Glom Is a Novel Mitochondrial DNA Packaging Protein in *Physarum polycephalum* and Causes Intense Chromatin Condensation without Suppressing DNA Functions

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Mitochondrial DNA (mtDNA) is packed into highly organized structures called mitochondrial nucleoids (mt-nucleoids). To understand the organization of mtDNA and the overall regulation of its genetic activity within the mt-nucleoids, we identified and characterized a novel mtDNA packaging protein, termed Glom (a protein inducing agglomeration of mitochondrial chromosome), from highly condensed mt-nucleoids of the true slime mold, *Physarum polycephalum*. This protein could bind to the entire mtDNA and package mtDNA into a highly condensed state in vitro. Immunostaining analysis showed that Glom specifically localized throughout the mt-nucleoid. Deduced amino acid sequence revealed that Glom has a lysine-rich region with proline-rich domain in the N-terminal half and two HMG boxes in C-terminal half. Deletion analysis of Glom revealed that the lysine-rich region was sufficient for the intense mtDNA condensation in vitro. When the recombinant Glom proteins containing the lysine-rich region were expressed in *Escherichia coli*, the condensed nucleoid structures were observed in *E. coli*. Such in vivo condensation did not interfere with transcription or replication of *E. coli* chromosome and the proline-rich domain was essential to keep those genetic activities. The expression of Glom also complemented the *E. coli* mutant lacking the bacterial histone-like protein HU and the HMG-boxes region of Glom was important for the complementation. Our results suggest that Glom is a new mitochondrial histone-like protein having a property to cause intense DNA condensation without suppressing DNA functions.

INTRODUCTION

Packaging of DNA into a compact structure is a universal and significant phenomenon in the cell. In the eukaryotic nucleus, the fundamental unit of chromatin is the nucleosome, in which the DNA is wrapped approximately twice around the histone core (Wolffe, 1998). The bacterial genome is packed into a beaded polynucleosome-like structure, and the abundant histone-like protein HU can induce negative supercoiling in circular DNA molecules and form nucleosome-like structures in vitro (Rouviere-Yaniv and Gros, 1975; Rouviere-Yaniv *et al.*, 1979). Like genomic DNA, mitochondrial DNA (mtDNA) is packed with proteins into a highly organized structure called the mitochondrial nucleoid (mt-nucleoid) or nucleus (re-

viewed by Kuroiwa, 1982; Miyakawa *et al.*, 1987). The mt-nucleoid is thought to be a functional unit of replication and transcription of the mitochondrial genome. However, it is poorly understood how the various functions of the mitochondrial genome are performed within the highly organized mt-nucleoids.

In numerous animals, plants, and fungi, the mt-nucleoids can usually be observed as tiny spots or visualized in mitochondria using fluorescence microscopy with a DNA-specific binding fluorochrome such as 4',6'-diamidino-2-phenylindole (DAPI). It has been estimated that each mt-nucleoid of human ovarian carcinoma cell line A2780 and *Saccharomyces cerevisiae* contains only 1–2 and 2–8 mtDNA molecules, respectively (Miyakawa *et al.*, 1984; Satoh and Kuroiwa, 1991). The small amount of mtDNA in the mt-nucleoid hampers any detailed analysis of the organization of mtDNA in mt-nucleoids. Even under electron microscopy, the majority of mtDNA is embedded in the electron-dense mitochondrial matrix, and some of the mtDNA is observed as clumped or thickened fibers in an electron-transparent spherical area (Nass and Nass, 1963a, 1963b; Nass *et al.*, 1965).

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In comparison to other organisms, the true slime mold *Physarum polycephalum* has several advantages for the study of the organization and function of mtDNA in the mt-nucleoid. First, the mt-nucleoid of *P. polycephalum* contains an extraordinarily large amount of mtDNA and has a simple rod shape (Kuroiwa, 1974). MtDNA of *P. polycephalum* is a 62,862-base pair circular molecule (Takano *et al.*, 2001). Each mt-nucleoid contains ~40 and 80 copies of mtDNA molecule at the mitochondrial G1 and G2 phases, respectively (Kuroiwa and Kuroiwa, 1980). The replication of high-copy mtDNA molecules in the mt-nucleoid is regulated within groups of adjacent mtDNA molecules, referred to as mitochondrial replicon clusters (Sasaki *et al.*, 1994, 1998).

Second, the mt-nucleoids of *P. polycephalum* maintain a higher degree of mtDNA organization than those of the other eukaryotes. Under electron microscopy, the mt-nucleoids in this organism can be easily seen as an electron-dense, rod-shaped structure at the central region of a mitochondrion throughout its mitochondrial division cycle, including mitochondrial M, G1, S, and G2 phases (Kuroiwa *et al.*, 1977). A similarly electron-dense structure of the mt-nucleoid is found in trypanosomes (Paulin, 1975), but their mtDNA (kinetoplast DNA) is one of the most unusual DNAs found in nature. It consists of ~5000 minicircles and 20–30 maxicircles catenated into a single network of interlocked circles (reviewed by Simpson, 1986).

Third, we previously established a method to isolate the highly purified mt-nucleoid from *P. polycephalum*, without contamination by cell nuclei (Suzuki *et al.*, 1982; Sasaki *et al.*, 1998). Electron and fluorescence microscopic observations indicate that the isolated mt-nucleoids have the same shape, size, and DNA content as in vivo (Suzuki *et al.*, 1982). Furthermore, the isolated mt-nucleoids retained the high capacity of replication and transcription of their own mtDNA (Sasaki *et al.*, 1998 and unpublished data). Because the replication of the isolated mt-nucleoids is regulated in the mitochondrial replicon cluster, the isolated mt-nucleoids have the potential to reflect the in vivo states of mtDNA replication (Sasaki *et al.*, 1998).

Identification of mt-nucleoid proteins of *P. polycephalum* that are involved in the organization of mtDNA should facilitate understanding of the overall regulation of genetic activity within the highly organized mt-nucleoid. Several lines of evidence from the analysis of mt-nucleoids of *P. polycephalum* have suggested that mtDNA molecules are organized by several basic proteins (Suzuki *et al.*, 1982), the genes of which have yet to be identified. In mitochondria of yeast, Abf2p is an abundant basic DNA-binding protein, required for the maintenance of the mitochondrial genome (Diffley and Stillman, 1991; Megraw and Chae, 1993; Zelenaya-Troitskaya *et al.*, 1998). Abf2p can bend and wrap DNA in vitro (Caron *et al.*, 1979; Diffley and Stillman, 1992; Fisher *et al.*, 1992), suggesting that Abf2p also plays an important role for packaging and organization of mtDNA. Abf2p is a member of the chromosomal nonhistone high mobility group (HMG) proteins (reviewed by Landsman and Bustin, 1993) and contains two HMG box domains (Diffley and Stillman, 1991). Similar HMG box proteins have been identified as the mitochondrial transcription factor A (TFAM/mtTFA) in human (Parisi and Clayton, 1991), mouse (Larsson *et al.*, 1996), rat (Pierro *et al.*, 1999), *Xenopus* (Antoshechkin and Bogenhagen, 1995) and *Drosophila* (Takata *et al.*, 2001). The remarkable abundance of TFAM in human (Takamatsu *et al.*, 2002) and *Xenopus* (Antoshechkin and Bogenhagen, 1995) suggests that they might also play an important role for packaging and organization of mtDNA in

those organisms. On the other hand, in the trypanosomatid *Crithidia fasciculata*, the genes of four basic proteins (p16, p17, p18, and p21) have been identified as those most likely to play a role in the organization of the kinetoplast DNA (Xu *et al.*, 1996; Hines and Ray, 1998). These proteins are lysine rich and have homology with histone H1 proteins.

