Mitochondrial processing peptidase activity is controlled by the processing of α -MPP during development in *Dictyostelium discoideum*

Koki Nagayama^{1,2†} and Tetsuo Ohmachi²

Correspondence

Tetsuo Ohmachi

tohmach@cc.hirosaki-u.ac.jp

¹Science of Bioresources, United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate 020-8551, Japan

²Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, 036-8561, Japan

We investigated the expression of the α subunit of the *Dictyostelium* mitochondrial processing peptidase (Dd α -MPP) during development. Dd α -MPP mRNA is expressed at the highest levels in vegetatively growing cells and during early development, and is markedly downregulated after 10 h of development. The Dd α -MPP protein is expressed as two forms, designated α -MPP_H and α -MPP_L, throughout the *Dictyostelium* life cycle. The larger form, α -MPP_H, is cleaved to produce the functional α -MPP_L form. We were not able to isolate mutants in which the α -mpp gene had been disrupted. Instead, an antisense transformant, α A2, expressing α -MPP at a lower level than the wild-type AX-3 was isolated to examine the function of the α -MPP protein. Development of the α A2 strain was normal until the slug formation stage, but the slug stage was prolonged to ~24 h. In this prolonged slug stage, only α -MPP_H was present, and α -MPP_L protein and MPP activity were not detected. After 28 h, α -MPP_L and MPP activity reappeared, and normal fruiting bodies were formed after a delay of approximately 8 h compared with normal development. These results indicate that MPP activity is controlled by the processing of α -MPP_H to α -MPP_L during development in *Dictyostelium*.

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INTRODUCTION

In eukaryotes, mitochondria are important as the energy-generating organelles and also play important roles in cellular functions, including the synthesis of metabolites, the metabolism of lipids, nucleotides and iron, and signalling pathways (Lill & Kispal, 2000; Newmeyer & Ferguson-Miller, 2003). Cells possess a mitochondrial genome as well as a nuclear genome. Yeast and human mitochondrial genomes encode only eight and 13 proteins, respectively, which are the subunits of the respiratory chain and oxidative phosphorylation (Foury *et al.*, 1998; Scarpulla, 2006). The other proteins in mitochondria are encoded by the nuclear genome and have to be imported into the organelles from the cytosol.

The majority of mitochondrial proteins encoded by nuclear genes are synthesized on cytoplasmic ribosomes as larger

precursors with a presequence called the mitochondrial targeting signal (MTS) sequence at the N terminus, which is a signal for targeting the protein to mitochondria. During or after import of the precursor proteins into the mitochondria, the presequences are removed by the mitochondrial processing peptidase (MPP; EC 3.4.24.64) to form the mature proteins (Ito, 1999; Gakh *et al.*, 2002; Koehler, 2004). Some precursor proteins are first cleaved by MPP, and then the remaining octapeptide of the presequence is removed by an additional mitochondrial intermediate peptidase (MIP; EC 3.4.24.59), resulting in the mature proteins (Hendrick *et al.*, 1989; Gavel & von Heijne, 1990; Branda & Isaya, 1995; Ito, 1999). MPP is a heterodimeric metalloendopeptidase consisting of two non-identical subunits called α - and β -MPP. α -MPP participates in the recognition of the N-terminal presequences (Luciano *et al.*, 1997; Shimokata *et al.*, 1998), and β -MPP functions as a catalytic subunit and contains a metal (Zn^{2+})-binding motif at the active site (Kitada *et al.*, 1995, 1998; Luciano *et al.*, 1998). The α - and β -MPP subunits act cooperatively to remove the N-terminal presequences of the precursor proteins (Kojima *et al.*, 1998).

The cellular slime mould *Dictyostelium discoideum* grows vegetatively as unicellular amoebae. Once nutrients are

[†]Present address: Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK.

Abbreviations: AP, alkaline phosphatase; COX IV, cytochrome c oxidase subunit IV; GST, glutathione S-transferase; MIP, mitochondrial intermediate peptidase; MPP, mitochondrial processing peptidase; MTS, mitochondrial targeting signal.

The GenBank/EMBL/DBJ accession number for the α -MPP cDNA sequence of *Dictyostelium discoideum* is AB213513.

depleted, the amoebae aggregate and eventually form a fruiting body containing spores supported on a stalk. Because of its unique life cycle, *D. discoideum* has been used as a model organism to study developmental processes. During the life cycle, mitochondria of *D. discoideum* exhibit marked morphological changes that appear to be closely related to the regulation of development (Ashworth *et al.*, 1969; Cotter *et al.*, 1969; Maeda, 1971; George *et al.*, 1972). Mitochondrial dysfunction is caused by mutations in nuclear genes encoding mitochondrial proteins. In *Dictyostelium*, deficiencies in certain nuclear-encoded mitochondrial proteins cause defects in chemotaxis to cAMP (van Es *et al.*, 2001), in cell-type differentiation (Kotsifas *et al.*, 2002), and in spore maturation (Morita *et al.*, 2005). Also, antisense RNA inhibition of nuclear gene-encoded mitochondrial proteins results in a phototaxis deficiency (Kotsifas *et al.*, 2002; Bokko *et al.*, 2007), and depletion of mitochondrial DNA (mtDNA) causes deficiencies in cell aggregation (Inazu *et al.*, 1999) and in cell-type differentiation (Wilczynska *et al.*, 1997; Chida *et al.*, 2004). Disruption of the *rps4* gene in mtDNA causes a failure of *Dictyostelium* cells to initiate differentiation (Chida *et al.*, 2008). Recently, Nagayama *et al.* (2008) showed that mitochondrial dysfunction caused by β -mmp antisense inhibition is compensated in a retrograde signalling manner. Despite increasing focus on novel mitochondrial functions in development, little is known about basic mitochondrial biogenesis in *D. discoideum*.

