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Autocatalytic Processing of *m*-AAA Protease Subunits in Mitochondria

Mirko Koppen,*† Florian Bonn,* Sarah Ehses,* and Thomas Langer*‡

*Institute for Genetics, Centre for Molecular Medicine (CMMC), Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, 50674 Cologne, Germany; and ‡Max-Planck-Institute for Biology of Aging, 50931 Cologne, Germany

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***m*-AAA proteases are ATP-dependent proteolytic machines in the inner membrane of mitochondria which are crucial for the maintenance of mitochondrial activities. Conserved nuclear-encoded subunits, termed paraplegin, Afg3l1, and Afg3l2, form various isoenzymes differing in their subunit composition in mammalian mitochondria. Mutations in different *m*-AAA protease subunits are associated with distinct neuronal disorders in human. However, the biogenesis of *m*-AAA protease complexes or of individual subunits is only poorly understood. Here, we have examined the processing of nuclear-encoded *m*-AAA protease subunits upon import into mitochondria and demonstrate autocatalytic processing of Afg3l1 and Afg3l2. The mitochondrial processing peptidase MPP generates an intermediate form of Afg3l2 that is matured autocatalytically. Afg3l1 or Afg3l2 are also required for maturation of newly imported paraplegin subunits after their cleavage by MPP. Our results establish that mammalian *m*-AAA proteases can act as processing enzymes in vivo and reveal overlapping activities of Afg3l1 and Afg3l2. These findings might be of relevance for the pathogenesis of neurodegenerative disorders associated with mutations in different *m*-AAA protease subunits.**

INTRODUCTION

Dysfunction of mitochondria, essential multifunctional organelles, has severe cellular consequences, and is associated with neurodegeneration and aging (Chan, 2006). Various intraorganellar proteases protect mitochondria against damage and ensure cellular integrity (Tatsuta and Langer, 2008). AAA proteases, conserved and membrane-bound ATP-dependent peptidases, are central for protein quality surveillance in the inner membrane (Koppen and Langer, 2007). Mutations in subunits of these proteolytic complexes lead to axonal degeneration and neuronal cell death in human, illustrating their central role for mitochondrial integrity (Casari *et al.*, 1998; Cagnoli *et al.*, 2008; DiBella *et al.*, 2008).

AAA proteases comprise a conserved protein family ubiquitously distributed in eukaryotic cells (Koppen and Langer, 2007). Mitochondria harbor at least two AAA protease complexes, which differ in their topology in the inner membrane: *i*-AAA proteases active in the intermembrane space and *m*-AAA proteases with catalytic sites exposed to the matrix space. They are built up of homologous subunits that assemble into homo- or hetero-oligomeric complexes. In contrast to the yeast *m*-AAA protease, which is composed of Yta10 and Yta12 subunits, several isoenzymes with variable subunit composition have been identified in mammalian mitochondria (Arlt *et al.*, 1996; Koppen *et al.*, 2007). Homologous paraplegin and Afg3l2 subunits form a hetero-oligomeric *m*-AAA protease, but homo-oligomeric assemblies composed of Afg3l2 only are also present in the inner membrane (Koppen *et al.*, 2007). Mouse mitochondria harbor an additional *m*-AAA protease subunit, Afg3l1 (Kremmidiotis *et al.*, 2001), which forms homo-oligomeric complexes as well as hetero-oligomeric complexes with Afg3l2 and paraplegin (Koppen *et al.*, 2007).

Tissue-specific differences in the relative expression of *m*-AAA protease subunits do exist (Koppen *et al.*, 2007; Martinelli *et al.*, 2009). The formation of different isoenzymes may therefore explain different pathogenic effects of mutations in *m*-AAA protease subunits. Loss of function of paraplegin (encoded by the *SPG7* gene) causes an autosomal recessive form of hereditary spastic paraplegia (HSP; Casari *et al.*, 1998), a neurodegenerative disorder characterized by retrograde degeneration of cortical motor axons and peripheral sensory neurons (Rugarli and Langer, 2006; Salinas *et al.*, 2008). Mutations in *Afg3l2*, on the other hand, impair axonal development in mice (Maltecca *et al.*, 2008) and have been associated recently with the degeneration of Purkinje cells in autosomal dominant spinocerebellar ataxia (SCA) type 28 (Cagnoli *et al.*, 2008; DiBella *et al.*, 2008). The molecular pathogenesis of these diseases, however, remains to be elucidated.

m-AAA proteases exert versatile activities within mitochondria, making an interpretation of phenotypes associated with mutations in proteolytic subunits difficult (Koppen and Langer, 2007). Most importantly, *m*-AAA proteases are not only quality control enzymes completely degrading misfolded polypeptides to peptides, but can also act as processing enzymes specifically cleaving mitochondrial proteins with regulatory functions. This was first recognized in yeast where the *m*-AAA protease mediates maturation of the ribosomal protein MrpL32, a prerequisite for mitochondrial

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† Present address: Department of Cell Biology, University of Geneva, 1207 Geneva, Switzerland.

