1983, Sasaki *et al.* 1994). The mitochondrial membrane-DNA complex of *P. polycephalum* was isolated and restriction endonuclease analysis of it identified a specific region of mtDNA involved in interaction with the mitochondrial membrane (Kawano and Kuroiwa 1985, Kuroiwa *et al.* 1994). This membrane-binding region (MBR) was mapped to a 19.6-kbp tandem duplication located on both ends of the 86-kbp linear mtDNA molecule (Takano *et al.* 1990, Kuroiwa *et al.* 1994).

To clarify the organization of these multiple MBRs in the mt-nucleus, we localized the sites of the MBRs in the mt-nucleus by fluorescence *in situ* hybridization (FISH) with a DNA probe specific for the MBR. We found that the MBRs were clustered at a few discrete sites along the mt-nucleus and each cluster contained the MBRs of 10 mtDNA molecules.

Materials and methods

Cultures of cells

An axenic culture of amoebae cell of *P. polycephalum*, strain LU352, was a kind gift from Dr. J. Dee. The amoebae cells were cultured with shaking in axenic medium at 22°C as described by Dee (1986).

Microplasmodia of *P. polycephalum*, Colonia isogenic strain KM 182×KM 187, were cultured in liquid medium at 23°C as described by Daniel and Baldwin (1964).

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Observation of cells

Cells were fixed on a glass slide in 0.6% glutaraldehyde and stained with 1 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI) in S buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.7, 1 mM EDTA, pH 7.5, 7 mM 2-mercaptoethanol, 0.75 mM spermidine and 0.4 mM PMSF) and observed under an epifluorescence microscope equipped with a phase-contrast objective (BHS-RFC; Olympus Optical Co., Tokyo, Japan).

In situ hybridization and signal detection

Cells were washed with distilled water three times and fixed with Carnoy's solution (25% glacial acetic acid in ethanol) for 10 min. A drop of fixed cells was then spread on a coverslip, and the coverslip was passed rapidly through a flame to burst and fix the samples. *In situ* hybridization was performed by the method of Hizume *et al.* (1992). A 4.7-kbp *Xba*I/*Sph*I fragment that overlaps a 1.7-kbp region of the MBR of *P. polycephalum* mtDNA (Takano 1990) was used as a probe. DNA was labeled with digoxigenin (DIG)-labeled deoxyuridine-triphosphate using a random primer DNA labeling system (Boehringer Mannheim Biochemicals, Mannheim, Germany). The sample was heat-denatured at 70°C for 5 min and then hybridized with the DIG-labeled DNA probes at 37°C for 12 h. After hybridization, the sample was washed with 50% formamide in 2 $\times$ SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for 10 min at 37°C and with 2 $\times$ SSC for 10 min twice. The sample was washed three times in PBS (0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.2) and blocked with 15 μl of blocking solution (PBS containing 5% BSA and 0.02% Tween 20) at 25°C for 30 min. Then the sample was incubated at 37°C for 1 h with primary anti-digoxigenin mouse monoclonal antibody (Boehringer Mannheim Biochemicals, Mannheim, Germany), which was diluted to 1:40 in blocking solution. After washing and blocking again, the sample was incubated at 37°C for 30 min with secondary anti-mouse-immunoglobulin goat antibody conjugated with fluorescein isothiocyanate (FITC, Tago Inc., CA, USA) which was diluted to 1:40 in blocking solution. The sample was then washed in PBS, stained with DAPI and observed with an epifluorescence microscope as described above.

Estimation of the amount of mtDNA and the length of the mt-nucleus

Cells were stained with DAPI as described above. The fluorescence intensity emitted from the mt-nuclei was measured with a video-intensified microscope photon-counting system (VIMPCS; Hamamatsu Photonics Ltd. Hamamatsu, Japan) connected to an epifluorescence microscope, as described previously (Kuroiwa *et al.* 1986). To evaluate the fluorescence intensity of mt-nuclei, T4 phage was used as a DNA standard marker (1T=170 kbp: Freifelder 1970). Since the *P. polycephalum* mtDNA is 86 kbp in size (Takano *et al.* 1990), 1T corresponds to about 2 copies of mtDNA. At the same time, the length of each mt-nucleus was measured on the video frame of VIMPCS.

Results and discussion

The life cycle of *P. polycephalum* is represented by two distinct forms; the haploid uninucleate amoebae and the diploid syncytial plasmodium (reviewed by Goodman 1980). Figures 1a and 1b show DAPI-stained DNA fluorescence images of the amoeba and the plasmodium, respectively. In both the amoeba and the plasmodium, the rod-shaped mt-nuclei were observed around the cell nuclei. The mt-nucleus of the amoeba and the plasmodium contains about 18–24 and 32 mtDNA molecules, respectively, at the logarithmic phase of growth (Kawano *et al.* 1983, Sasaki *et al.* 1994).