In this paper, we report the cloning of a cDNA encoding the α subunit of *Dictyostelium* MPP (Dd α -MPP), the structural features of Dd α -MPP, and the expression of the α -MPP gene (*mppA*) during *Dictyostelium* development. We also show that the processing of α -MPP is necessary for MPP activity expression and for development in *Dictyostelium*.

METHODS

Strain, culture and development of *Dictyostelium*. *D. discoideum* strain AX-3 was grown axenically in HL-5 medium (Sussman & Sussman, 1967) supplemented with 100 μ g streptomycin ml⁻¹ at 22 °C on a reciprocal shaker (150 r.p.m.). Vegetative amoebae at a density of $\sim 5.0 \times 10^6$ cells ml⁻¹ were harvested, washed twice in 12 mM sodium phosphate buffer (pH 6.7), and resuspended in development buffer PDF (13 mM KCl, 10 mM NaH₂PO₄, 25 mM NaH₂PO₄, 0.8 mM CaCl₂, 5 mM MgSO₄) at 1.5×10^8 cells ml⁻¹. Approximately 1.0×10^7 cells were plated onto nitrocellulose filters (Millipore, type HABP, 0.45 μ m pore-size, diameter 47 mm) for multicellular development.

Isolation of nuclei, nucleic acids and mitochondria. The isolation of nuclei, genomic DNA and total RNA from *Dictyostelium* amoebae was performed using general methods as previously described (Nagayama *et al.*, 2008). Mitochondria were isolated from vegetatively growing cells, as previously described (Nagayama *et al.*, 2008).

Screening of α -MPP cDNA clone, sequencing and analysis. A 3' region (604 bp long) of α -subunit cDNA (contig U14151-1) of *Dictyostelium* MPP has been reported by the *Dictyostelium* cDNA

project (<http://dictycdb.biol.tsukuba.ac.jp/>). To amplify a part of the α -MPP gene, two oligonucleotide primers, mpp- α 1 (5'-GAGGATC-CTTGTTGGGTGGTGGCAGTTC-3', containing 20 nucleotides from positions 1441 to 1460 of α -mpp cDNA) and mpp- α 2 (5'-GAGAA-TTCATGTCTAGCGATATCATCAC-3', containing 20 nucleotides complementary to the sequence from positions 1751 to 1771), were designed. These primers contained *Bam*HI and *Eco*RI sites (underlined) at the 5' ends. PCR amplification was performed on *Dictyostelium* genomic DNA using these primers, as described previously (Nagayama *et al.*, 2008). The amplified fragment of 330 bp was sequenced for confirmation. By screening a λ ZAP II cDNA library (kindly provided by Dr Herbert L. Ennis, Columbia University) using the digoxigenin-dUTP-labelled PCR fragment as a probe, a positive clone (pBzmpp) was isolated, which is a plasmid containing the full-length α -mpp cDNA inserted between the *Eco*RI and *Xho*I sites of Bluescript. The nucleotide sequencing and analysis of pBzmpp were performed as described previously (Nagayama *et al.*, 2008).

Construction of plasmids pD α mpp237-gfp, pD α mpp237-gst and pET α mpp237-gst. The *Eco*RI fragment (encoding the first 237 amino acids) from pBzmpp was subcloned into the *Eco*RI site of pBluescript SKII(–) to yield plasmid pBzmpp237. The GFP gene was PCR-amplified using pUC118H-gfp as a template with the forward primer (5'-GGAAACAGCTATGACCATGATTTACG-3') and the reverse primer (5'-GCTCTCGAGTTACTTGTACAGCTCGTC-3'). The underlining in the reverse primer indicates the *Xho*I site introduced for subcloning. The PCR product was digested with *Hind*III and *Xho*I, and then fused in-frame at the 5' end with the 3' end of the α mpp237 in pBzmpp237, to generate the construct pBzmpp237-gfp. The *Bam*HI/*Xho*I fragment (containing an α mpp237-gfp fusion gene) from pBzmpp237-gfp was subcloned between the *Bam*HI and *Xho*I sites of the *Dictyostelium* expression vector pDNeo67, to yield the construct pD α mpp237-gfp. The PCR-amplified GFP gene was also subcloned into pDNeo67 to obtain pDgfp. To construct plasmid pD α mpp237-gst, the glutathione S-transferase (GST) gene was amplified using pGEX-2T (Pharmacia Biotech) as a template with a set of primers (forward primer, 5'-CAGAAGCTTATGTCCCCCTATACTAGG-3'; reverse primer, 5'-TCTCTCGAGTTAACGCGGAACCAGATCC-3'). The underlined bases in the two primers indicate *Hind*III and *Xho*I sites, respectively. Using the amplified PCR product, a plasmid, pD α mpp237-gst, was constructed in a manner similar to the construction procedure of pD α mpp237-gfp described above. For the expression of α MPP237-GST in *Escherichia coli*, the α mpp237-gst fragment from pD α mpp237-gst was subcloned into a pET15b vector (Novagen) to make plasmid pET α mpp237-gst.