Address correspondence to: Thomas Langer (Thomas.Langer@uni-koeln.de).

protein synthesis (Nolden *et al.*, 2005). Both hetero- and homo-oligomeric mammalian isoenzymes are able to substitute for the yeast *m*-AAA protease demonstrating functional conservation (Nolden *et al.*, 2005; Koppen and Langer, 2007). Pathogenic defects caused by mutations in *AFG3L2* or in *SPG7* may therefore result from an impaired processing of specific mitochondrial proteins, rather than from a damaging effect of misfolded polypeptides accumulating within mitochondria. However, in vivo evidence for cleavage of specific substrates by mammalian *m*-AAA protease isoenzymes is limited. Both *m*-AAA and *i*-AAA proteases have been linked to the processing of the dynamin-like GTPase OPA1, a central component of the mitochondrial fusion machinery, at two distinct sites (Ishihara *et al.*, 2006; Duvezin-Caubet *et al.*, 2007; Griparic *et al.*, 2007; Song *et al.*, 2007). However, in particular the role of the *m*-AAA protease is controversially discussed, because OPA1 processing occurs normally in paraplegin-deficient $Spg7^{-/-}$ mice and as yet another protease, the rhomboid protease PARL, was proposed to cleave OPA1 at the same site (Cipolat *et al.*, 2006).

Here, we have examined the processing of various mouse *m*-AAA protease subunits upon import into mitochondria. Our results provide direct evidence for a role of Afg3l1 and Afg3l2 as processing enzymes within mitochondria. They mediate not only their own maturation in an autocatalytic manner, but process newly imported paraplegin subunits.

MATERIALS AND METHODS

Cloning Procedures

Full-length paraplegin, Afg3l1, and Afg3l2 were expressed under the control of the *YTA10* promoter in yeast using the multicopy vectors YEplac181, YEplac195, and YEplac112, respectively. To facilitate purification and subsequent protein sequencing, *m*-AAA protease subunits harboring a C-terminal hexahistidine-tag were generated and overexpressed in yeast. To this end, the corresponding DNA sequences were amplified by PCR and cloned into YEplac181 or YEplac112 under the *ADH1* promoter. About 250 base pairs of the 3′UTR of the *YTA10* gene were introduced downstream of the respective gene to increase expression. Site-directed mutagenesis was performed as described previously (Koppen *et al.*, 2007). For transcription in vitro, *Spg7* and *Afg3l1* were amplified by PCR and cloned into pGEM4 (Promega, Madison, WI) under the control of the SP6 promoter, whereas the *Afg3l2* gene was transcribed using the T7 promoter. Truncated variants of paraplegin lacking amino acids 1–48 (Δ1–48) or 1–105 (Δ1–105) were amplified by PCR, a paraplegin variant lacking amino acids 48–105 (Δ48–105) was generated by site-directed mutagenesis.

Yeast Strains and Growth Conditions

Yeast strains expressing mouse *m*-AAA protease subunits were generated by transformation of Δ*yta10*Δ*yta12* cells (YKO200; Koppen *et al.*, 2007) with yeast expression plasmids encoding paraplegin, Afg3l1, or Afg3l2, or mutant variants thereof. Yeast cells were cultivated at 30°C either in YP medium or synthetic complete (SC) medium supplemented with 2% (wt/vol) glucose or 2% (wt/vol) galactose and 0.5% (wt/vol) lactate.

Isolation of Mitochondria

Yeast and mouse mitochondria were isolated as described previously (Tatsuta and Langer, 2007). To isolate mitochondria from mouse embryonic fibroblasts (MEFs), cells were resuspended in homogenization buffer (250 mM sucrose, 10 mM HEPES/KOH, pH 7.4) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and disrupted by 15 strokes with a Teflon homogenizer at 800 rpm. The homogenate was centrifuged twice for 5 min at 600 × *g* and 4°C to remove unbroken cells and nuclei. Mitochondria were sedimented by centrifugation for 10 min at 10,000 × *g* and 4°C and resuspended in 250 mM mannitol, 0.5 mM EGTA/Tris, and 5 mM HEPES/KOH, pH 7.4.

Protein Import into Isolated Mitochondria

The cell-free synthesis of ^{35}S-labeled precursor proteins of paraplegin, Afg3l1, and Afg3l2 was performed using the TNT Sp6 or T7 Coupled Reticulocyte lysate system (Promega) according to the manufacturer's instructions. For in vitro protein import, mitochondria isolated from murine liver or MEFs were resuspended to a concentration of 1 mg/ml in import buffer (250 mM sucrose, 5 mM MgAc, 80 mM KAc, 10 mM sodium succinate, and 20 mM HEPES/KOH, pH 7.4) supplemented with 2.5 mM ATP and 1 mM fresh DTT or additionally 0.5 μM valinomycin to dissipate the membrane potential. Samples were incubated at 30°C for 3 min before ^{35}S-labeled precursor proteins were added. Import was performed for 30 min at 30°C and halted by the addition of 0.5 μM valinomycin. If indicated, samples were further incubated at 30°C for 60 min (chase). Nonimported precursor proteins were degraded by trypsin (50 μg/ml). After an incubation at 4°C for 20 min, trypsin was inhibited with soybean trypsin inhibitor (1 mg/ml). Mitochondria were washed with SHKCl buffer (0.6 M sorbitol, 50 mM HEPES/KOH, pH 7.4, and 80 mM KCl) and analyzed by SDS-PAGE, immunoblotting, and autoradiography.