In this study, we have identified a new mtDNA packaging protein that contains both a lysine-rich region and two HMG-box domains. This protein, termed Glom, was specifically localized to the entire mt-nucleoid and was capable of packaging mtDNA into a highly condensed state in vitro. Deletion analysis showed that the lysine-rich region is sufficient for severe condensation of DNA. Interestingly, the expression of Glom in *Escherichia coli*-induced nucleoid condensation in vivo but does not affect transcription or replication in *E. coli* cells. We discuss the roles of Glom in genome organization and the overall regulation of genetic activity in the mt-nucleoid.

MATERIALS AND METHODS

Cell Culture

Microplasmidia of *P. polycephalum*, Colonia isogenic strain KM182 × KM187, were used for isolation of the mt-nucleoid. Microplasmidia were cultured in semidefined medium (Daniel and Baldwin, 1964) at 23°C. Cultures at the middle-exponential phases were used in this study. Myxamoebae of *P. polycephalum*, Colonia isogenic strain KM88, were used for immunolocalization analysis. Myxamoebae were cultured on PGY plates (Ohta *et al.*, 1993) with live bacteria (*Klebsiella aerogenes*) as food, at 23°C.

In Vitro Disassembly of mt-nucleoids

Mt-nucleoids were isolated from microplasmidia of *P. polycephalum* as described in Sasaki *et al.* (1998). The isolated mt-nucleoids were suspended in NE1-S buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 7.7, 1 mM EDTA, pH 7.5, 7 mM 2-mercaptoethanol, 0.4 mM spermidine, 0.4 mM PMSF) to a concentration of 1 µg protein/µl. To disassemble the mt-nucleoids, an equal volume of NE1-S buffer containing NaCl at twice the final concentration was added to the suspension of isolated mt-nucleoids, which was then incubated at 26°C for 1 h. For observation, the mt-nucleoids were attached onto the slide glass by centrifugation at 500 × g for 5 min using the centrifugal cell collector (SC-2, Tomy Seiko Co., Ltd., Tokyo, Japan) and then fixed with 0.6% glutaraldehyde and stained with 1 µg/ml DAPI in NE1-S buffer.

Purification of Glom

The 0.3 M NaCl disassembled mt-nucleoids were centrifuged at 18,500 × g for 15 min at 4°C. The resultant supernatant fraction was dialyzed against buffer A (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT) and was then applied to a HiTrap-SP column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with buffer A. Proteins were eluted with a 15-ml linear NaCl gradient (150–800 mM), and the fractions eluted with 450–500 mM NaCl and collected. The fractions were then dialyzed against buffer A and concentrated using Microcon (Millipore Corporation, Billerica, MA).

DNA Mobility Shift Assay

Purified Glom and rGlom proteins were analyzed for their DNA binding activity by electrophoretic mobility shift assay using *Xba*I-digested mtDNA and/or *Sty*I-digested λ phage DNA (Marker 6, Nippon Gene, Tokyo, Japan). Each 2 µl of purified Glom, rGlom, or BSA in buffer A was added to 8 µl of reaction buffer (50 mM HEPES, pH 7.5, 0.5 mM EDTA) containing 400 ng of DNA and incubated at room temperature for 3 h. The reaction mixtures were applied on a 1% agarose gel in 40 mM Tris-acetate (pH 8.0) and 2 mM EDTA. After electrophoresis, the gel was stained with ethidium bromide.

In Vitro Reassembly of mt-nucleoids

The isolated mt-nucleoids (5 µg protein) were disassembled at 0.3 M NaCl as described above. For preparation of the 0.3 M-NaCl supernatant-depleted fraction, the disassembled mt-nucleoids were centrifuged at 18,500 × g for 15 min at 4°C and then the pelletable fraction was resuspended in 10 µl of NE1-S buffer. To reassemble mt-nucleoids, fractions were dialyzed against NE1-S buffer at 4°C for 3 h using Slide-A-Lyzers (Pierce Biotechnology, Rockford, IL). To analyze the DNA packaging ability, the purified Glom or recombinant Glom (rGlom) proteins (1 µg protein) were added to the 0.3 M-NaCl supernatant-depleted fraction and then dialyzed against NE1-S buffer as described above.

Cloning of Glom cDNA

The Glom was electroblotted onto a PVDF membrane and sequenced from the N-terminus using a protein sequencing system (model G1005A, Hewlett-Packard, Palo Alto, CA), and the following 22-amino acid sequence was obtained: SVGKGKPTPKAVTPAKKAPPPP. To obtain the internal peptide sequences, the electroblotted Glom was digested with Achromobacter protease I in 1% hydrogenated Triton X-100, 10% CH₃CN, and 100 mM Tris-HCl (pH 9.0) for 16 h at 37°C. The digested peptides were separated by reverse-phase HPLC with a linear gradient of acetonitrile from 0 to 80% in 0.05% trifluoroacetic acid. The two peptides were sequenced using a protein sequencer (models PSQ-1, Shimadzu Corporation, Kyoto, Japan), and the following 16 and 14-amino acid sequences were obtained: ENPQLPVTAVLGEIAK and RALSAFSTIFVQENS.

PCR with genomic DNA of *P. polycephalum* was performed with two degenerate primers based on the amino acid sequences obtained: 5'-AARG-GHAARCCACICICIAARGCIGGT-3' and 5'-AAGAGAACCCICARYTBC-CCGTIACIGGC-3'. The resulting PCR fragment was used as a probe to screen a *P. Polycephalum* cDNA library that was kindly donated by Dr. Akio Nakamura.

Antibody Preparation

Full-length mature Glom was expressed in *E. coli* (XL1-Blue) as a 6× His-tagged protein using vector pQE-30 (Qiagen, Hilden, Germany). This protein was purified using Ni-NTA agarose and was then used to raise polyclonal rabbit antisera. Anti-Glom antibodies were purified from serum by Protein G affinity chromatography (MABTrap Kit; Amersham Pharmacia Biotech).