Preparation of α -MPP overexpressing and α -mpp antisense transformants. The *Bam*HI/*Xho*I fragment from pBzmpp was inserted between the same sites of pDNeo67 to generate pD α mpp. The constructed plasmid was introduced into AX-3 cells by electroporation by the method of Howard *et al.* (1988), and transformed cells were selected in HL-5 medium containing 10 μ g G418 ml⁻¹ to obtain the α -MPP-overexpressing transformants (the α^{OE} strain). The α -mpp antisense transformants of *Dictyostelium* were isolated as described in our previous paper (Nagayama *et al.*, 2008). Also, as a control, the α -mpp sense transformant was isolated (Nagayama *et al.*, 2008).

Analysis of gene expression by real-time quantitative PCR. Real-time quantitative PCR was performed for quantification of the α -MPP mRNA during development of the wild-type strain, as previously described (Nagayama *et al.*, 2008). The sense primer (5'-GTCCAGGTAAGGGTATGCAAAGTAG-3', corresponding to positions 1475–1499 in the cDNA sequence) and the antisense primer (5'-ATTGTTTCAGCAGTTAAGATTGGTTC-3', positions 1912–1923) were used. Amplification of the *rnl* gene was also performed under the

same conditions for use as an intrinsic normalization control. The amounts of cDNA synthesized from 0.1 µg total RNA were measured according to the manufacturer's protocols (Roche Diagnostics).

Preparation of antibodies. The preparation of the anti- α -MPP antibody against the N-terminally truncated α -MPP (α -MPP Δ N, 47 kDa) and anti-COX IV antibody has been described in our previous paper (Nagayama *et al.*, 2008). The antisera containing anti- α -MPP and anti-COX IV antibodies were tested by Western blot analysis.

Assay of enzyme activities

MPP activity. The substrate (precursor protein) for MPP activity assay was prepared as follows. DdCOX IV cDNA was PCR-amplified from the VSK134 cDNA clone for COX IV (*Dictyostelium* cDNA project) using the forward primer *cox4f*(nco) (5'-GAGAGCCATGGTTCG-TTTAAGATCAATTCG-3') and the reverse primer *cox4r*(bam) (5'-TAAGGATCCTTAGTTATTTAAACTTTGAC-3'). The underlined bases indicate the *Nco*I site in the forward primer and the *Bam*HI site in the reverse primer. The PCR product was digested with *Nco*I and *Bam*HI, and then inserted between the *Nco*I and *Bam*HI sites of the expression vector pET15b to yield the construct pETcox4. A His-tag was added to the 3' end of COX IV gene, using the oligonucleotide *c-his*(xho) (5'-GCCGCTCGAGCTAATGGTGGTGATGGTGGTGTGAGCAGCCGAACCGTA-3'; underlined bases indicate the *Xho*I site), which contains a His-tag sequence and bases complementary to the cloning site of pET15b. The primers *cox4f*(nco) and *c-his*(xho) were used to amplify a cDNA with a His-tag sequence at the 3' end from pETcox4. The PCR product was digested with *Nco*I and *Xho*I and inserted into the corresponding sites of pET15b to generate the construct pETcox4-his. *E. coli* AD494(DE3) was transformed with pETcox4-his, and the resultant transformant was cultured in the presence of 0.5 mM isopropyl- β -thio-D-galactopyranoside. The COX IV-His protein, strongly expressed in an inclusion body, was purified and used as a substrate (precursor protein) for the MPP enzyme activity assay. The precursor protein (COX IV-His) was incubated with the crude extract or the mitochondrial fraction (20 µg protein) in reaction buffer containing 30 mM Tris/HCl (pH 8.0) at 4 °C for 15 min. The reaction was stopped by adding SDS buffer (25 mM Tris/HCl, pH 6.5, 10 mM β -mercaptoethanol, 1% SDS, 10% glycerol, 0.01% bromophenol blue). The processing protein products were separated by SDS-PAGE and visualized by immunostaining with anti-COX IV antiserum or anti-His-tag antibody (Invitrogen), as described for Western blot analysis.

α -MPP_H processing activity. The extract containing α -MPP_H but no α -MPP_L was prepared from the α^{OE} strain cells developed at 16 h or the slug cells developed for 24 h on non-nutrient H₂O-agar plates according to the method of Fisher & Annesley (2006), and was used as a substrate to determine α -MPP_H processing activity. The extract (20 µg protein) containing α -MPP_H was incubated at 4 °C for 16 h with the crude extract (20 µg protein) prepared from vegetative or developing cells. The conversion protein products were separated by SDS-PAGE and visualized with anti- α -MPP antiserum and goat anti-mouse IgG-alkaline phosphatase (AP), as described for Western blot analysis.

Western blot analysis. Total proteins were extracted from the cells at various developmental stages by sonication or by grinding with glass beads. Protein concentration was measured by the Lowry method (Lowry *et al.*, 1951), using BSA as a standard. Samples of protein (20 µg; or 150 µg in the case of expression analysis of α -MPP) were separated by SDS-PAGE and then electroblotted onto PVDF membranes (Bio-Rad). Blots were blocked with 5% skimmed milk and then incubated with anti- α -MPP antiserum or anti-COX IV antiserum (1:500 dilution) for 90 min. The blots were then incubated with goat anti-mouse IgG-linked AP (1:1500 dilution; Dako Cytomation) for 60 min. Protein bands were visualized using

4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) as substrates. Precision Plus protein standards (Bio-Rad) and Prestained Protein Marker (New England Biolabs) were used as protein size markers.

