A Protein from a Parasitic Microorganism, Rickettsia prowazekii, Can Cleave the Signal Sequences of Proteins Targeting Mitochondria\textsuperscript{†}

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The obligate intracellular parasitic bacteria rickettsiae are more closely related to mitochondria than any other microbes investigated to date. A rickettsial putative peptidase (RPP) was found to resemble the \(\alpha\) and \(\beta\) subunits of mitochondrial processing peptidase (MPP), which cleaves the transport signal sequences of mitochondrial preproteins. RPP showed completely conserved zinc-binding and catalytic residues compared with \(\beta\)-MPP but barely contained any of the glycine-rich loop region characteristic of \(\alpha\)-MPP. When the biochemical activity of RPP purified from a recombinant source was analyzed, RPP specifically hydrolyzed basic peptides and presequence peptides with frequent cleavage at their MPP-processing sites. Moreover, RPP appeared to activate yeast \(\beta\)-MPP so that it processed preproteins with shorter presequences. Thus, RPP behaves as a bifunctional protein that could act as a basic peptide peptidase and a somewhat regulatory protein for other protein activities in rickettsiae. These are the first biological and enzymological studies to report that a protein from a parasitic microorganism can cleave the signal sequences of proteins targeted to mitochondria.

The endosymbiont hypothesis for the origin of mitochondria in eukaryotes is now widely accepted, accompanied by a growing interest in evolution as entire genomic sequences are revealed from various organisms. According to this hypothesis, a free-living bacterium as an organelle progenitor once entered an anaerobic organism, which is thought to be an archaebacterial ancestor, and established a constitutive endosymbiotic relationship with the host cells, mainly to supply ATP (7, 11, 17). Modern mitochondria originated when the parasitic invader lost most of its own genome and began to depend on nuclyarly encoded proteins for its biogenesis, although it retained control of the eukaryotic cell viability via metabolic and apoptotic pathways.

Most mitochondrial proteins are encoded in the nucleus and synthesized by cytoplasmic ribosomes as preproteins with N-terminal presequences that are required for targeting to mitochondria (3, 12). These preproteins are unfolded and imported into the mitochondrial matrix across the double membrane through protein translocation machinery comprising a translocase on the outer mitochondrial membrane and a translocase on the inner mitochondrial membrane (20, 24, 26, 27). Finally, the presequences are cleaved by a matrix-located metalloendopeptidase, i.e., mitochondrial processing peptidase (MPP) (10, 13, 16). Overall, this proteolytic processing is involved in maturation of the mitochondrial proteins and is essential for eukaryotic cell viability from unicellular (30) to multicellular (22) organisms. Therefore, the \(\alpha\) and \(\beta\) subunits of MPP (\(\alpha\)- and \(\beta\)-MPP, respectively) tightly regulate the protease action and specifically cleave the preproteins.

The genome sequences of the obligate intracellular parasitic bacteria rickettsiae (the agents that cause typhus) reveal gene profiles strikingly similar to those of mitochondria (2, 18, 23). Among the bacteria examined to date, rickettsiae are more closely related to mitochondria than any other bacteria analyzed at the genome level. Interestingly, Rickettsia prowazekii gene 219 (RP219) encodes a putative peptidase (rickettsial putative protease [RPP]) that is highly similar to MPPs (2), and corresponding genes have also been found in other rickettsial species (18, 23). Similar to \(\beta\)-MPPs, which are the catalytic MPP subunits (14, 15), RPPs have a zinc-binding motif, HxxEHx75E, in which the histidine residues and final glutamate residue presumably participate in metal binding, while the first glutamate residue could be involved in water activation for hydrolysis of the peptide bond. These active-site motifs are found in the M16 protease family of metalloendopeptidases characterized by Escherichia coli pitrilysin and insulinases from mammals and insects, which appear to diverge widely from bacteria to higher eukaryotes (25).