Immunofluorescence and Immunoelectron Microscopy Using Anti-Glom Antibody

For immunofluorescence staining, amoebae cells were fixed onto a coverslip with 3.7% formaldehyde in 10 mM KPB for 15 min and then permeabilized with 1% Triton X-100 in 10 mM KPB for 2 h. The *E. coli* cells were fixed and permeabilized as previously described (Hiraga *et al.*, 1998). The primary antibody was anti-Glom antibody at a dilution of 1:1000 (vol/vol) in blocking buffer (5% bovine serum albumin in PBS), and the secondary antibody was goat anti-rabbit IgG labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR) at a dilution of 1:500 (vol/vol) in blocking buffer.

Immunoelectron staining was performed as previously described (Sasaki *et al.*, 1998). The primary antibody was anti-Glom antibody at a dilution of 1:1000 (vol/vol) in blocking buffer (5% bovine serum albumin in PBS), and the second antibody was goat anti-rabbit IgG conjugated with 10-nm colloidal gold particles at a dilution of 1:50 (vol/vol) in blocking buffer. Samples were stained with uranyl acetate and observed with a JEM-1200EX electron microscope (JOEL, Akishima, Tokyo, Japan).

Purification of Recombinant Proteins

Recombinant Glom (rGlom) species were expressed as fusion proteins with glutathione-S-transferase (GST) using vector pGEX6P-1 (Amersham Pharmacia Biotech) in *E. coli* (XL1-Blue). All of the GST-fused rGlom proteins were soluble and were selectively retained on glutathione-Sepharose 4B columns. The retained proteins were released from the column by cleavage with Precision protease, which resulted in the addition of a pentapeptide (Gly-Pro-Leu-Gly-Ser) to the rGlom N-terminus. Recombinant proteins were further purified with HiTrap-SP columns as described above.

DNA Condensation Assay in *E. coli*

All rGlom proteins were expressed as nontagged proteins in *E. coli* (XL1-Blue) using vector pQE50 (Qiagen). Transformed strains were grown in LB-medium supplemented with 2% glucose and 50 µg/ml ampicillin, and IPTG (final 1–2 mM) was added at OD₆₀₀ of 0.5–0.6. For observation, cells were cultured for 3 h after IPTG induction. For analysis of cell proliferation, transcription, and replication, cells that had been precultured for 3 h in the presence of IPTG were used to inoculate fresh IPTG-containing LB medium at an OD₆₀₀ of 0.2–0.3. After 30 min, 12.5 µCi of [³H]thymidine and [³H]uridine was added to each 2.5-ml culture. After an additional 30 min, 1 ml of culture was centrifuged and 300 µl of nucleic acid extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 300 mM NaCl, 2% [wt/vol] Sarkosyl, 4% [wt/vol] SDS) was added to the resultant pellet. The pellet was resuspended and was then incubated at 65°C. After 15 min, 6 µl of 20 mg/ml Proteinase K was added, and incubation was continued for 15 h. Total nucleic acids were extracted with phenol/chloroform/isoamyl alcohol. After isopropanol precipitation, DNA and RNA pellets were suspended in 50 µl of distilled water. The amount of radioactivity in each aliquot was determined by liquid scintillation counting. The amount of DNA in each aliquot was determined using DyNA Quant (Amersham Pharmacia Biotech).

Complementation Analysis of HU-deficient *E. coli* Mutant with Glom

The wild-type (strain JR1669) and single *hupA* and *hupB* *E. coli* mutants (strains JR1670 and JR1671, respectively) were kindly provided by Dr. Josette Rouviere-Yaniv. The genotypes of JR1670 and JR1671 are *EchupA::Cm* and *EchupB::Km*, respectively. The double mutant (strain JR1672) was constructed by P1 transduction (Miller, 1972) starting from the single *EchupA* and *EchupB* mutants.

The rGlom proteins were expressed as nontagged proteins in the *E. coli* *hupA* and *hupB* double mutant (JR1672) using vector pQE50. Transformed strains were grown in LB medium, supplemented with 2% glucose, 50 µg/ml kanamycin, 12.5 µg/ml chloramphenicol, 50 µg/ml ampicillin, and 2 mM IPTG.

To examine influence of cold shock, the strains were grown into the log phase in LB medium containing 2% glucose, the appropriate antibiotics and 2 mM IPTG. They were cultured at 0°C for 0, 2, or 4 h and then spread on LB agar containing 2% glucose, the appropriate antibiotics, and 2 mM IPTG. The plates were incubated at 37°C overnight, and the number of colonies was counted.

RESULTS

Identification of Glom, a 41-kDa Protein Required for DNA Condensation in the mt-nucleoid

P. polycephalum has highly condensed, rod-shaped mt-nucleoids (Figure 1Aa). To identify the protein required for the packaging and organization of mtDNA, we studied which proteins were released from the mtDNA on the disassembly of isolated mt-nucleoids. We isolated the mt-nucleoid by dissolving the membrane of purified mitochondria with 0.5% Nonidet P-40, as described in a previous report (Sasaki *et al.*, 1998). DAPI staining showed that the isolated mt-nucleoids retained their morphological structure in vitro (Figure 1Ab). When the isolated mt-nucleoids were treated with various concentrations of NaCl at 26°C for 1 h, disassembly of mt-nucleoids was induced by increasing ionic strength (Figure 1A, c–f). The highly condensed structure of mt-nucleoids was retained in 0.1 M NaCl (Figure 1Ac). Disassembly began to occur when the NaCl concentration reached 0.2 M (Figure 1Ad), and complete disassembly occurred at 0.3 M NaCl (Figure 1Ae). These results suggest that the factors required for packaging of mtDNA are released from the mtDNA at 0.2–0.3 M NaCl.

To identify the proteins released from the mt-nucleoid at 0.2–0.3 M NaCl, the mt-nucleoids that were treated with NaCl were centrifuged and the supernatant and pelletable fractions were analyzed by SDS-PAGE (Figure 1B). Many proteins in the mt-nucleoid were released into the supernatant fraction at 0.1 M. A few proteins, such as 39-, 41-, and 56-kDa proteins, began to be released at salt concentrations above 0.2–0.3 M NaCl. The 41-kDa protein (p41) began to be released into the supernatant fraction at 0.2 M and was almost completely released at 0.3 M NaCl. In the mt-nucleoid fraction, the p41 was the most abundant and highly concentrated compared with the mitochondrial fraction.

We then purified p41 from the mt-nucleoid fraction. Because two-dimensional electrophoresis of mt-nucleoid proteins showed that p41 was the most basic among them (Suzuki *et al.*, 1982), we used the cation exchange chromatography for the purification of p41. Figure 2A shows the SDS-PAGE analysis of mt-nucleoid and purified p41. There are no other proteins in the purified p41 fraction. The intensities of the Coomassie-stained p41 bands in 1 µg of purified p41 were approximately similar to those in 5 µg of mt-nucleoid fraction. Because it has been determined that 5 µg of mt-nucleoid fraction contains ~0.45 µg mtDNA, it is calculated that the mt-nucleoid contained 2.2 µg of p41 per µg of mtDNA.