Fluorescence microscopy. Transformed cells expressing the α MPP237-GFP fusion protein were grown in HL-5 medium with shaking. The mitochondria were labelled with MitoTracker Red 580 (Invitrogen Molecular Probes) at a concentration of 100 nM in HL-5 medium. The cells were washed four times in fresh HL-5 medium to remove unbound MitoTracker Red. The cells were resuspended at 1×10^6 cells ml⁻¹ and mounted for fluorescence microscopy (Olympus BX50). The images were analysed using IPLab Scientific Imaging Software (Scanalytics).

RESULTS

Isolation of the α -MPP cDNA clone and characterization of Dd α -MPP

A full-length cDNA clone of Dd α -MPP (pBzmpm) was isolated from a *Dictyostelium* λ ZAP II cDNA library. The cDNA encoded a protein of 654 amino acids with a calculated molecular mass of 73 258. The gene (*mppA*) encoding α -MPP is located on chromosome 2, and its nucleotide sequence is available in contig DDB0232199 from the *Dictyostelium* genome project (<http://dictybase.org>).

The amino acid sequence of Dd α -MPP was compared with those of the known α -MPPs from other organisms. Dd α -MPP had amino acid identities of 25–27% and similarities of 43–47% with known α -MPPs. The domain structure of Dd α -MPP is shown in Fig. 1(a). A highly conserved glycine-rich domain (GGSSYSTGGPKGM), which is predicted to form a loop that might be involved in substrate binding and/or product release (Nagao *et al.*, 2000), is conserved in Dd α -MPP. The glutamate/aspartate residues that are well-conserved in other α -MPPs, and which might recognize the distal basic amino acid of the MTS peptide (Shimokata *et al.*, 1998), are also conserved in Dd α -MPP as the sequence D⁵⁷⁹D⁵⁸⁰. Dd α -MPP contains an asparagine-rich region [poly(N) cluster] from positions 51 to 100 in the N-terminal region. An extra sequence containing a glutamine stretch [poly(Q) cluster] and a high level of acidic amino acids is located from positions 388 to 450. These repeated sequences are common in *Dictyostelium* proteins, and their functions remain unknown (Eichinger *et al.*, 2005).

Subcellular localization of α -MPP

Dd α -MPP does not contain a typical MTS sequence at the N terminus, which is rich in basic, hydrophobic and hydroxylated amino acids, and forms an amphiphilic α -helix (von Heijne *et al.*, 1989; von Heijne, 1990). However, fractionation of mitochondria showed that Dd α -MPP localizes in the mitochondrial matrix fraction (Nagayama *et al.*, 2008). In order to confirm that α -MPP has a targeting signal and can be imported into mitochondria, an α MPP237-GFP fusion protein, in which the N-terminal

Fig. 1. Domain structure of Dd α -MPP and intracellular localization of α MPP237-GFP. (a) Schematic representation of the domain structure of Dd α -MPP. The map shows the positions of the functional domains of Dd α -MPP. The well-conserved Gly-rich domain (GGGSSYSTGGPKGM) and distal basic amino acid recognition site (DD) are located in the C-terminal half of the Dd α -MPP molecule. There is a poly(N) cluster in the N-terminal region and a poly(Q) and acidic amino acid-rich region in the middle region. (b) Localization of α MPP237-GFP, a fusion protein in which the N-terminal 237 amino acids of α -MPP are fused to GFP. The fluorescence image of *Dictyostelium* cells transformed with constructs pD α mpp237-gfp (upper row) and pDgfp (lower row) are shown. The first column shows the GFP fluorescence image, and the second column the MitoTracker Red fluorescence image. The last column shows the merged image for the localization of α MPP237-GFP fusion protein. The localization of GFP alone, as a control, is also shown by the fluorescence image. A schematic map of α MPP237-GFP is shown above the panels.

region (the first 237 amino acids) of α -MPP is fused at the C-terminal end to GFP, was generated (Fig. 1b). α MPP237-GFP was located in the mitochondria labelled with MitoTracker Red (Fig. 1b). GFP alone, as a negative control, was localized in the cytosol (Fig. 1b). These results suggest that the N-terminal region of α -MPP harbours a targeting signal to directly import α -MPP into the mitochondria. The MTS sequence of α -MPP remains to be identified.

Mitochondrial processing activity in *Dictyostelium* cells

Cytochrome *c* oxidase subunit IV (COX IV), a protein localized in the inner membrane of mitochondria, is synthesized in the cytosol as a precursor protein with an MTS sequence at the N terminus, imported into the mitochondria, and subsequently processed by MPP and MIP to yield the mature form (Maarse *et al.*, 1984; Branda & Isaya, 1995; Gakh *et al.*, 2002). Fig. 2(a) shows the N-terminal amino acid sequence of DdCOX IV and its putative MPP and MIP cleavage sites. We measured the processing activities of mitochondria using a recombinant DdCOX IV precursor (17 kDa) as a substrate. As shown in Fig. 2(b), MPP cleaved the precursor to generate the intermediate form (14.3 kDa). When incubated at temperatures over 20 °C, the intermediate form was further cleaved by MIP to yield the mature form (13.4 kDa). MPP activity was also detected at the relatively low temperatures of 0 and 4 °C. So, MPP activity was measured at 4 °C to

eliminate MIP activity in the crude extracts. MPP activity was present in the mitochondrial soluble (matrix) fraction but not in the mitochondrial insoluble (membrane) fraction (Fig. 2c).