In the present study, we analyzed the structural characteristics and biochemical activities of the RPP from R. prowazekii. The RPP primary structure resembled those of both of the MPP subunits, since the N- and C-terminal regions of RPP were similar to the N domains of \(\beta\)-MPPs and C domains of \(\alpha\)-MPPs, respectively. The biphasic structure of RPP seemed to reflect dual functions, namely, catalytic and regulatory actions toward basic peptides and preprotein processing, respectively. Thus, the characteristics of the structures and functions of RPP and the MPP subunits could be inherited from a common ancestor protein in the parasite that led to the endosymbiotic evolution of mitochondria. Here, we discuss the evolutionary and functional relationships between proteins that resemble components of the mitochondrial transport-process...
MATERIALS AND METHODS

Genetic analyses. The amino acid sequences of MPP and RPP were assembled from the Swiss-Prot and Genome databases and aligned using the CLUSTAL W program (29). From this multiple sequence alignment, a consensus sequence for the active site of MPP was derived. The phylogeny was inferred by using the bootstrap method with 1,000 trials, and a bootstrap tree was drawn using TreeView 1.6.6. Genomic blast searches to identify microbial MPP-like proteins were carried out using the BLAST website for microbial genomes at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), using the whole protein sequence of yeast α- or β-MPP. The searches were performed with the EXPECT parameter set to 0.0001 and the FALTER set to default. In the resultant proteins, we collected MPP-like proteins with expected values under 1E−10 and confirmed whether each sequence had a glycine-rich loop (GRL) region.

Culture of rickettsiae and RPP expression analyses. Rickettsia typhi, Rickettsia conorii, and Rickettsia japonica were grown in Vero cells at 34°C and purified as described previously (31). Total RNA was extracted from purified R. typhi cells using a total RNA isolation system (Promega) according to the manufacturer's instructions. Next, cDNAs were synthesized from the total RNA using reverse transcriptase (Promega) and the primers 5'TTGGTACCATCAATAAATCCATTAAGATCATTCTG-3′ for rpp and 5′-TTGAAAGTCCATACCCACCC-3′ for groEL. Amplification of the DNA fragments was performed by PCR using the primer pairs 5′-ATGATAATCTGATGACC-3′ and 5′-TTCTCTTGACTTATGAGG-3′ for the genomic clone including RP219 inserted into lambda-Zap (a generous gift from S. G. Andersson and C. G. Kurland [University of Uppsala]) was a kind gift from S. G. Andersson and C. G. Kurland [University of Uppsala]) was used as the source of the RPP gene. The DNA fragment for the open reading frame of RPP was amplified by PCR using an RPP-specific pair of 5′ and 3′ primers and ligated into the expression vector pET-23d (Novagen), which produces proteins with a C-terminal His tag, thereby constructing pET-RPP. E. coli BL21 cells were cotransformed with pET-RPP and pKY206, a plasmid encoding groEL (H11032/H11032/11032/H11032) for R. prowazekii. Amplification of the DNA fragments was performed by PCR using the primer pairs 5′-ATGATAATCTGATGACC-3′ and 5′-TTCTCTTGACTTATGAGG-3′ for the genomic clone including RP219 inserted into lambda-Zap (a generous gift from S. G. Andersson and C. G. Kurland [University of Uppsala]) was used as the source of the RPP gene. The DNA fragment for the open reading frame of RPP was amplified by PCR using an RPP-specific pair of 5′ and 3′ primers and ligated into the expression vector pET-23d (Novagen), which produces proteins with a C-terminal His tag, thereby constructing pET-RPP. E. coli BL21 cells were cotransformed with pET-RPP and pKY206, a plasmid encoding the E. coli GroEL operon. After the cells were cultured in LB medium at 30°C for 24 h, the proteins were extracted and purified using a nickel-chelating Sepharose (Amersham Biosciences) column as described previously (15). The RPP-containing fractions eluted from the affinity column were pooled and incubated with 5 mM ATP at 0°C for 60 min to release the GroEL and ES associated with RPP. The protein solution was diluted by more than 10-fold with buffer A (20 mM Tris-Cl, pH 7.5, 30% glycerol, and 0.01% Tween 20) and loaded onto a DEAE-Sepharose (Amersham Biosciences) column equilibrated with buffer A. After the column was washed, RPP eluted with buffer A containing 100 mM NaCl. The last purification step was repeated once. Size exclusion chromatography was performed, using TSK-GEL SUPER SW 3000 (Tosoh) in 20 mM Tris-Cl (pH 7.5) containing 100 mM NaCl. The gene for PRR (H9250 Esq) was genetically close to either α-MPP or β-MPP and were more closely related to prokaryotic MPPs than to other eukaryotic MPPs (Fig. 1). The processing products were separated by SDSPAGE and visualized using an imaging analyzer (Voyager PR-HR Biopsctrometry). The processing efficiency was determined by quantifying the radioactivity of the cleaved protein relative to that of the total protein using Image Gauge 3.0 (Fujifilm Photofilm Co., Ltd., and Koshin Graphic Systems).