Figure 1. Analysis of the disassembly of mt-nucleoid induced by the treatment with NaCl. (A) DAPI-fluorescence micrographs showing the disassembly of mt-nucleoids with increasing concentrations of NaCl. (a) Plasmodium at log-growth phase; (b) isolated mt-nucleoids; (c-f) isolated mt-nucleoids treated with 0.1 M (c), 0.2 M (d), 0.3 M (e), and 0.5 M (f) of NaCl for 1 h at 26°C. Scale bar, (a) 2 μm and (b-f) 1 μm. (B) SDS-PAGE analysis of mt-nucleoid proteins released from mtDNA during the disassembly. The isolated mt-nucleoid (8 μg) was treated with various concentrations of NaCl and then centrifuged at $18,500 \times g$ for 15 min at 4°C. Proteins from mitochondria (Mt; 8 μg), mt-nucleoids (MN; 8 μg), pelletable (ppt) and supernatant fractions (sup) of mt-nucleoids treated with various concentrations of NaCl, were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

We confirmed the DNA binding ability of p41 by DNA-mobility shift assays. To examine the protein-mtDNA interaction, we used *Xba*I-digested mtDNA of *P. polycephalum*. The *Xba*I-fragments of mtDNA were incubated with various amounts of the purified p41 and analyzed by agarose gel electrophoresis (Figure 2B). The *Xba*I fragments were not shifted when they were incubated with a high amount of BSA, which was used as a negative control. All *Xba*I fragments were clearly shifted at high amount of p41, indicating that p41 could bind to each mtDNA fragment. The DNA-binding affinity of p41 to each mtDNA fragment might be slightly different, because the 18.4-kbp *Xba*I fragment showed slower migration rate than other fragments. Such a difference of migration rate was not observed when *Sty*I-digested λ phage DNA was used (Figure 2C).

We then analyzed the DNA-packaging ability of p41. As indicated in Figure 1A, the mt-nucleoids were disassembled

Figure 2. The in vitro DNA binding and packaging ability of p41. (A) Coomassie-stained gel after SDS-PAGE of the mt-nucleoids (5 μg) and purified p41 (1 μg). (B and C) DNA-mobility shift assay of p41 using *Xba*I-digested mtDNA (B) and *Sty*I-digested λ phage DNA (C). Each digested DNA (400 ng) was incubated without (–) or with various amounts (800, 400, 200, 100 ng) of p41 and 800 ng of BSA as described in MATERIALS AND METHODS. The band of 18.4-kbp *Xba*I-fragment of mtDNA (*) shifted more extensively than that of other fragments. The DNA-fragment sizes of marker are 19.3, 7.7, 6.2, 4.3, 3.5, 2.7, 1.9, 1.5 kbp from the top. (D) DAPI-fluorescence micrographs showing the reassembly of mt-nucleoids by dialysis. The isolated mt-nucleoids (5 μg protein) were disassembled at 0.3 M NaCl and then 0.3 M NaCl supernatant-depleted fractions were prepared as described in MATERIALS AND METHODS. 0.3 M NaCl disassembled mt-nucleoids fraction (a), 0.3 M-NaCl supernatant-depleted fraction (b), and 0.3 M NaCl supernatant-depleted fraction with 1 μg of p41 (c), were dialyzed against NE1-S buffer at 4°C for 3 h. Scale bar, 2 μm. (E) Electron micrograph of isolated mt-nucleoid (a) and reassembled mt-nucleoids induced by the addition of p41 (b) after negative staining. Scale bar, 500 nm.

by treatment with 0.3 M NaCl. This disassembly process was reversible: subsequent dialysis of the 0.3 M NaCl sample induced reassembly of the mt-nucleoids into a condensed, rod-shaped form (Figure 2Da). Thus, the factors required for DNA condensation can reassociate with mtDNA to reassemble mt-nucleoid structures upon dilution from high-salt solutions. Conversely, when the 0.3 M NaCl sample was depleted by centrifugation and the resultant supernatant fraction removed before dialysis, reassembly was severely

Figure 3. (A) Predicted amino acid sequence of Glom. The three peptides obtained from purified Glom are underlined. The N-terminus of Glom is indicated by an open triangle. The boxed amino acids represent the polyproline tracts. The accession number of Glom in DNA Databank of Japan (DDBJ) database is ABO49419. (B) Schematic model for Glom. Dotted box, targeting peptide; black box, polyproline tracts; shaded box, HMG boxes 1 and 2.

inhibited (Figure 2Db). Therefore, we investigated whether the addition of purified p41 into the 0.3 M NaCl supernatant-depleted fraction induced the reassembly of the mt-nucleoid. It is known that the ratio of protein/DNA affects the packaging of DNA; excess amounts of DNA-binding protein cause progressive condensation of DNA. In this experiment, the amount of p41 added to disassembled mt-nucleoid was the same as that released from the mt-nucleoid during disassembly. After dialysis, the highly condensed nucleoids were reassembled (Figure 2Dc). The reassembled mt-nucleoids did not appear to be rod-shaped but were instead agglomerated. Electron microscopy revealed that bead-like structures, similar to those observed in intact mt-nucleoids, were formed in the reassembled mt-nucleoids (Figure 2E). These results demonstrate that p41 is able to package the mt-nucleoid into a highly condensed state in vitro. We have therefore given p41 the name Glom (a protein inducing agglomeration of mitochondrial chromosomes).

Primary Structure of Glom

We then cloned a cDNA encoding Glom. An N-terminal peptide sequence and two internal peptide sequences of Glom were obtained by microsequencing. The sequence information was then used to design degenerate oligonucleotide primers for PCR amplification. Using the PCR product as a probe, a cDNA clone containing an open reading frame (ORF) of 362 amino acids was isolated from a *P. polycephalum* cDNA library (the accession number in the DNA Databank of Japan database is ABO49419). The predicted amino acid sequence of the resulting cDNA clone is shown in Figure 3A. All three sequences of peptides obtained with the purified Glom were present in

the ORF, indicating that the cDNA clone contained the Glom gene. Because no homology has been found in the mitochondrial genome sequence (Takano *et al.*, 2001), this gene must be encoded by the nuclear genome. The mature amino terminus of the protein is located 31 residues after the putative initiation codon. Therefore, the first 30 amino acids are assumed to be a mitochondrial transit peptide resulting in a mature protein of 330 amino acids.

The predicted amino acid sequence of Glom indicates that the mature form of this protein is rich in basic amino acids, especially lysine residues (21.7%), and has an estimated isoelectric point of 10.4. The calculated molecular mass of Glom is 37,900, which is smaller than the apparent molecular mass determined by SDS-PAGE. The anomalous migration of Glom on SDS-PAGE probably results from the highly basic nature of this protein. A search of the BLAST and BEAUTY protein databases revealed that the C-terminal half (amino acids 217–356) of Glom contains two homologous HMG boxes (Figure 3B). Although the N-terminal half (amino acid 31–216) has no sequence homology with other known proteins, this region is considerably rich in lysine residues (26.3%) and contains five characteristic polyproline tracts.