Expression of *mppA* is developmentally regulated

To examine the levels of α -MPP mRNA during *Dictyostelium* development, Northern blotting was performed. However, no band was detected, suggesting that the expression level of α -MPP mRNA is quite low. To measure the amounts of α -MPP mRNA, real-time quantitative PCR was performed on cDNA from developing cells at the indicated times. Expression of mitochondrial large subunit rRNA (*rnl*), which is constitutively expressed by a mitochondrial gene, was used as an internal control (Early *et al.*, 1988). Fig. 3 shows the normalized relative amounts of α -MPP cDNA synthesized from 0.1 μ g total RNA at the indicated times. The expression of α -MPP mRNA was highest in vegetative cells (0 h) and at the early development stage (10 h), and then markedly decreased at 14 h of development, indicating that transcription of the α -MPP gene is strongly down-regulated at this time.

Processing of an immature form of Dd α -MPP is necessary for MPP enzyme activity

To examine whether the level of α -MPP protein is similarly regulated throughout development, Western blot analysis was performed using anti- α -MPP antiserum against the

Fig. 2. Processing peptidase activities of MPP and MIP. (a) N-terminal amino acid sequence of the DdCOX IV precursor. The MPP and MIP cleavage sites are indicated by an arrowhead and an arrow, respectively. (b) Mitochondrial processing activities. The precursor of DdCOX IV (tagged with a His-tag at the C terminus) was incubated at temperatures between 0 and 50 °C for 15 min with the mitochondrial fraction (20 µg protein) isolated from vegetative cells in reaction buffer, as described in Methods. Processing peptides were subjected to SDS-PAGE, followed by Western blot analysis and immunostaining using the anti-His-tag antibody. Lane S contains substrate alone. Numbers indicate temperature (°C) of the reaction. (c) MPP activity in the mitochondrial subfractions. The mitochondria isolated were disrupted by sonication and then centrifuged at 14 000 *g* for 1 h, yielding the soluble and insoluble fractions. MPP activity of these fractions was measured using DdCOX IV precursor as a substrate, as described above. Lane 1, substrate alone; lanes 2 and 3, soluble and insoluble fractions, respectively.

N-terminally truncated α -MPP. In vegetative cells (0 h), two major bands of 65 and 60 kDa were detected by the antiserum. The 60 kDa protein was expressed at slightly higher levels than the 65 kDa protein (Fig. 4a). The 65 kDa protein was designated α -MPP_H, and the smaller 60 kDa protein as α -MPP_L. α -MPP_H was expressed at the same level in developing cells from 0 to 16 h, and then the amount of this protein in developing cells decreased rapidly after 20 h. By comparison, there was little change in the amount of α -MPP_L in developing cells from 0 to 8 h, a decrease at 12 h, and then a marked increase thereafter.

To examine the expression of MPP activity during development, the enzyme activity at various developmental stages was measured using the COX IV precursor as a substrate. MPP activity was largely constant at all stages of development, although cells at 12 h of development exhibited slightly lower activity (Fig. 4b), suggesting a correlation between the expression pattern of MPP activity and that of α -MPP_L. These results for the expression of α -

Fig. 3. Expression of α -MPP mRNA during development. Normalized relative amounts of α -MPP cDNA from total RNA at the indicated developmental times. Real-time PCR was performed as described in Methods. The mean values of the results obtained from two independent experiments are shown.

MPP and MPP activity during development were confirmed reproducibly in several experiments. On the other hand, β -MPP is expressed at a size of 52 kDa at almost the same level throughout development, as described previously (Nagayama *et al.*, 2008).

In order to investigate the relationship between α -MPP_L and MPP activity, we focused on slug-stage cells because the beginning of the slug stage is at about 12 h of development. To completely separate the slug cells from cells at other stages of development, the wild-type cells were allowed to develop on non-nutrient H₂O-agar plates, according to the method of Fisher & Annesley (2006). Aggregation mounds were formed at about 12 h of development, and the slug was subsequently formed. When developed for an additional 12 h, the slug did not proceed to the following developmental stage and the slug-stage state was maintained, as described by Newell *et al.* (1969). As shown in Fig. 4(c, d), in the slug cells that had developed for 24 h [slug (24 h)], only α -MPP_H was present and MPP activity was not detected, suggesting that α -MPP_L, not α -MPP_H, is the mature active form of α -MPP that associates with the β -MPP subunit to form the functional MPP with its enzyme activity.

α -MPP_H is processed to α -MPP_L by removing its N-terminal region

To examine whether the N-terminal region of α -MPP_H is removed or not, α MPP237-GST was expressed in *Dictyostelium* cells, because α MPP237-GFP is located in the mitochondria as described above. When α MPP237-GST was expressed in *Dictyostelium* cells, two protein bands of 42 and 38 kDa were detected by anti-GST antibody (Fig. 5a), whereas, when expressed in *E. coli*, a 51 kDa