Purification of m-AAA Protease Subunits for Edman Sequencing

Yeast mitochondria (40 mg) harboring mouse *m*-AAA protease subunits containing a C-terminal hexahistidine (6His) tag were solubilized in 1% (vol/vol) NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (wt/vol) glycerol, 50 mM imidazole, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche) at a concentration of 8 mg/ml. After incubation for 20 min at 4°C, insoluble material was removed by centrifugation (20 min, 30,000 × *g* at 4°C). The supernatant was applied to 300 μl Ni Sepharose High-Performance beads (GE Healthcare, Waukesha, WI) and incubated for 2 h at 4°C under gentle mixing. Subsequently, the beads were washed three times with 5 ml washing buffer [0.2% (vol/vol) NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (wt/vol) glycerol, 70 mM imidazole, and 1 mM PMSF]. Bound proteins were eluted incubating the beads twice with 500 μl 0.2% (vol/vol) NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (wt/vol) glycerol, and 300 mM imidazole, subjected to TCA precipitation and analyzed by SDS-PAGE. After transfer to Sequi-Blot PVDF membranes (Bio-Rad) using CAPS electroblotting buffer [10 mM CAPS/NaOH, pH 11, and 10% (vol/vol) methanol] and staining with 0.1% (wt/vol) Coomassie R-250 in 40% (vol/vol) methanol, intermediate and mature forms of paraplegin, Afg3l1, and Afg3l2 were excised and analyzed by Edman sequencing using an ABI Procise 491 sequencer (Columbia, MD).

Cell Culture and Down-Regulation of Afg3l1 and Afg3l2

MEFs were cultivated in Dulbecco's minimum essential medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids, and 1 mM sodium pyruvate at 37°C in 90% humidified air and 5% CO_2. Primary MEFs were established from E14.5 embryos derived from intercrosses of time-mated $Spg7^{+/+}$ or $Spg7^{-/-}$ mice and immortalized by SV40 transformation (Ferreirinha *et al.*, 2004). RNA interference (RNAi)-mediated down-regulation of gene expression was performed using specific Stealth RNAi and the nontargeting Stealth RNAi Negative Control (Invitrogen). The sequences are 5′-CCUGCCUCCGUACGCUCUAUCAAUA-3′ for AFG3L2 5′-GCGAAACCAUGGUGGAGAAGCCAUA-3′ for AFG3L1. MEFs were transfected twice with 10 nM of each small interfering RNA (siRNA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells were harvested ~2.5 d after transfection for subsequent lysis or isolation of mitochondria, respectively. Down-regulation was monitored by immunoblot analysis using Afg3l1- and Afg3l2-specific antisera described previously (Koppen *et al.*, 2007).

Miscellaneous

The following procedures have been performed as described previously: coimmunoprecipitation of *m*-AAA protease complexes using subunit-specific antibodies (Koppen *et al.*, 2007); blue-native gel electrophoresis (BN-PAGE) analysis (Wittig and Schägger, 2008); purification of recombinant yeast mitochondrial processing peptidase (MPP; Luciano and Geli, 1996); and MPP cleavage assay in vitro (Nolden *et al.*, 2005).

RESULTS

Maturation of Paraplegin Depends on Afg3l1 and Afg3l2

We used RNAi in MEFs to transiently down-regulate *m*-AAA protease subunits individually or in combination. To exclude unspecific effects, we examined three different oligonucleotides specific for the respective gene and scrambled RNAi as a control. Efficient depletion of Afg3l1, Afg3l2, and paraplegin was verified by immunoblotting (Figure 1A). Depletion of MEFs from Afg3l1, Afg3l2, or paraplegin did not affect the steady-state concentration of the remaining *m*-AAA protease subunits (Figure 1A). However, we observed the accumulation of an additional form of paraplegin upon simultaneous down-regulation of Afg3l1 and Afg3l2