Glom Is Specifically Localized to the Entire mt-nucleoid

We then raised a polyclonal antibody against a bacterially expressed His-tag protein containing full-length mature Glom protein. Western analysis showed that this antibody specifically recognized a single polypeptide of 41-kDa in total cell extract (our unpublished results). Using this antibody, we examined the intracellular localization of Glom in the whole cell (Figure 4A). The cell was also counterstained with DAPI to visualize both the nucleus and mt-nucleoid (Figure 4Aa). Immunofluorescent staining revealed that Glom was located in all mt-nucleoids, but was not in the nucleus and that Glom was located uniformly throughout the mt-nucleoid (Figure 4Ab). We also analyzed the localization of Glom in the mt-nucleoid at the ultrastructural level by immunoelectron microscopy (Figure 4B). The electron-dense mt-nucleoid was observed at the central region of the mitochondria, and gold particles were distributed throughout the mt-nucleoid. This finding is consistent with the immunofluorescence study (Figure 4A). Therefore, it is clear that Glom interacts with the entire mtDNA in the mt-nucleoid.

The Lysine-rich Region of Glom Is Required for DNA Condensation In Vitro

To clarify the functional region of Glom that is involved in the packaging and condensation of mt-nucleoids, several forms of recombinant Glom (rGlom), including the full-length mature protein, were constructed as shown in Figure 5A. These proteins were overexpressed as GST fusion proteins in *E. coli*. All of the expressed proteins were found in the soluble fraction of crude *E. coli* lysates. The proteins were partially purified from crude lysates using a glutathione-Sepharose column and were subsequently eluted by cleavage of the GST tag with precision protease. We further purified the proteins by cation exchange chromatography. The purity of the recombinant proteins was checked by SDS-PAGE (Figure 5B).

Before examining the condensation ability of purified rGlom proteins, we tested the mtDNA binding ability of them by DNA-mobility shift assays (Figure 5C). Each rGlom protein was incubated with the *Xba*I fragments of mtDNA. It was clear that all of the rGlom proteins were able to bind to mtDNA. We then investigated the DNA condensation ability of each rGlom protein by the in vitro disassembly and

Figure 4. Intracellular immunolocalization of Glom. (A) Immunofluorescence micrograph showing the localization of Glom to the mt-nucleoids. The amoeba cell was stained with anti-Glom antibody and fluorescein isothiocyanate-conjugated second antibody (b). The same cell is also stained with DAPI (a). Scale bar, 2 μ m. (B) Immunoelectron micrograph showing the localization of Glom to the mt-nucleoid. The section of the plasmodium cell was stained with anti-Glom antibody and Gold-conjugated second antibody. Gold particles are distributed throughout the electron-dense mt-nucleoid in a mitochondrion. Scale bar, 500 nm.

reassembly analysis used in Figure 2D (Figure 5D). When rGlom proteins were added to the 0.3 M NaCl supernatant-depleted fraction before dialysis, rGlom proteins containing the N-terminal lysine-rich region (rGlom II, IV, V, and VIII) induced condensation of mt-nucleoids to the same extent as the full-length protein (rGlom I). However, rGlom proteins containing only the C-terminal HMG-box region (rGlom III, VI, and VII) caused only minimal condensation. These results suggest that the lysine-rich region of Glom plays a more important role in the condensation of the mt-nucleoid than the HMG-box region.

Overexpression of Glom Can Induce *E. coli* Nucleoid Condensation without DNA Dysfunction

In vitro DNA-binding assay using Southwestern analysis showed that Glom could also bind to *E. coli* DNA (our unpublished results). Therefore, we investigated whether *E.*

coli nucleoid condensation can be induced in vivo by the overexpression of Glom (Figure 6A). In this experiment, the full-length mature Glom (rGlom I) protein was expressed as a nontagged protein, to prevent abnormal effects due to a tag. In fact, the His- and GST-tags did affect the nucleoid condensation (our unpublished results). When cells were cultured for 3 h in the presence of the inducer IPTG, the nucleoids in Glom-expressing strains were observed to be condensed. Such a change was not observed in control cells expressing parent vector sequences only. In induced cells, the amount of Glom expressed in *E. coli* was $\sim 1.8 \mu$ g protein per μ g *E. coli* DNA. This amount was comparable to that in mt-nucleoids of *P. polycephalum* (2.2 μ g protein per μ g mtDNA). Electron microscopic observations of these strains confirmed that the presence of compact nucleoid structures was specific to the Glom-expressing strain (Figure 6B). Electron-dense nucleoids similar to mt-nucleoids of *P. polycephalum* were also observed in the Glom-expressing strain. Furthermore, analysis of the immunolocalization of Glom showed specific localization in the condensed nucleoids of *E. coli* (Figure 6C). These results suggest that Glom directly induces the *E. coli* nucleoid condensation in vivo.

Subsequently, we investigated the effects of Glom-mediated DNA condensation on DNA function. Although cells having condensed nucleoids were observed 3 h after IPTG induction, these cells entered stationary growth phase during the IPTG induction. Therefore, in this study, cells were precultured for 3 h in the presence of IPTG, cells having condensed nucleoids subsequently being transferred to fresh IPTG-containing medium at an OD₆₀₀ of 0.2–0.3. Surprisingly, despite considerable nucleoid condensation, the Glom-expressing strain continued to grow at a rate similar to the control strain expressing parent vector sequences (Figure 6D). We also confirmed that the Glom-expressing strain grew normally after 12 generations (our unpublished results). The effects on DNA synthesis and RNA synthesis were examined by monitoring the incorporation of [³H]thymidine and [³H]uridine into total nucleic acids. The data show that Glom-expressing cells had replication and transcription levels similar to the control cells (Figure 6E). Thus, nucleoid condensation induced by Glom had no influence on cell proliferation, replication, or transcription.

Proline-rich Region Prevents DNA Dysfunction in Condensed Nucleoids

We also expressed various regions of Glom in *E. coli* cells for 3 h in the presence of IPTG (Figure 7A). The rGlom proteins containing the lysine-rich region (rGlom II, V, and VIII) induced nucleoid condensation in *E. coli*. However, the rGlom proteins containing the HMG-box region (rGlom III, VI, and VII) did not induce nucleoid condensation. In those strains, the amount of expressed protein in *E. coli* was ~ 1.8 –3.3 μ g protein per μ g *E. coli* DNA. In these experiments, we could not characterize the effects of expression of rGlom IV, because the amount of expressed protein was too low ($<0.1 \mu$ g protein per μ g *E. coli* DNA).