Fig. 4. Expression of α -MPP and MPP activity during development in the wild-type strain. (a) Crude extracts (150 μ g protein) from developing cells of the wild-type strain at the indicated times were separated on a 9.0% SDS-PAGE gel and electroblotted onto PVDF membranes. Blots were visualized by immunostaining using anti- α -MPP antiserum and anti-mouse IgG-AP. Lane M indicates marker proteins (75 and 50 kDa). (b) For MPP activity assay, each fraction (20 μ g protein) of crude extract from developing cells at the indicated times was incubated with the DdCOX IV precursor at 4 °C for 15 min, and then subjected to SDS-PAGE (12.5% gel). This was followed by Western blot analysis and immunostaining using anti-COX IV antiserum. Lane S, substrate alone; asterisk, non-specific band. (c, d) Wild-type cells were developed on non-nutrient H₂O-agar plates according to the method of Fisher & Annesley (2006). Crude extracts were prepared from the vegetative cells (0 h), cells at 12 h of development (12 h), and slug cells (24 h). The slug (24 h) was maintained at the slug stage even after an additional 12 h of development. The expression of α -MPP (c) and MPP activity (d) in these cells was examined.

protein was detected, which is consistent with the size predicted from the amino acid sequence (Fig. 5b). These two 42 and 38 kDa proteins appear to correspond to α -MPP_H and α -MPP_L, respectively. Taken together, these results indicate that α MPP237-GST is synthesized in the cytosol as a precursor of 51 kDa and imported into the mitochondria to form 42 and 38 kDa proteins, and that α -

MPP_L is produced from α -MPP_H by removing the N-terminal region.

α -MPP_L and MPP activity completely disappears around the slug stage in an α -MPP-overexpressing strain

The expression level of α -MPP protein appeared very low, and detection of the protein by Western blotting was rather difficult, requiring large amounts of sample to be loaded (150 μ g total protein per lane) and long staining times (overnight). We generated an α -MPP-overexpressing (α^{OE}) strain which expressed full-length α -MPP under the control of the actin-6 promoter and actin-8 terminator. The strain did not show any notable phenotypic differences in development (data not shown). Western blotting with the crude extract (20 μ g protein) from the α^{OE} strain easily detected α -MPP protein and its expression level in the α^{OE} strain was much higher (12 times or more) than the wild-type strain (Fig. 6a). α -MPP_H was highly and almost constantly expressed at about the same level throughout development of the α^{OE} strain. On the other hand, α -MPP_L disappeared at 12 and 16 h of development, and reappeared in culminating and fruiting body-forming stages of development (Fig. 6b, ~20–24 h). Corresponding to the absence of α -MPP_L at 12 and 16 h of development, MPP activity was also lost during this period (Fig. 6c). This suggests that α -MPP_L is the active

Fig. 5. Expression of α MPP237-GST in *D. discoideum* and in *E. coli*. The crude extracts (20 μ g protein) from a *Dictyostelium* transformant (pD α mpp237-gst) (a) and an *E. coli* transformant (pET α mpp237-gst) (b) were separated on a 9.0% SDS-PAGE gel and electroblotted onto PVDF membranes. The blots were immunostained using goat anti-GST antibody (GE Healthcare) and rabbit anti-goat IgG-AP (Santa Cruz). Lanes 1 and 3, wild-type *D. discoideum* and *E. coli*, respectively; lanes 2 and 4, transformants of *D. discoideum* (pD α mpp237-gst) and *E. coli* (pET α mpp237-gst), respectively; lane M, marker proteins (58 and 46 kDa).

(a) Expression of α -MPP in the wild-type and α -MPP^{OE} strains **(b) α -MPP** **(c) MPP activity**

form of α -MPP. Since β -MPP is expressed at almost constant levels throughout development (Nagayama *et al.*, 2008), these results indicate that MPP activity is controlled via the maturation of α -MPP from the α -MPP_H form to α -MPP_L.

 α -MPP_H processing activity is lost at the slug stage

When the crude extract (20 μ g protein) prepared from wild-type vegetative cells was added to the extract (20 μ g protein) from the α ^{OE} cells developed for 16 h, which contained only α -MPP_H (Fig. 6b, lane 16), α -MPP_H was converted to α -MPP_L (Fig. 7, lane 0). As shown in Fig. 6a, since the amount of wild-type α -MPP is much less than that of the α ^{OE} strain, the amount of α -MPP derived from the wild-type crude extract in the assay mixture is negligible. To examine the expression of processing activity of α -MPP_H to α -MPP_L (α -MPP_H processing activity) during development, the enzyme activity at various developmental stages was measured using α -MPP_H from α ^{OE} (16 h) cells as a substrate. As shown in Fig. 7, the processing activity was highest in cells at late development (20–28 h), moderate in vegetative cells (0 h), and very weak in cells at early stages (4 and 8 h). No processing activity was detected in cells at 12 and 16 h of development. The expression pattern of α -MPP_H processing activity during development is therefore similar to that of MPP activity and of α -MPP_L, even though MPP activity

and α -MPP_L have not completely disappeared in cells at 12 h development in the wild-type strain (Figs 4 and 7).

Development of an α -mmp antisense strain is delayed

To investigate the effects of α -MPP on development of *Dictyostelium*, we attempted to isolate knockout mutants of the *mppA* gene by homologous recombination. Consistent with similar studies of the *mppB* gene (Nagayama *et al.*, 2008), we were unable to isolate a *mppA*-null strain, suggesting that disruption of the *mppA* gene is lethal in *Dictyostelium* and that MPP plays an essential role in mitochondrial biogenesis and cell proliferation. So, in order

Fig. 7. Expression of α -MPP_H processing activity during development in the wild-type strain. Each crude extract from developing cells at the indicated times was incubated at 4 °C for 16 h with α -MPP_H prepared from the α ^{OE} cells developed at 16 h. α -MPP_H processing activity for the conversion of α -MPP_H to α -MPP_L was analysed by SDS-PAGE, followed by Western blotting using anti- α -MPP antiserum, as described in Methods.

to reduce the level of α -MPP in cells, we generated an antisense construct to express antisense RNA of the *mppA* gene under the control of the actin 6 promoter. A sense construct was also generated as a control (see Methods).