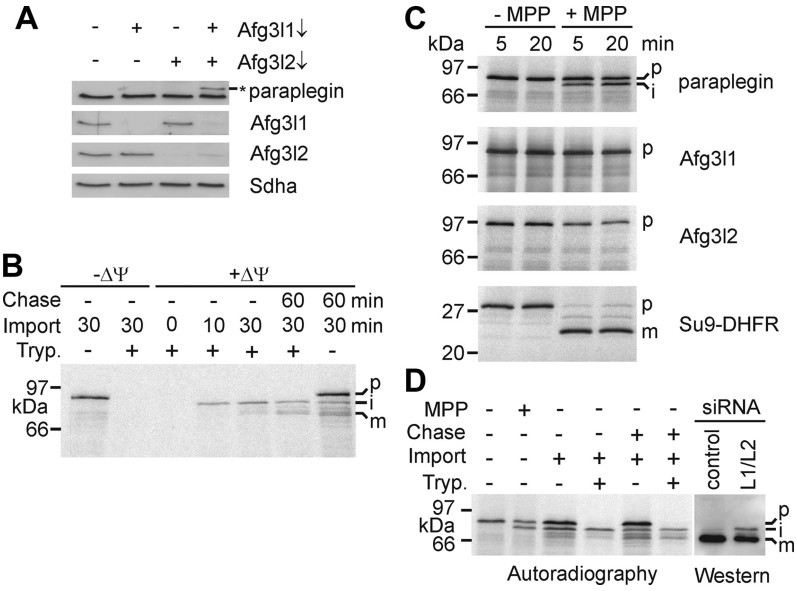

Figure 1. Paraplegin is processed by MPP and Afg3l1/Afg3l2 upon import into mitochondria. (A) Impaired processing of paraplegin upon down-regulation of Afg3l1 and Afg3l2. MEFs were transfected twice with scrambled siRNA or siRNAs directed against Afg3l1, Afg3l2, or both. After 48 h, cells were lysed and analyzed by SDS-PAGE and immunoblotting using paraplegin-, Afg3l1-, and Afg3l2-specific antibodies and, as a gel loading control, with antibodies directed against the 70-kDa subunit of succinate dehydrogenase (Sdha). A larger paraplegin variant, which accumulates upon down-regulation of Afg3l1 and Afg3l2, is marked with an asterisk. (B) Two-step processing of newly imported paraplegin. ^{35}S-labeled paraplegin was imported into isolated mouse liver mitochondria at 30°C in the absence ($-\Delta\Psi$) or presence ($+\Delta\Psi$) of a mitochondrial membrane potential. Import was halted at various time points by the addition of valinomycin (0.5 μM). Samples were incubated further at 30°C for 60 min when indicated (Chase). Nonimported precursor proteins were removed by treatment with trypsin (Tryp, 50 μg/ml). Samples were analyzed by SDS-PAGE and autoradiography. (C) The mitochondrial processing peptidase (MPP) cleaves paraplegin in vitro. ^{35}S-labeled precursor proteins were incubated at 30°C for 5 or 20 min in the absence or the presence of purified MPP. ^{35}S-labeled Su9 (1–69)-DHFR served as a positive control. Samples were analyzed by SDS-PAGE and autoradiography. (D) Accumulation of the intermediate form of paraplegin upon down-regulation of Afg3l1 and Afg3l2. The electrophoretic mobility of different paraplegin forms accumulating after incubation of ^{35}S-labeled paraplegin with MPP in vitro, after import of ^{35}S-labeled paraplegin into isolated liver mitochondria, and in MEFs after transfection with control siRNA or siRNA directed against Afg3l1 and Afg3l2 (L1/L2) were compared using SDS-PAGE. Paraplegin was detected either by autoradiography or immunoblotting (Western) using paraplegin-specific antibodies. p, precursor; i, intermediate form; m, mature form.

but not in control cells (Figure 1A). This paraplegin species was significantly larger than mature paraplegin suggesting that maturation of paraplegin is impaired in the absence of Afg3l1 and Afg3l2. Notably, paraplegin was processed normally when only Afg3l1 or Afg3l2 were down-regulated, indicating overlapping activities of both *m*-AAA protease subunits (Figure 1A).

Two-Step Processing of Paraplegin upon Import into Mitochondria

These findings prompted us to examine the processing of paraplegin upon import into mitochondria. We synthesized the precursor form of mouse paraplegin in a cell-free translation system in the presence of [^{35}S]methionine and incubated the protein with energized mitochondria isolated from mouse liver (Figure 1B). Import of paraplegin occurred in a membrane potential–dependent manner and was accompanied by the formation of a protease-protected form, which was smaller than the precursor form. An even smaller form of paraplegin was generated upon further incubation of mitochondria after completion of import (Figure 1B). This form comigrated with endogenous paraplegin detected by immunoblotting with a paraplegin-specific antiserum (data not shown) and thus corresponds to mature paraplegin. We therefore conclude that paraplegin is cleaved upon import in at least two steps, giving rise first to an intermediate form that is subsequently converted into the mature protein.

Paraplegin possesses a typical N-terminal mitochondrial targeting signal characterized by the ability to form an amphipathic, positively charged helix. To test for the involvement of the general MPP in paraplegin processing, we incubated the ^{35}S-labeled precursor form of paraplegin with recombinant MPP purified from *Escherichia coli*. Paraplegin was converted to a smaller form in the presence of MPP demonstrating that MPP cleaves the precursor form of paraplegin in vitro (Figure 1C). Similarly, the model substrate Su9 (1–69)-DHFR, which contains the presequence of subunit 9 of the *Neurospora crassa* F_1F_O-ATPase including two MPP cleavage sites (Ungermann *et al.*, 1994), was matured by MPP (Figure 1C).

A comparison of the electrophoretic mobility of different paraplegin species accumulating in import studies, siRNA experiments, and the in vitro MPP assay revealed that similar-sized paraplegin species are generated at early stages of mitochondrial import, upon down-regulation of Afg3l1 and Afg3l2 in MEFs, and upon MPP cleavage of paraplegin in vitro (Figure 1D). We therefore conclude that paraplegin is cleaved by MPP upon import into mitochondria and converted to an intermediate form. Down-regulation of Afg3l1 and Afg3l2 results in the accumulation of this intermediate form and thus specifically affects the subsequent maturation of paraplegin.