We then measured cell proliferation, replication, and transcription in each strain (Figure 7, B and C). The rGlom II-expressing strain with highly condensed nucleoids continued to grow at a rate similar to the control strain, expressing parent vector sequences. This strain had replication and transcription levels similar to that of the control cells. On the other hand, other strains exhibited lower levels of cell proliferation, replication, and transcription. Especially, the strains lacking the five polyproline tracts at the N-terminus of Glom (rGlom V, VIII), which had highly condensed nucle-

Figure 5. The in vitro DNA packaging and condensation ability of rGlom proteins. (A) Constructs of rGlom proteins. Each region is indicated as same as that in Figure 3B. (B) Coomassie-stained gel after SDS-PAGE of purified rGlom proteins (1 μg each). Lane numbers correspond to the rGlom construct numbers in A. (C) DNA-mobility shift assay of rGlom proteins using *Xba*I-digested mtDNA. The digested DNA (400 ng) was incubated without (–) or with various amounts (800, 400, 200 ng) of each purified rGlom protein as described in MATERIALS AND METHODS. The DNA-fragment sizes of marker are same as in Figure 2, B and C. Numbers on the lane correspond to the rGlom construct numbers in A. (D) Condensation of mt-nucleoids by rGlom proteins. The 0.3 M NaCl supernatant-depleted fraction was dialyzed after addition of each rGlom protein (1 μg each). Resultant nucleoids were examined by DAPI fluorescence microscopy. Panel numbers correspond to the rGlom construct numbers in A. Scale bar, 1 μm.

oids, exhibited severe inhibition of cell proliferation, replication, and transcription. These results suggest that the polypoline tracks prevent DNA dysfunction in condensed nucleoids.

Glom Can Complement the HU-deficient E. coli Mutant

The *E. coli* HU protein is an abundant nonspecific DNA-binding protein with roles in replication, recombination, and chromosome segregation. HU is a heterodimer with two subunits encoded by the *hupA* and *hupB* genes. Mutations in either *hupA* or *hupB* genes in *E. coli* produce no apparent phenotype. However, null mutations of both genes result in cells with filamentous and cold-sensitive phenotypes (Wada *et al.*, 1988). It has been reported that expression of other mtDNA packaging proteins, such as Abf2p, p16, p17, and p18, rescue the filamentous phenotype in the HU-deficient *E. coli* mutant (Megraw and Chae, 1993; Xu *et al.*, 1996). To investigate the ability of Glom to complement the HU-deficient *E. coli* mutant, we expressed the full-length of mature Glom (rGlom I) in double mutant cells (Figure 8). The wild-type cells were short and homogeneous in size (Figure 8Aa), whereas the double mutant cells showed the characteristic filamentous phenotype of HU-deficient cells (Figure 8Ab). Although expression of parent vector only in the double

mutant cells did not affect the filamentous phenotype (Figure 8Ac), expression of rGlom I resulted in cells that resemble the wild type (Figure 8Ad). Furthermore, the expression of rGrom I also rescued the cold-sensitive phenotype (Figure 8B). These results indicate that Glom is also functionally similar to HU protein. We then expressed the lysine-rich region (rGlom II) and HMG-box region (rGlom III) in double mutant cells to address which region of Glom is important for complementation of HU-deficient *E. coli* mutant. Morphologically, the rGlom III-expressing strain exhibited the wild phenotype (Figure 8Af) and rescued the cold-sensitive phenotype (Figure 8B). However, the expression of rGlom II did not rescue both phenotypes (Figure 8, Ae and B). Therefore, the HMG-boxes region in Glom is important for complementation of HU-deficient mutant.

DISCUSSION

Glom Is the mtDNA-packaging Protein That Organizes the Entire mt-nucleoid

The highly organized mt-nucleoid of *P. polycephalum* has provided a unique opportunity for the cytological and biochemical analysis of the mt-nucleoid (reviewed by Kuroiwa, 1982 and Kuroiwa *et al.*, 1994; Sasaki *et al.*, 1998). In this

Figure 6. Effects of expression of Glom proteins in *E. coli*. (A) Effect of Glom on the structure of *E. coli* nucleoids. *E. coli* cells expressing parent vector (a) and the rGlom I (b) were stained with nucleic acid stain SYTO 11 (Molecular Probes) to avoid artifacts associated with fixation and visualized by phase-contrast and fluorescence microscopy. For comparison of the differences in condensation, both cells are mixed and then stained (c). Scale bar, 3 μ m. (B) Transmission electron micrographs of Glom-expressing *E. coli*. In the vector-only strain (a), the nucleoid (electron-transparent area) localized throughout the cytoplasm. In Glom-expressing strains (b–d), the nucleoids were more compact and some cells had an electron-dense nucleoid as shown in d. Scale bar, 300 nm. (C) Immunolocalization of Glom in *E. coli* cells. The *E. coli* cells expressing parent vector (a and b) and the rGlom I (c and d) were stained with DAPI (a and c) and anti-Glom antibody (b and d) as described in Figure 4A. (D) Effect of nucleoid condensation mediated by Glom on *E. coli* cell proliferation. OD₆₀₀ were measured for *E. coli* cultures expressing the vector only (●) and rGlom I (□). (E) Effect of nucleoid condensation mediated by Glom on replication and transcription. DNA synthesis and RNA synthesis were examined by monitoring incorporation of [³H]thymidine (■) and [³H]uridine (□) into total nucleic acid, respectively.

study, we investigated the mt-nucleoid proteins that are involved in the packaging and organization of mtDNA in *P. polycephalum*, by using a disassembly-reassembly system of isolated mt-nucleoids. SDS-PAGE analysis showed that at least 70–80 polypeptides were associated with the mt-nu-

Figure 7. Effects of expression of truncated Glom in *E. coli*. (A) Effect of truncated Glom on the structure of *E. coli* nucleoids. *E. coli* cells expressing rGlom II (a), rGlom III (b), rGlom V (c) and rGlom VIII (d) were observed as described in Figure 6A. The strain expressing rGlom VI and VII exhibited the similar observations to the strain expressing rGlom III (our unpublished results). Scale bar, 3 μ m. (B) Effect of nucleoid condensation mediated by truncated Glom on *E. coli* cell proliferation. OD₆₀₀ were measured for *E. coli* cultures expressing the vector only (●), rGlomII (×), rGlomIII (○), rGlomV (△), rGlomVI (■), rGlomVII (▲), and rGlomVIII (◇). (C) Effect of nucleoid condensation mediated by truncated Glom on replication and transcription. DNA synthesis and RNA synthesis in the rGlom-expressing strains were examined as described in Figure 6E.

cleoid (Figure 1B, lane 2). Most of these proteins may not be important for the packaging of mtDNA, because they are released from the mt-nucleoids before the disassembly of the mt-nucleoid structure (Figure 1). In association with the disassembly of mt-nucleoids, several types of proteins, including Glom, begin to be released from the mt-nucleoid. The purified Glom induced the reassembly of highly condensed mt-nucleoids (Figure 2D). In the reassembled mt-nucleoid, a poly-beaded-like structure similar to that of nontreated mt-nucleoid was also observed (Figure 2E). These results suggest that Glom is sufficient to package mt-nucleoids into highly condensed states.