We used FISH with a DIG-labeled DNA probe specific for the MBR to visualize the sites of the MBRs in the mt-nucleus of amoebae and plasmodia (Figs. 1c–n). When the mt-nuclei were stained with DAPI after FISH, uneven fluorescence intensities were seen in the mt-nuclei (Figs. 1c–

Fig. 1. (a, b) DAPI-stained DNA fluorescence image of amoebae (a) and the plasmodium (b) of *P. polycephalum*. The arrowheads indicate a dividing mt-nucleus. CN, cell nucleus; MN, mt-nucleus. Bar, 3 μ m. (c–n) The localization of MBRs in the mt-nucleus of amoebae (c–h) and plasmodium (i–n). The sites of MBRs in the mt-nucleus were visualized by the FISH with DIG-labeled DNA probe specific for MBR. Sites of MBRs in mt-nuclei (f–h and l–n) are shown together with DAPI-stained DNA fluorescence images (c–e and i–k). The sites of MBRs were detected as a few globular fluorescence along the mt-nucleus. The number of fluorescence in a mt-nuclei increased with an increase in the length of the mt-nucleus. Bar, 1 μ m.

e, i–k), which probably reflected sites at which DNA probes had hybridized to denatured mtDNA because DAPI stains double stranded DNA. Fluorescence intensities were uniform after FISH without DNA probes (data not shown). In spite of high copy numbers of mtDNA in the mt-nucleus, the sites at which MBR probes had hybridized were observed as a few fluorescence globules distributed along the mt-nuclei (Figs. 1f–h, 1l–n). This indicates that MBRs of mtDNA are not distributed randomly throughout the mt-nucleus but are clustered at a few discrete sites along the mt-nucleus.

The number of sites of fluorescence in a mt-nucleus increased with an increase in the length of the mt-nucleus (Figs. 1c–n). Since the length of the mt-nucleus increases as the amount of mtDNA in the mt-nucleus increases (Sasaki *et al.* 1994), the amount of mtDNA in the mt-nucleus can be estimated from the length. Therefore, we measured the length of the mt-nucleus and quantified the amount of mtDNA in 3600 mt-nuclei as described in Materials and methods. It was determined that a mt-nucleus of one- μ m in length contained about 12 mtDNA molecules. Fixation with Carnoy's solution for FISH, however, decreased 0.6 time the length of the mt-nuclei such that one- μ m mt-nucleus contained about 20 mtDNA molecules. In order to quantify the number of MBRs within a MBR cluster in both amoebae and plasmodia, we investigated the relationship between the number of MBR clusters and the copy number of mtDNA per mt-nucleus (Fig. 2). In both amoebae and plasmodia, the number of MBR clusters per mt-nucleus was proportional to the amount of mtDNA per mt-nucleus and one MBR cluster consisted of the MBRs of about 10 mtDNA molecules.

Figure 3 is a model of the organization of multiple mtDNA molecules in a mt-nucleus of *P. polycephalum*. The MBR clusters consisting of MBRs of 10 mtDNA molecules are distributed at a few discrete sites along the mt-nuclei. While the 3.5-kbp MBRs are located in the 19.6-kbp tandem duplication region on opposite ends of mtDNA molecule (Takano *et al.* 1990, Kuroiwa *et al.* 1994). Two MBRs of a single mtDNA probably lie adjacent to each other at an MBR cluster. Electron microscopy of serial sections of intact cells indicated that some mtDNA molecules were apparently attached to the mitochondrial inner membrane (Nass *et al.* 1965, Kuroiwa *et al.* 1977). Therefore, the

Fig. 2. Relationship between the number of MBR cluster and the copy number of mtDNA in the mt-nuclei of amoebae (○) and plasmodia (●), respectively. The copy number of mtDNA per mt-nucleus was calculated from the length of the mt-nucleus, which is exactly proportional to its DNA content. Values are presented as the mean $\pm$ s.d.

Fig. 3. A model for the organization of multiple mtDNA molecules in the mt-nucleus. The mitochondrion of *P. polycephalum* has a mt-nucleus containing 18–32 mtDNA molecules. There are a few units consisting of adjacent 10 mtDNA molecules in the mt-nucleus and each unit associates with the membrane at one site of inner membrane.

MBRs of 10 mtDNA molecules may be clustered at one site of inner membrane. The multiple mtDNA molecules in the mt-nucleus should be organized into a few units, each of which is composed of 10 mtDNA molecules, with one MBR cluster. This organization of mtDNA in the mt-nucleus may not change throughout the life cycle of *P. polycephalum*.

The replication of multiple mtDNA molecules in the mt-nucleus is regulated separately in groups of 10 mtDNA molecules termed mitochondrial replicon clusters in both amoebae (Sasaki *et al.* 1994) and plasmodia (unpublished data). Since the copy number in the unit of mtDNA molecules with one MBR cluster is similar to that in the mitochondrial replicon cluster, these may repre-

sent the same mt-nuclear structure. This suggests that the organization of mtDNA in the mt-nucleus described here may be important for the regulation of mtDNA replication.

The MBR appears to play an important role in the segregation of mtDNA because the association of mtDNA with the membrane is important for the regulation of mt-nuclear division (Kawano and Kuroiwa 1985) and the MBR is very rich in A-T base pairs, like the centromere which functions in the segregation of chromosomes in the cell nuclei (Alberts *et al.* 1989). Therefore, the organization of MBRs described here may be important for the regulation of segregation of multiple mtDNA molecules in the mt-nucleus.

Summary

MtDNA of *Physarum polycephalum* has duplicated specific regions that bind tightly to the mitochondrial membrane. In this study, we visualized the localization of the membrane-binding regions (MBRs) of mtDNA in the mitochondrial nucleus (mt-nucleus) by fluorescence *in situ* hybridization with a DNA probe specific for the MBR. The mt-nucleus of *P. polycephalum* contains 18–32 mtDNA molecules. In spite of the high copy number of mtDNA, the sites of MBRs were detected as a few globular fluorescence that were distributed along the mt-nucleus. This indicates that MBRs were clustered at a few discrete sites along the mt-nucleus. Furthermore, the number of MBR cluster in a mt-nucleus was proportional to the number of copies of mtDNA; one MBR cluster consisted of the MBRs of about 10 mtDNA molecules.

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