Maturation of Paraplegin Can Occur upon Assembly into m*-AAA Protease Complexes*

To further define the role of Afg3l1 and Afg3l2 for maturation of paraplegin, we examined the assembly of newly imported paraplegin molecules with other preexisting *m*-AAA protease subunits in coimmunoprecipitation experiments (Figure 2A). Mitochondrial membranes were solubilized with digitonin after completion of import, and extracts were incubated with antisera specific for Afg3l1 or Afg3l2 and, for control, with preimmune serum. Newly imported paraplegin was precipitated with Afg3l1 and Afg3l2 antibodies but not with preimmune serum (Figure 2A). Notably, both intermediate and mature forms of paraplegin were found to interact with Afg3l1 or Afg3l2.

In further experiments, we assessed the oligomeric state of paraplegin newly imported into liver mitochondria by BN-PAGE (Figure 2B). ^{35}S-labeled paraplegin was almost exclu-

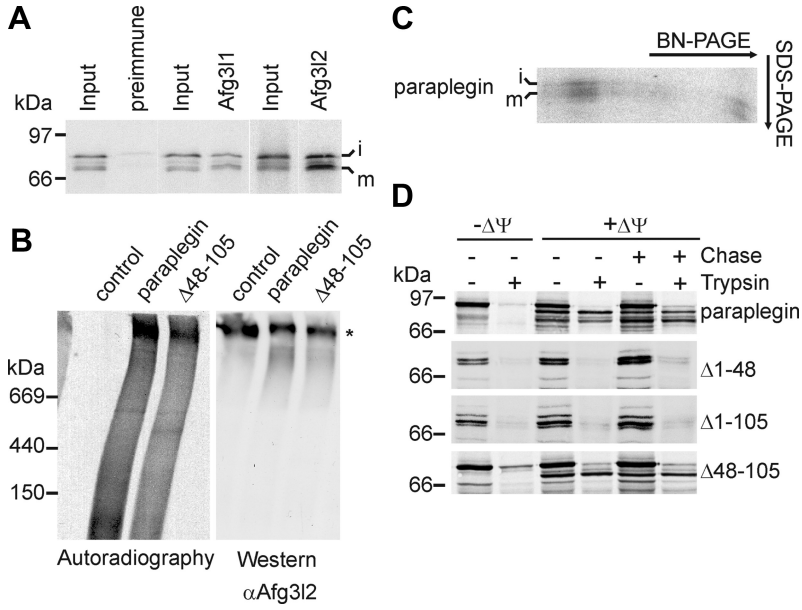

Figure 2. Assembly of newly imported paraplegin with Afg3l1 and Afg3l2. (A) Coimmunoprecipitation of newly imported ^{35}S-labeled paraplegin with subunits of the *m*-AAA protease. Murine liver mitochondria (200 μg) containing ^{35}S-labeled, newly imported paraplegin were lysed in digitonin. After removal of an input control (10%), extracts were subjected to coimmunoprecipitation using affinity-purified polyclonal antibodies directed against Afg3l1 or Afg3l2. Preimmune serum was used as a negative control. Precipitates were analyzed by SDS-PAGE and autoradiography. (B) Assembly of ^{35}S-labeled paraplegin variants into high-molecular-weight complexes in mitochondria. After import of ^{35}S-labeled paraplegin, a paraplegin variant lacking amino acid residues 48–105 (Δ48–105) or an unrelated precursor protein (control) into mouse liver mitochondria (100 μg), membranes were solubilized with digitonin and extracts were analyzed by BN-PAGE and autoradiography (left) or immunoblotting (right) using Afg3l2-specific antiserum (αAfg3l2). *m*-AAA protease complexes are marked with an asterisk. Thyroglobulin (669 kDa), apoferritin (440 kDa), and alcohol dehydrogenase (150 kDa) were used for calibration. (C) Paraplegin assembly does not require its processing to the mature form. *m*-AAA protease complexes containing newly imported paraplegin were isolated from BN-PAGE and fractionated by SDS-PAGE in a second dimension. Paraplegin species were detected by autoradiography. i, intermediate form of paraplegin; m, mature form. (D) Mitochondrial targeting of paraplegin depends on amino acid residues 1–48. ^{35}S-labeled paraplegin or variants thereof (Δ1–48; Δ1–105; Δ48–105) were incubated with isolated mouse liver mitochondria at 30°C for 30 min in the absence (−ΔΨ) or presence (+ΔΨ) of a mitochondrial membrane potential. Import was halted by the addition of valinomycin (0.5 μM) and samples were incubated further at 30°C for 60 min when indicated (Chase). Nonimported precursor proteins were removed by treatment with trypsin (50 μg/ml). Samples were analyzed by SDS-PAGE and autoradiography.

sively part of a high-molecular-mass complex (Figure 2B), which corresponds in size to assembled *m*-AAA proteases containing Afg3l1 and Afg3l2 (Koppen *et al.*, 2007). To define which forms of paraplegin are present in this complex, its components were resolved by SDS-PAGE in a second dimension (Figure 2C). Both the intermediate form of paraplegin generated upon MPP cleavage as well as mature paraplegin was detected as part of the high-molecular-weight assembly, indicating that both forms of paraplegin are assembly-competent.