The DNA-mobility shift assay showed that Glom bound to all *Xba*I-fragments of mtDNA and *Sty*I-fragments of λ phage DNA (Figure 2, B and C). This suggests that Glom binds to DNA with no-sequence specificity. Because the

Figure 8. Complement of HU-deficient *E. coli* mutants by rGlom proteins. (A) Phase-contrast micrographs showing the wild-type strain (a), mutants lacking HU (b), mutants lacking HU expressing parent vector (c), rGlom I (d), rGlom II (e), and rGlom III (f). SYTO 11-stained cells are shown in the insets. Scale bar, 3 μ m. (B) Effect of rGlom proteins on the cold-sensitive growth phenotype of HU-lacking *E. coli* mutant. Wild-type strain (Δ), mutant lacking HU ($\diamond$), mutants lacking HU expressing parent vector ($\bullet$), rGlom I ($\square$), rGlom II ($\times$), and rGlom III ($\circ$) were transferred to 0°C for various periods and plated on LB agar containing appropriate antibiotics and IPTG. The plates were incubated at 37°C overnight, and the number of colonies was scored. The colony number without transfer to 0°C was calculated as 100%.

binding of Glom to DNA was affected by ionic strength and Glom is a highly basic protein (pI 10.4), the primary DNA-Glom interaction appears to be electrostatic. We also showed that Glom preferentially bound to the 18.4-kbp *Xba*I fragment of mtDNA. It is known that mtDNA packaging proteins in other species, such as Abf2p (yeast), xl-TFAM (*Xenopus*), and h-TFAM (human), bind to DNA both nonspecifically and specifically at sequences associated with major regulatory region for mtDNA transcription and replication (Fisher *et al.*, 1987; Diffley and Stillman, 1992; Ghivizzani *et al.*, 1994; Antoshechkin and Bogenhagen, 1995). Although the regulatory region of mtDNA has not been identified in *P. polycephalum*, the 18.4-kbp *Xba*I fragment contains the membrane-binding region of mtDNA (Kawano and Kuroiwa, 1985; Kuroiwa *et al.*, 1994; Takano *et al.*, 2001). In bacteria, it has been suggested that the chromosome is attached to the cell membrane and the attachment may play a role in chromosome replication, transcription, and segregation (Leibowitz and Schaechter, 1975; Firshein, 1989) and that the membrane recognition site is close to the replication origin (Hoshino *et al.*, 1987; Yung and Kornberg, 1988). Therefore, it might be possible that the 18.4-kbp *Xba*I fragment contains the regulatory region of mtDNA.

Glom is the most abundant protein in the mt-nucleoid. The quantitative analysis of Glom revealed that mt-nucleoids contained $\sim 2.2 \mu$ g of Glom per 1 μ g of mtDNA. Thus, there is enough Glom to bind to every 20 base pairs of mtDNA. The mtDNA packaging proteins in other species are also abundant mitochondrial proteins. It has been calculated that Abf2p (Diffley and Stillman, 1992), xl-TFAM (Antoshechkin and Bogenhagen, 1995), and h-TFAM (Takamatsu *et al.*, 2002) bind to every 15–30, 7–90, and 10–18 base pairs of mtDNA, respectively. These proteins can bind to the entire mtDNA, because one molecule occupies 25–30 base pairs of mtDNA (Fisher and Clayton, 1988; Diffley and Stillman, 1992; Antoshechkin and Bogenhagen, 1995). Comparison of the amount of Glom with the levels of those mtDNA packaging proteins suggests that Glom is also able to bind to the entire mtDNA. Such abundance and nonspecific DNA binding properties of Glom could be important for packaging of the entire mtDNA in the mt-nucleoid.

The immunolocalization analyses by fluorescence and electron microscopy using anti-Glom antibody provided clear evidence that Glom is associated with the entire mt-nucleoid in vivo (Figure 4). In trypanosome, the p16, p17, p18, and p21 proteins have been identified as proteins that may play a role in condensation and organization of kinetoplast DNA (Xu *et al.*, 1996; Hines and Ray, 1998). The immunofluorescence analyses of those proteins showed that p16, p17, and p18 localized throughout kinetoplast DNA disk (mt-nucleoid) and that p21 surrounded the disk. Ultrastructural observation by electron microscopy showed that p16 was often localized in two opposite edges of the kinetoplast DNA disk, which is the region of the replication machinery. In human, immunofluorescence analysis with anti-TFAM showed that TFAM localized in intramitochondrial foci which were colocalized with sites of BrdU-incorporation (Alam *et al.*, 2003; Garrido *et al.*, 2003). In yeast, the subcellular fractionation showed that Abf2p was found in both purified mitochondria and the nucleus. Although Abf2p-GFP fusion protein was shown to specifically colocalize with mt-nucleoids (Zelenaya-Troitskaya *et al.*, 1998), immunofluorescence analysis with anti-Abf2p antibody failed to show clearly the localization of Abf2p in the mt-nucleoid (Diffley and Stillman, 1991). Our analyses clearly showed that Glom was specifically localized to mt-nucleoids, but not to the nucleus. Moreover, Glom was distributed uniformly throughout the entire mt-nucleoid. Together with the biological properties of Glom as described above, it is strongly suggested that Glom is an important structural protein that organizes the entire mt-nucleoid in vivo.

Glom Is a New Type of Mitochondrial HMG Protein That Has a Lysine-rich Region for Severe DNA Condensation

The Glom gene is encoded in the nuclear genome. Like many nuclear genome-encoded proteins that are present in mitochondria, the N-terminal sequence of Glom suggests that this protein is synthesized as a precursor protein and that the targeting peptide is cleaved within the mitochondria. Comparison of the deduced amino acid sequence of

Glom with databases revealed that C-terminal half of Glom contains two HMG-box domains, which are similar to those of Abf2p (Diffley and Stillman, 1991), xl-TFAM (Antoshechkin and Bogenhagen, 1995), and h-TFAM (Parisi and Clayton, 1991). In Abf2p, xl-TFAM, and h-TFAM, two HMG-box domains comprise most of the molecule. In comparison, Glom has a long extended lysine-rich region containing five polyproline tracts in the N-terminal half. The lysine composition of this region (26.3%) is comparable to that of histone H1, which binds to linker DNA and facilitates chromatin condensation (Thoma *et al.*, 1979; Allan *et al.*, 1986). The p16, p17, p18, and p21 proteins in trypanosomes are known as histone H1-like proteins because of the high content of lysine and alanine residues (Xu *et al.*, 1996; Hines and Ray, 1998). However, those proteins do not contain any HMG-box domains. Thus, Glom is a new type of mtDNA packaging protein that contains both HMG-boxes and a lysine-rich region in a single protein.

Each region of Glom, namely, the N-terminal polyproline tracts, the lysine-rich region, and two HMG boxes had DNA binding ability (Figure 5C). It is suggested that Glom can interact with mtDNA at multiple sites. The *in vitro* disassembly and reassembly analysis using rGlom proteins showed that the lysine-rich region of Glom was important for packaging mtDNA into a highly condensed state (Figure 5D). It is known that p16, p17, p18, and p21 of trypanosome assemble the kinetoplast DNA into a highly condensed state *in vitro* (Xu *et al.*, 1996), even though they have no homology to the lysine-rich region of Glom. We suggest that the highly positive charged lysine-rich region might be important to neutralize the negative charge of DNA, allowing DNA to be packaged into a highly condensed state. On the other hand, the rGlom proteins containing only an HMG-box region caused minimum DNA condensation. This result is consistent with the data that Abf2p, which contains only HMG-box domains, assembles the mtDNA into a less tightly packed state *in vitro* (Miyakawa *et al.*, 1995).