RESULTS

Evolutionary and structural characteristics of RPP. RPPs were identified in the genomes of several rickettsial species and were found to be highly conserved with each other. When three RPPs from R. prowazekii, R. typhi, and R. conorii were compared with MPP subunits (from prokaryotic to mammalian) using statistical genetics, the potential phylogenetic relationship between them revealed that RPPs were genetically close to either α-MPP or β-MPP and were more closely related to prokaryotic MPPs than to other eukaryotic MPPs (Fig. 1). Figure 2A shows structural schematic diagrams of R. prowazekii RPP and the yeast Saccharomyces cerevisiae MPP subunits. It was of interest to note that the N- and C-terminal regions of the RPP resembled the terminal domains of β-MPP and α-MPP (N and C domains), respectively. In particular, the N-terminal region of the RPP included an active-site motif, HxEXX$_2$E, that was previously characterized in a subfamily of M16 proteases. Moreover, the rickettsial amino acid sequences around the motif were highly conserved in the active-site sequences of β-MPPs (Fig. 3), indicating that RPPs potentially possess peptidase activity. On the other hand, the GRLs that are typically preserved in the C domains of α-MPPs were mostly lacking in the C domains of RPPs, whereas the rickettsial...
amino acid sequences corresponding to regions around the GRLs were comparatively similar to the primary structures of \( H9251 \)-MPPs (Fig. 3), suggesting a lesser importance for the GRL function among RPPs. The three-dimensional structure of yeast MPP complexed with a synthetic presequence peptide is represented in Fig. 2B according to the coordination data reported by Taylor et al. (28). Each yeast MPP subunit contains N and C domains of \( \sim 210 \) residues with nearly identical folding topologies, which are related by an approximately two-fold rotation. Thus, the higher-order structure of RPPs appears to resemble those of the MPP subunits and is probably more similar to that of \( \beta \)-MPP, such that RPPs would be expected to show peptidase activity.

**Gene and protein expression of RPP.** Since \( R. \) prowazekii is unavailable in Japan, we used another rickettsial species, \( R. \) typhi, whose RPP gene is closely related to the \( R. \) prowazekii one (Fig. 1). The RPP gene expression in \( R. \) typhi infecting simian cells was examined by reverse transcription-PCR (RT-PCR). Reverse transcripts from \( R. \) typhi total RNA extracts were amplified using specific primer pairs designed for the RPP-coding region, while RT-PCR for the GroEL-coding region served as a control. DNA fragments of 680 and 500 bp for the RPP and GroEL genes, respectively, were amplified from the transcripts in a reverse transcriptase-dependent manner (see Fig. S1A in the supplemental material). The DNA sequences of these fragments revealed that the RT-PCR products were the same as the corresponding part of each gene, indicating the existence of RPP mRNA in parasitic rickettsial cells. Three species of rickettsiae appeared to produce RPP proteins (see Fig. S1B in the supplemental material). Immunoactive proteins of around 50 kDa were detected, although the rickettsial proteins were slightly smaller than the recombinant RPP, probably due to the additional C-terminal His\(_6\) tag.

To characterize the RPP, we tried to purify the recombinant protein from an extract of \( E. \) coli cells transformed with a protein expression vector carrying the RPP gene. After centrifugation of the cell lysate, the RPP was barely recovered in the soluble protein fraction (see Fig. S1C in the supplemental material). However, we succeeded in producing the soluble protein by coexpression of the \( E. \) coli molecular chaperons GroEL and GroES (see Fig. S1C in the supplemental material). The recombinant RPP was purified using a combination of nickel affinity and ion exchange chromatographies, and the protein preparation was estimated to show \( \sim 99\% \) purity by SDS-PAGE and protein staining (see Fig. S1C in the supplemental material). Size exclusion chromatography revealed that the non-denatured molecular size was approximately 50 kDa (data not shown), suggesting that the recombinant RPP was in a monomeric state.