To substantiate this conclusion, we deleted parts of the mitochondrial presequence of paraplegin. The paraplegin variants were synthesized in a cell-free system in the presence of [^{35}S]methionine and imported into isolated mouse liver mitochondria (Figure 2D). Deletion of amino acid residues 1–48 or 1–105 abrogated import of paraplegin into mitochondria, demonstrating that the first 48 amino acid residues are required for mitochondrial targeting of paraplegin. In contrast, a paraplegin variant lacking amino acids 48–105 was imported into mitochondria in a membrane-potential–dependent manner and subsequently converted into the mature form (Figure 2D). Moreover, it assembled into high-molecular-weight complexes as revealed by BN-PAGE analysis of mitochondrial extracts after import (Figure 2B), indicating that amino acids 48–105 are not required for the assembly of paraplegin into *m*-AAA protease complexes.

The proteolytic activity of paraplegin depends on its assembly with Afg3l1 and/or Afg3l2 into heteromeric *m*-AAA protease complexes (Koppen *et al.*, 2007). It is therefore conceivable that paraplegin is autocatalytically processed upon assembly with Afg3l1 and/or Afg3l2. To investigate if the proteolytic activity of newly imported paraplegin is essential for its processing, we replaced glutamate 575 within the proteolytic center of paraplegin by glutamine (Koppen *et al.*, 2007). Paraplegin and paraplegin^{E575Q} were synthesized in a cell-free system in the presence of [^{35}S]methionine and imported into isolated mouse liver mitochondria. Paraplegin^{E575Q} was processed as efficiently as wild-type paraplegin upon mitochondrial import (Supplemental Figure S1). To exclude a role of preexisting, endogenous paraplegin for maturation, we isolated mitochondria from liver derived from paraplegin-deficient *Spg7*$^{-/-}$ mice (Ferreirinha *et al.*, 2004). The absence of paraplegin within mitochondria did not impair the import and processing of ^{35}S-labeled paraplegin, which occurred to a similar extent in *Spg7*$^{+/+}$ and *Spg7*$^{-/-}$ liver mitochondria (Supplemental Figure S1). Similarly, processing of proteolytically inactive paraplegin^{E575Q} was not affected upon import into *Spg7*$^{-/-}$ mitochondria (Supplemental Figure S1). These experiments unambiguously demonstrate that maturation of paraplegin does not depend on its own proteolytic activity, and the presence of heteromeric *m*-AAA proteases containing paraplegin, highlighting the role of Afg3l1 and/or Afg3l2 for maturation.

Determination of Processing Sites in Paraplegin

To define the N-terminal amino acid residue of mature paraplegin and the cleavage site of MPP within the precursor form of paraplegin, we expressed a paraplegin variant harboring a hexahistidine peptide at its C-terminal end from a multicopy plasmid in yeast cells. Both intermediate and mature forms of paraplegin accumulated in these cells, most likely because of overexpression of paraplegin (Supplemental Figure S2A). Previous complementation studies revealed functional activity of paraplegin in yeast mitochondria, indicating that the biogenesis occurs normally in the heterologous environment (Koppen *et al.*, 2007). The electrophoretic mobility of mature paraplegin generated in yeast was indeed indistinguishable from endogenous paraplegin as re-