As described in our previous studies (Nagayama *et al.*, 2008), crude extracts prepared from vegetative cells of antisense, sense and the wild-type strains were subjected to SDS-PAGE and then immunostained with anti- α -MPP antiserum. α -MPP_H and α -MPP_L were detected at the same level in the wild-type and sense strains. Several antisense transformants (α A1 to about α A12) expressed α -MPP (α -MPP_H and α -MPP_L) at almost the same level as in the wild-type strain or at a slightly lower level. Of these transformants, the α A2 strain, which expressed α -MPP at a slightly lower level than the wild-type strain, was selected for further analysis, as described in previous work (Nagayama *et al.*, 2008). Determination of the α -MPP mRNA by semiquantitative RT-PCR revealed a slightly lower level (0.7-fold) of α -MPP mRNA in the α A2 strain relative to the wild-type strain, whereas the mRNAs encoding other mitochondrial proteins, β -MPP and COX IV, and the actin mRNA (control) were expressed at the same level as in the wild-type strain, as described previously (Nagayama *et al.*, 2008).

Next, we investigated whether the α A2 strain exhibited any physiologically significant changes in growth and development compared with the wild-type strain. The growth

rate of the α A2 strain was almost the same as that of the wild-type strain (data not shown). Fig. 8 shows the morphological changes of the α A2 strain during development compared with the wild-type and sense strains. Development of the sense α S strain was the same as that of the wild-type strain (Fig. 8a, b). In contrast, although development of the α A2 strain was almost normal until the mound-formation stage (about 12 h), the slug stage was lengthened; culmination started late at 24–28 h of development, and fruiting bodies were only formed at 32 h (Fig. 8c). Development of the α A2 strain was therefore delayed by approximately 8 h compared with the wild-type strain. Other antisense strains with less reduction of α -MPP than the α A2 strain exhibited similar development to the α A2 strain, except for the ~2–4 h delay (data not shown).

Fig. 9 shows the expression of α -MPP protein, MPP activity and α -MPP_H processing activity throughout development in the α A2 strain. Although α -MPP_L was expressed in cells until 8 h of development, it was not expressed in cells during late development from 12 to 24 h (Fig. 9a). Only α -MPP_H was present during this late period of development. MPP activity was high in vegetative cells and then decreased markedly by 8–12 h of development. MPP activity was not detected from 16 to 24 h of development, but reappeared at 28 h (Fig. 9b). The expression pattern of α -MPP_H processing activity was similar to that of MPP activity (Fig. 9c).

Fig. 8. Morphology of developing cells of the α A2 strain. Growing cells of the wild-type (a), sense α S (b) and antisense α A2 (c) strains were plated onto nitrocellulose filters, developed under standard conditions, and then photographed at the indicated times.

Fig. 9. Analysis of α -MPP expression, MPP activity and α -MPP_H processing activity during development in the $\alpha A2$ strain. (a) Crude extracts were prepared from the developing cells of the $\alpha A2$ strain at the indicated times. Western blotting and immunostaining were performed by the procedure described in Fig. 4. (b) The measurements of MPP activity in crude extracts were performed using the DdCOX IV precursor, as described in Fig. 4. (c) The measurements of α -MPP_H processing activity in crude extracts were performed using α -MPP_H as a substrate, as described in Methods.

Taken together, these results show that α -mpp antisense RNA causes a prolonged slug stage at which α -MPP_L protein, MPP activity and α -MPP_H processing activity are absent. When these activities reappear, the delay is eventually overcome to allow the continuation of development and the formation of fruiting bodies.

DISCUSSION

Prior to this study of *Dictyostelium* MPP, there was no information on α -MPP in the *Dictyostelium* Database. The present study focused on characterizing α -MPP and *mppA*, the gene encoding α -MPP. The *Dictyostelium* genome has a single copy of *mppA* (data not shown), and the *Dictyostelium* genome project revealed that the gene encoding α -MPP (DDB0232199, *mppA*) is located on chromosome 2 (<http://dictybase.org>) (Eichinger *et al.*, 2005).

As shown in Figs 3 and 4(a), expression of α -MPP mRNA is downregulated after early development, whereas, on the whole, its protein is expressed at a constant level as two forms, α -MPP_H and α -MPP_L, throughout the life cycle, except at 12 h of development. Expression patterns of α -MPP mRNA and its protein during development are similar to those of some other mitochondrial proteins, such as β -MPP (Nagayama *et al.*, 2008), succinyl-CoA synthetase α -subunit (Birney & Klein, 1995), adenine nucleotide translocator (Bof *et al.*, 1999), chaperonin 60 (Kotsifas *et al.*, 2002) and DNA topoisomerase (Komori *et al.*, 1997), suggesting the stable nature of mitochondrial proteins in *Dictyostelium* development.

Western blot analysis using an anti- α -MPP antiserum, which recognizes the C-terminal region (amino acid residues 238–654) of α -MPP, showed two major cross-reactivity bands, α -

MPP_H (65 kDa) and α -MPP_L (60 kDa). These two forms might be produced from the *mppA* gene, which is a single copy in the *Dictyostelium* genome, by alternative splicing. However, the fact that the *mppA* gene has no apparent intron and that, in Fig. 7, α -MPP_L appears to be produced by processing of α -MPP_H indicates that α -MPP_H and α -MPP_L are not the products of a single-copy *mppA* gene produced by alternative splicing.