**Peptidase activity of RPP.** Since RPPs showed conservation of the active-site sequence of metalloendopeptidases of the M16 protease family, we expected that the recombinant RPP would show proteolytic activity. To investigate whether RPP inherently hydrolyzes bound peptides, several synthetic peptides were incubated with the purified RPP in vitro and analyzed by reverse-phase HPLC. Basic peptides from various sources, namely dynorphin A, vasoactive intestinal peptide, mastoparan, and \( \alpha \)-MSH amide, produced detectable amounts of fragments (Table 1; also see Fig. S2 in the supplemental material) following incubation with RPP, whereas cleavage of neutral and acidic peptides (peptides E to H in Table 1) by RPP was undetectable. Thus, RPP showed preferential cleav-
TABLE 1. RPP cleaves basic peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide sequence and sites of cleavage by RPP</th>
<th>Degradation (%)</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>YGGF1LRR1IRP1K1L1KWDNQ</td>
<td>76.4</td>
<td>+4</td>
</tr>
<tr>
<td>B</td>
<td>HSDAVF1TDN1YT1R1L1ROQ1M1</td>
<td>36.2</td>
<td>+3</td>
</tr>
<tr>
<td>C</td>
<td>AKYLNSILN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>AIP112IS1LY1NS1NK1A11AA</td>
<td>7.2</td>
<td>+3</td>
</tr>
<tr>
<td>E</td>
<td>VNTPEHV1YPYG1LS1PES1</td>
<td>&lt;1.0</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>CET1OFT1NL1D1LO1TS1</td>
<td>&lt;1.0</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>LEN1LE1E1AT1P1L1P</td>
<td>&lt;1.0</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>P1Hy1GP1W1L1E1EE1AY1GW1MD1F1H1</td>
<td>&lt;1.0</td>
<td>6</td>
</tr>
</tbody>
</table>

- Peptide A, dymorphin A; B, vasoactive intestinal peptide; C, mastoparan; D, αMSH amide; E, big endotheline; F, peptide fragment of somatostatin receptor SST2A; G, peptide fragment of μ opioid receptor MOR1A; H, gastrin I.
- Arrows denote RPP cleavage sites for peptides, which were identifiable.
- Basic residues are indicated in italics. 
- Cleavage efficiencies are determined as described in Materials and Methods.
- Net charges are calculated from acidic and basic amino acid residues in each peptide in the reaction condition.

TABLE 2. RPP cleaves mitochondrial presequence peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide sequence and sites of cleavage by RPP and MPP</th>
<th>Degradation by RPP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LS1ALARPV1G1AA1LRR1FS1T1SA1Q1NN1AK1VA</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>M1LAAK1N1L1R11NSS1L1S111F11RIAT11R111NST11Q1G1QS1A</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>LS1RV1AK1RA1F11F1S1FT1ST1VP1N1P11</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>ML1RA1L1T111TT1V1R1GP11P1L11R1S111L1S11A1A1S1A1SA1V</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>M1FS1K1L1A1HL1Q11RP1A11V1LS1R1G1</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>MLS1A1R1L1Q1A1FO1AQ11G1V1SR11KV1D1APT11KI1ST1LA</td>
<td>9,5</td>
</tr>
<tr>
<td>7</td>
<td>GR1W1R11L1VR1PR1AG1AG111L1G1R1S11GP11G1L1G1G1G1AV1AT1R1</td>
<td>7,3</td>
</tr>
<tr>
<td>8</td>
<td>L1S1L1R1Q1S1R11F1FK1P1AT11R11L1C1S1ST1Y1L11</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

- Peptide 1, mouse MDH2-26; 2, yeast heat shock protein SSCI (amino acids 1 to 32); 3, yeast MDH2-17; 4, mouse aldehyde dehydrogenase (amino acids 1 to 29); 5, human ornithine aminotransferase (amino acids 1 to 25); 6, yeast ubiquinol cytochrome c reductase subunit 2 (amino acids 1 to 32); 7, bovine ADX18-57; 8, yeast COXIV2-25.
- Arrows (↑) denote RPP cleavage sites for peptides that can be detected or MPP cleavage sites (↓).
- Cleavage efficiencies are determined as described in Materials and Methods.