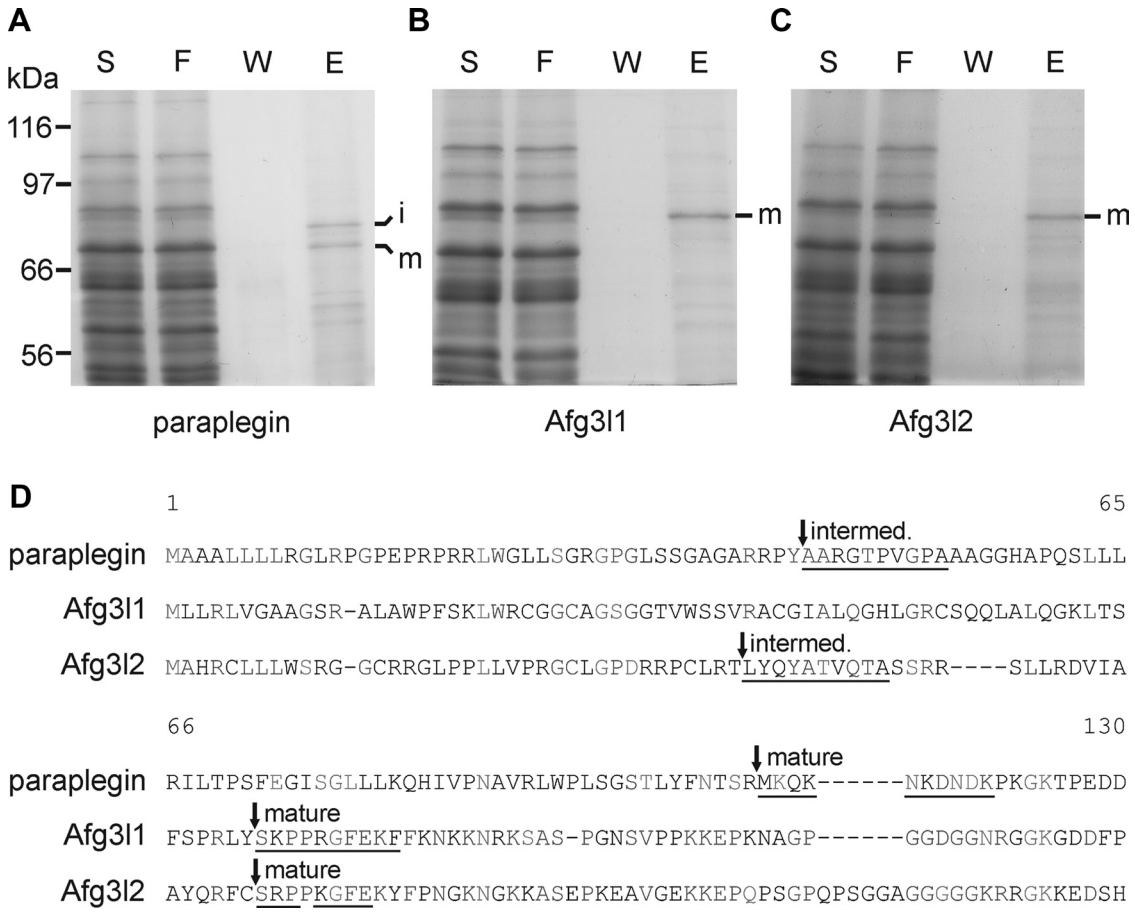

Figure 3. Determination of processing sites in mouse *m*-AAA protease subunits. (A–C) Affinity purification of paraplegin (A), Afg3l1 (B), and Afg3l2 (C) expressed in yeast. Mitochondria harboring a C-terminal hexahistidine tagged murine *m*-AAA protease subunits were isolated (40 mg) and solubilized in NP-40 and extracts were fractionated by metal chelating chromatography. Different fractions were analyzed by SDS-PAGE and Coomassie staining. S, supernatant after solubilization; F, flow through; W, washing fraction (0.5% of each fraction); E, elution fraction (6.25%); i, intermediate form; m, mature form. (D) Alignment of the N-terminal amino acid sequence of mouse paraplegin, Afg3l1, and Afg3l2. Amino acid residues determined by Edman sequencing of intermediate and mature forms are underlined. The arrows indicate the processing sites.

vealed by immunoblotting of mouse mitochondria and yeast mitochondria expressing paraplegin (Supplemental Figure S2A). Moreover, import of paraplegin into yeast mitochondria was accompanied by the formation of similar-sized bands as observed in liver mitochondria (data not shown), substantiating that paraplegin is processed in a similar manner in yeast and mouse mitochondria. We purified intermediate and mature paraplegin by metal chelating chromatography and determined N-terminal amino acids by Edman sequencing (Figure 3A). The N-terminus of the intermediate form of paraplegin was found to correspond to alanine 44 of the precursor form (Figure 3D). In agreement with the observed cleavage of paraplegin by MPP in vitro (Figure 1C), an arginine residue is present at position −3 relative to the cleavage site, a characteristic property of MPP cleavage sites (R-3 motif; Gakh *et al.*, 2002). Methionine 106 of paraplegin was identified as the N-terminus of mature paraplegin, indicating processing in a region that does not correspond to known cleavage motifs of mitochondrial processing enzymes. We therefore conclude that MPP cleaves off 43 N-terminal amino acids of newly imported paraplegin generating an intermediate form. This form is subsequently converted into mature paraplegin upon removal of 52 additional N-terminal amino acid residues by Afg3l1 and/or Afg3l2.

Processing of Newly Imported Afg3l1 and Afg3l2

The role of Afg3l1 and Afg3l2 for maturation of paraplegin prompted us to investigate the processing of these *m*-AAA protease subunits upon import into mitochondria. We synthesized ^{35}S-labeled Afg3l1 and Afg3l2 in a cell-free translation system and performed import experiments using isolated mouse liver mitochondria (Figure 4A). Import of both Afg3l1 and Afg3l2 depended on the membrane potential across the mitochondrial inner membrane. Afg3l1 was converted into a smaller, protease-protected form within mitochondria (Figure 4A). This form comigrated with endogenous Afg3l1 detected by immunoblotting and was therefore identified as mature Afg3l1 (data not shown). On the other hand, two processed, similar-sized forms accumulated within mitochondria upon import of Afg3l2 (Figure 4A). Further incubation of the mitochondria after completion of import led to the accumulation of the smaller form (Figure 4A), which corresponded in size to mature, endogenous Afg3l2 (data not shown). These experiments suggest that