The HMG-boxes region of Glom complemented HU-deficient *E. coli* mutant (Figure 8). HU protein participates in a number of cellular mechanisms such as replication, recombination, and chromosome segregation (reviewed by Drlica and Rouviere-Yaniv, 1987; Schmid, 1990). Another mitochondrial HMG-box protein, Abf2p, can also complement the HU-deficient *E. coli* mutant (Megraw and Chae, 1993). It has been reported that Abf2p plays an important role for mtDNA maintenance, segregation, and recombination (Diffley and Stillman, 1991; Zelenaya-Troitskaya *et al.*, 1998). The HMG-boxes region of Glom may also have such similar functions. Overexpression of HMG-box region in *E. coli* induced the inhibition of cell proliferation, replication, and transcription (Figure 7), but not in the HU-deficient *E. coli* mutant (Figure 8). These results suggested that coexistence of HMG-box protein and HU protein affected function of the nucleoid, probably due to a dosage problem or some interaction among them.

N-terminal Polyproline Tracts of Glom Keep the Condensed Nucleoid Active

In vivo, the mt-nucleoid of *P. polycephalum* is more tightly packed than the *E. coli* nucleoid. It was deduced that $\sim 2.4 \times 10^6$ base pairs mtDNA is compacted in each *P. polycephalum* mt-nucleoid, the volume of which is estimated to be $0.037 \mu\text{m}^3$ at the mitochondrial G1 phase (Kuroiwa and Kuroiwa, 1980). This packaging ratio is ~ 10 times than that of *E. coli*, in which 5×10^6 base pairs of DNA is compacted in $0.9 \mu\text{m}^3$ (reviewed by Schmid, 1990). The expression of rGlomI re-

sulted in extreme condensation of *E. coli* nucleoids (Figure 6, A and B). The specific localization of Glom in the condensed nucleoids of *E. coli* suggests that Glom induces nucleoid condensation through direct interaction with *E. coli* DNA (Figure 6C). Furthermore, the amount of Glom in *E. coli* was comparable to that in mt-nucleoids of *P. polycephalum*. These results suggest that the condensation of the *E. coli* nucleoid by Glom might reflect the ability of Glom to induce DNA condensation in the mt-nucleoid.

In general, the condensed chromatin is much less accessible to *trans*-acting factors than the less compacted chromatin and hence undergoes lower levels of transcription and replication (Wolffe, 1998). It has been reported that transfection of genes that encode proteins involved in chromosome condensation induces DNA dysfunction in the host cell (Sun *et al.*, 1989; Barry *et al.*, 1993). For example, chlamydial histone H1-like protein (Hc1) is expressed only in metabolically inert elementary bodies (EB) with an electron-dense core of condensed chromatin and plays a role in the condensation of chromatin (Hackstadt *et al.*, 1991). Expression of HC1 in *E. coli* induces nucleoid condensation of the *E. coli* and down-regulation of cell proliferation, replication, transcription, and translation (Barry *et al.*, 1992, 1993). However, our experiments showed that nucleoid condensation of *E. coli* by Glom had no influence on cell proliferation, replication, or transcription (Figure 6, D and E). This result suggests that DNA condensation by Glom must allow for the activation of transcription and replication. To the best of our knowledge, this type of DNA-condensation protein has not been previously reported. This unique property of Glom may be important for the activation of transcription and replication of mtDNA in the highly condensed mt-nucleoids of *P. polycephalum*.

Deletion analysis showed that the polyproline tracts at the N-terminal of Glom were essential for the activation of transcription and replication in the condensed nucleoid (Figure 7). The proline-rich regions are known as a novel class of transcriptional activation domain in a number of eukaryotic transcription factors (Mermod *et al.*, 1989; Gerster *et al.*, 1990; Tanaka *et al.*, 1994). The proline-rich domain of the transcription factor CTF/NF-1 in humans enhances transcription by directly interacting with the transcriptional activation machinery (Kim and Roeder, 1994; Chiang and Roeder, 1995) and histone H3 (Alevizopoulos *et al.*, 1995). Besides transcription factors, proline-rich regions of proteins occur widely in both prokaryotes and eukaryotes and generally act as a "sticky" arm, binding rapidly and reversibly to a wider range of ligands such as proteins, polyphenols, and DNA (reviewed by Williamson, 1994). Such flexibility of the binding of the proline-rich region might contribute to the accessibility of various proteins to the condensed chromosome and hence keep the chromosome active.

The Molecular Structure of Glom May Reflect the Evolution of the Mitochondrial Histone-like Protein

It is believed that mitochondria and chloroplasts arose from prokaryotic endosymbionts. In chloroplasts, the homologue of the bacterial histone-like protein HU gene is encoded in the chloroplast genome of some primitive red algae. These homologues play an important role for DNA packaging in chloroplast nucleoids (Kobayashi *et al.*, 2002). On the other hand, no HU homologue has been identified in mitochondria, although HU has been found in one of the closest relatives of the ancestor of mitochondria, *Rickettsia* (Anderson *et al.*, 1998). Here, we have identified Glom as a mtDNA packaging protein from a lower eukaryote *P. polycephalum*.

As in the case of yeast Abf2p (Megraw and Chae, 1993) and trypanosomatid p16, p17, and p18 (Xu *et al.*, 1996), Glom could complement the HU null mutant of *E. coli* (Figure 8). Therefore, it appears HU protein was replaced in mitochondria by functional homologue at an early stage in evolution.

Existence of various types of mtDNA packaging proteins suggests that the mitochondrial histone-like protein changed during the evolution. Differences in the mtDNA packaging proteins must affect the structure of the mt-nucleoid. In some protists that have electron-dense mt-nucleoid, such as *P. polycephalum* and trypanosomes, proteins with lysine-rich regions were found to be acting as the mtDNA packaging proteins (Xu *et al.*, 1996; Hines and Ray, 1998). However, in higher eukaryotes that have the electron-transparent mt-nucleoid, HMG box proteins without lysine-rich regions have been found (Diffley and Stillman, 1991; Parisi and Clayton, 1991; Antoshechkin and Bogenhagen, 1995). This is consistent with our result that the lysine-rich region caused more intensive condensation of DNA than the HMG box region. It is possible that the lysine-rich region or protein was lost from the mtDNA packaging proteins during the evolution of the eukaryote, which was accompanied by the change of the electron transparent appearance of the mt-nucleoid. The molecular structure of Glom comprises both of the HMG boxes and the lysine-rich region, which may represent a transitional phase in the evolution of the mitochondrial histone-like protein.

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