Regulation of preprotein processing by RPP. Next, we examined whether RPP could cleave mitochondrial preproteins using mouse precursor of MDH (preMDH) and bovine precursor of ADX (preADX), which contain different presequence lengths of 16 and 58 residues, respectively. RPP alone could not cleave either preMDH or preADX (Fig. 4A). To our surprise, however, a stoichiometric mixture of RPP and yeast β-MPP (RPP–β-MPP) processed preMDH, whereas a similar stoichiometric mixture of RPP and yeast α-MPP did not. On the other hand, preADX was not cleaved by either RPP–β-MPP or RPP–α-MPP. A functional association between RPP and β-MPP was established, since β-MPP alone did not show any preprotein processing activities. However, the stable RPP–β-MPP interaction was not demonstrated by β-MPP pull-down assays using the His6 tag of RPP and affinity beads (data not shown).
preMDH processing, RPP and other. To address which active sites were involved in the cleavage of long presequences, we tried to construct some variants of the GRL-inserting RPP and to examine whether these variants could process preADX. However, the variant proteins were misfolded due to the protein inclusion in E. coli, and they seemed to be unstable during the refolding in vitro.

**DISCUSSION**

In the present study, we addressed the peptidase activity of a eubacterial homologue of MPP and found a functional and evolutionary relationship between RPP and MPP. The RPP cleaved basic peptides, including mitochondrial targeting presequence peptides, with partial specificity for MPP cleavage sites. The most notable finding was that RPP was able to activate eukaryotic β-MPP and subsequently render it able to process preproteins. Thus, RPP retains not only the structural characteristics of the processing peptidases but also the bifunctional hallmarks of the catalytic and regulatory subunits of MPP. Considering mitochondrial evolution on the basis of the endosymbiont theory, RPP appears to have the closest remaining structural and functional hallmarks of a processing peptidase in primitive mitochondria.

Although RPP is expressed in rickettsial cells, its functions in vivo remain unknown. For instance, what is its function in rickettsiae and what is its physiological substrate? One of the reasons for this lack of knowledge is the difficulty associated with elucidating the exact actions of RPP in vivo, since rickettsiae can only live within other cells. However, its actions in vivo may be speculated on based on the functions of the RPP-like proteins from the parasitic eubacteria. Analyses of a S. aureus mu- cysteine protease (AprG), the major serine protease secreted by S. aureus, revealed that the major serine protease secreted by S. aureus has a functional and evolutionary relationship with RPP.

**GRL is required for cleavage of long presequences.** Since RPP behaved as a regulatory subunit toward preMDH processing, similar to α-MPP, we next investigated why RPP–β-MPP was unable to cleave preADX. As mentioned above, there is a marked difference in the presequence lengths between the two preproteins, and RPP lacks a GRL region. GRL in yeast α-MPP was previously shown to play an essential role in cleaving a synthetic preMDH peptide (19). Here we analyzed whether an enzyme comprised of GRL deletion variants of yeast α-MPP (α-MPP<sup>GRL</sup>) and β-MPP (MPP<sup>GRL</sup>) could process the preproteins. MPP<sup>GRL</sup> was able to cleave preMDH, albeit with a lower processing activity than that of wild-type MPP, but showed inefficient preADX processing (Fig. 4C). An excess amount of α-MPP<sup>GRL</sup> in relation to β-MPP was null for the processing efficiency (data not shown). A pull-down assay of α-MPP<sup>GRL</sup> by β-MPP-His<sub>6</sub> previously revealed a stoichiometric association of α-MPP<sup>GRL</sup> with β-MPP (19). These results therefore suggest that the GRL region is required for the cleavage of long presequences but does not influence the association between the subunits. The RPP–β-MPP complex behaved as MPP<sup>GRL</sup> at least for processing of preMDH and preADX. The reason for the inability of the RPP–β-MPP complex to process preADX could be lack of GRL in RPP. To confirm whether the GRL is sufficient to cleave long presequences, we tried to construct some variants of the GRL-inserting RPP and to examine whether these variants could process preADX. However, the variant proteins were misfolded due to the protein inclusion in E. coli, and they seemed to be unstable during the refolding in vitro.
second, it could indirectly modulate the activity of a transcriptional regulator of aprE, possibly through proteolysis. Considering the homology of RPP to YmxG and the RPP peptidase/regulatory activities revealed in the present study, RPP may play a key role in regulating protein expressions through its protease activity. Even though the RPP functions in vivo remain unknown, the fact that the eubacterial MPP-like protein YmxG is not essential for viability or cell growth is interesting (4) when we consider the origin of these preprotein processing enzymes according to mitochondrial endosymbiont evolution, as discussed below.