α -MPP_H and α -MPP_L are present in the mitochondrial matrix fraction (Nagayama *et al.*, 2008). Since α -MPP_H is smaller than the predicted size of the precursor form, 73 kDa, it is considered that α -MPP_H is an intermediate. α -MPP_L appears to be the mature form of α -MPP, because MPP activity is lost when α -MPP_L is absent in the cells (Figs 4, 6 and 9). This is also supported by the results of expression of α MPP237–GST in *D. discoideum* and in *E. coli* (Fig. 5).

Since the expression pattern of α -MPP_H processing activity during development is similar to that of MPP activity (Figs 4, 7 and 9), it is possible that the processing activity is due to the self-processing of MPP. Some modification of α -MPP_H, such as phosphorylation/dephosphorylation or glycosylation/deglycosylation, might activate its self-processing. When the MPP activity and α -MPP_L disappeared at the slug stage, another form, which was slightly smaller than α -MPP_H, was detected (Fig. 6). The appearance of this form might reflect differences in the phosphorylated and/or glycosylated state of the α -MPP molecule species. At present, however, we have no information on this form. There was a considerable difference in kinetics of the processing activities using COX IV precursor and α -MPP_H as substrates, when the activities were measured at low temperatures such as 0 or 4 °C. The COX IV precursor was

processed to intermediate within 1 min, while α -MPP_H processing activity was not detectable after 1 h incubation at these temperatures (data not shown). Since it is possible that MPP shows different kinetics on different substrates, the possibility of self-processing could not be excluded. Alternatively, α -MPP_H could be cleaved by an unidentified protease. Since an α MPP237–GST fusion protein containing only the N-terminal 237 residues of α -MPP was also found to be expressed as two protein bands, this suggests that processing of α -MPP_H to α -MPP_L is unlikely to result from self-processing. If this is the case, MPP activity could be controlled by developmentally regulated expression or activation/inactivation of the unknown protease.

In the α A2 strain, the effects of the α -*mpp* antisense RNA were not observed in vegetatively growing cells and in cells during early development. The developmental phase of the slug stage, however, was prolonged. MPP enzyme activity was not detected during the prolonged period of the slug stage (Fig. 9b). The accumulation of α -MPP_H at the slug stage of the α A2 strain appears to be caused by the absence of α -MPP_H processing activity (Fig. 9a, c); that is, the defect in α -MPP_H processing activity and repression of the re-expression of α -MPP_L and MPP activity at the slug stage resulted in a prolonged slug stage (Figs 8 and 9). These results indicate that the re-expression (or reactivation) of α -MPP_H processing activity at the late slug stage appears to be necessary for the formation of a fruiting body.

Defective mitochondria resulting from mutation of the mitochondrial genome cause defects in cell-type differentiation, a prolonged slug stage, and a failure to form fruiting bodies (Wilczynska *et al.*, 1997; Chida *et al.*, 2004, 2008). Also, deficiencies in nuclear-encoded mitochondrial proteins cause defects in *Dictyostelium* development (van Es *et al.*, 2001; Kotsifas *et al.*, 2002; Morita *et al.*, 2005). Especially, mitochondrial chaperone Hsp90 (TRAP-1) has been shown to be involved in late development, including spore differentiation (Morita *et al.*, 2005). Mitochondrial defects caused by a decrease of MPP activity by α -*mpp* antisense RNA inhibition may prolong the slug stage to delay late development. Despite this similar phenotype, the relationship between DdTRAP-1 and α -MPP remains to be determined. Taken together, however, these studies highlight the importance of mitochondrial function in *Dictyostelium* development.

Although antisense RNA of the *mppA* gene resulted in a fairly weak inhibition of α -MPP expression in vegetative cells (Nagayama *et al.*, 2008), there was no effect during early development (up to ~10 h), when the α -MPP mRNA is strongly expressed. After 10 h of development, transcription of the *mppA* gene is downregulated to less than one-tenth of that in vegetative cells (Fig. 3). Although the reason why the repression of *mppA* resulted in such a prolonged slug stage is largely unknown, it is possible that the rather weak repression has greater effects at the slug

stage, where the mRNA level of α -MPP is much lower than that in vegetatively growing cells. The expression of α -*mpp* antisense RNA was controlled by an actin 6 promoter. Actin genes are highly expressed during growing phases and early development (Alton & Lodish, 1977; Margolskee & Lodish, 1980). Since expression of actin genes decreases during late development, expression of α -*mpp* antisense RNA might also decrease during culmination. As a result, after the prolonged slug stage, culmination might start to give rise to formation of a fruiting body.

In this report, we show that a novel mechanism controls MPP activity via the processing of α -MPP by an unidentified protease activity in *Dictyostelium*. Although much research on MPP of other organisms has been reported, there has been no report, to our knowledge, that MPP activity is controlled by the processing of the constituent subunit, α -MPP or β -MPP. So, the MPP activity-controlling mechanism via the processing of α -MPP might be specific to *Dictyostelium*. At present, however, the mechanism underlying the processing of α -MPP_H to α -MPP_L has not been fully elucidated. To determine the processing mechanism of α -MPP and the controlling mechanism of MPP activity, the purification and characterization of the unidentified protease necessary for the processing of α -MPP_H to α -MPP_L and the identification of the N-terminal residues of α -MPP_H and α -MPP_L are in progress.

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