Genes for proteins containing zinc-binding HxxEH motifs characteristic of M16 proteases and sharing high degrees of similarity with MPPs were identified by sequence searches of many bacterial genomes. BLAST (1) searches of prokaryotic genomes revealed homologues of yeast MPP subunits (see Table S1 in the supplemental material). Notably, there were nearly 500 putative proteins resembling β-MPP in the 511 genomes of eubacteria examined, whereas proteins showing slight homology to MPP with genetic significance were encoded in 29 genomes of archaeabacteria. In particular, BLAST searches of rickettsial genomes from 20 species identified 33 genes encoding β-MPP-like proteins, indicating that at least one RPP gene could be carried on each rickettsial genome. Notably, no putative MPP-like proteins carrying GRLs were found in the bacterial genomes. Considering the genomic distributions of MPP-like genes described above, and since it is widely accepted that mitochondria originated from the α-proteobacterial order Rickettsiales based on phylogenetic analyses comparing the sequences of bacterial and mitochondrial genes (2, 9, 18, 23), MPP is unlikely to have originated from proteins in the host cells of an archaeabacterial ancestor and is most likely to have arisen from an RPP-like progenitor in a parasitic bacterium. During the endosymbiotic evolution of mitochondria, the gene encoding the progenitor of MPP, which may be nonessential, similar to ymxG in B. subtilis, could be transferred from the endosymbiont to the host cell. At this stage, the nonessential gene conversion presumably succeeded during mitochondrial evolution due to the maintained viability of the imperfect symbiotic organelle. Alternatively, the genes may exist in both the endosymbiont and host genomes under the predominant circumstance of the immature eukaryotic cells. In any case, during or after the gene transfer, gain of the signal sequences and procurement of the protein transport-processing system must be one of the critical stages toward the success of endosymbiotic evolution in the primitive eukaryotic cells.

Precise recognition of signal sequences by the protein translocation and processing system must be one of the critical stages for the biogenesis of mitochondria, plastids, and hydrogenosomes, which are thought to have arisen from endosymbiotic bacteria. Although it is unclear how the system was acquired during endosymbiotic evolution, the central components of the mitochondrial translocases may be genetically converted from eubacterial pore/channel-forming proteins and chaperones (8). The relationship between MPP and RPP suggests the same situation, as mentioned above. Since MPP primarily bears the traits of a parasitic bacterial peptidase, it appears that MPP and RPP have a common progenitor in ancient parasitic bacteria. During the evolution stage from endosymbiont to mitochondria, the progenitor gene was duplicated and the proteins were converted into two distinct components of the processing enzyme, which are now α- and β-MPP (see Fig. S6 in the supplemental material). It appears that this protein dimerization was required for the peptidase regulation and that gain of the GRL region was particularly involved in the efficient processing of longer transport signal sequences, since these lengths have tended to become greater. Interestingly, the stromal processing peptidase that cleaves the signal peptide of the plastidial protein precursor has an M16 protease active site and shares homology to MPPs, although it is active as a single polypeptide and carries no GRL region. To decipher the complex histories of preprotein transport and processing systems in endosymbiotic organelles, broad genetic investigations and biochemical analyses are required for parasitic organisms and some lower eukaryotes, since it was recently reported that Giardia mitosomes and Trichomonad hydrogenosomes share a common mode of protein targeting (6).

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REFERENCES