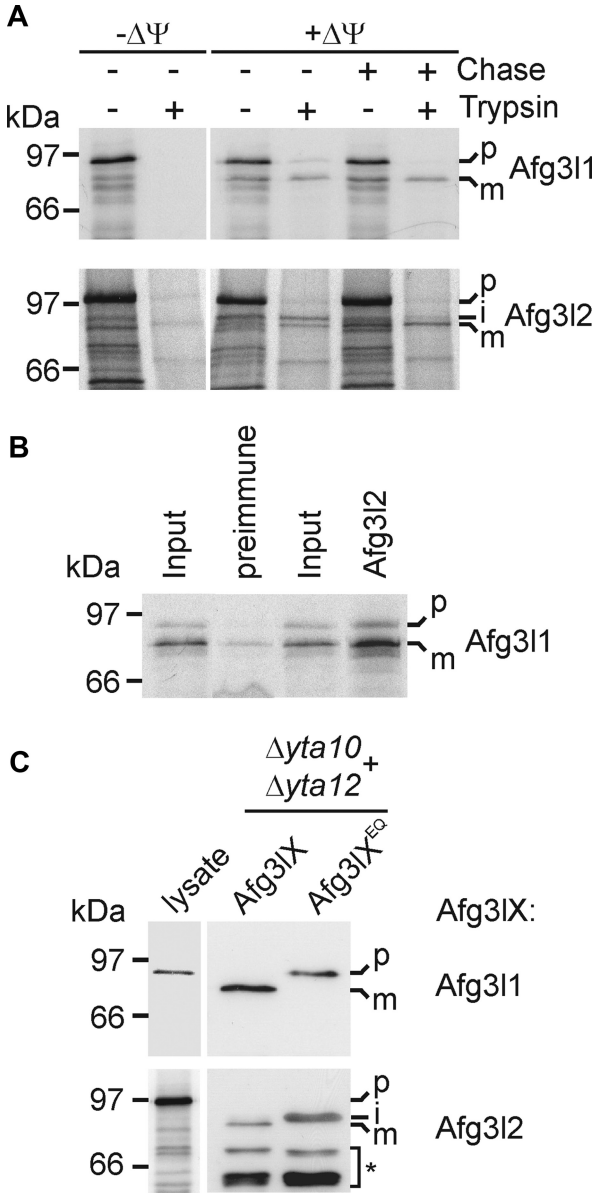

Figure 4. Mitochondrial import and processing of Afg3l1 and Afg3l2. (A) Afg3l1 and Afg3l2 are processed differently upon import into mitochondria. ^{35}S-labeled Afg3l1 and Afg3l2 were imported for 30 min at 30°C into mouse liver mitochondria in the absence ($-\Delta\Psi$) or the presence ($+\Delta\Psi$) of a mitochondrial membrane potential. Import was halted by the addition of valinomycin (0.5 μM), and mitochondria were incubated further for 60 min at 30°C when indicated (Chase). Samples were further analyzed as described in Figure 1B. (B) Assembly of newly imported Afg3l1 with Afg3l2. ^{35}S-labeled Afg3l1 was imported into mouse liver mitochondria (200 μg), which were lysed subsequently with digitonin. Ten percent of the sample was removed for control (Input). Coimmunoprecipitations were carried out using either preimmune serum or affinity-purified polyclonal Afg3l2-specific antibodies. Immunoprecipitates were analyzed as described in Figure 2A. (C) Processing of Afg3l1 and Afg3l2 in yeast. Afg3l1, Afg3l2 (Afg3lX), or their proteolytically inactive variants Afg3l1^{E567Q} and Afg3l^{E574Q} (Afg3lX^{EQ}) were expressed individually in Δ*yta10*Δ*yta12* yeast cells. Mitochondria isolated from these yeast strains were analyzed by SDS-PAGE and immunoblotting with Afg3l1- and Afg3l2-specific antibodies. ^{35}S-labeled precursor forms of Afg3l1 and Afg3l2 were examined in parallel by SDS-PAGE and autoradiography (lysate). The asterisk indicates degradation products of Afg3l2. p, precursor; i, intermediate form; m, mature form.

processing of Afg3l1 proceeds in one step, whereas maturation of Afg3l2 requires two distinct cleavage events.

We examined if newly imported Afg3l1 assembles with Afg3l2 molecules in mitochondria by coimmunoprecipitation experiments. After import of Afg3l1 in mouse liver mitochondria, membranes were solubilized with digitonin and extracts were incubated with an antiserum specific for Afg3l2. Afg3l1 was precipitated specifically with Afg3l2 antibodies but not with preimmune serum, demonstrating a direct interaction of newly imported Afg3l1 with preexisting Afg3l2 (Figure 4B). Notably, both mature and precursor forms of Afg3l1 bind to Afg3l2 (Figure 4B). BN-PAGE analysis confirmed the assembly of newly imported Afg3l1 into *m*-AAA protease complexes with a size of ~1 MDa (data not shown).

Autocatalytic Processing of Afg3l1 and Afg3l2 upon Import into Mitochondria

To examine a potential involvement of MPP in maturation of Afg3l1 and Afg3l2, we synthesized both *m*-AAA protease subunits in a cell-free system in the presence of [^{35}S]methionine and incubated the precursor forms with purified MPP (Figure 1C). However, unlike paraplegin, neither Afg3l1 nor Afg3l2 were processed by MPP in vitro (Figure 1C). It is conceivable that tight folding of the Afg3l1 and Afg3l2 precursor proteins in vitro may render them inaccessible to MPP. We therefore denatured paraplegin, Afg3l1, and Afg3l2 in 8 M urea and 100 mM DTT before incubation with purified MPP. Although paraplegin was processed by MPP under these conditions, MPP did not cleave Afg3l1 or Afg3l2 (data not shown), confirming that MPP is not involved in the maturation of these *m*-AAA protease subunits.

We therefore analyzed processing of Afg3l1 and Afg3l2 after expression in yeast. Both *m*-AAA protease subunits were previously shown to be functional in the heterologous environment indicating that their biogenesis occurs normally (Koppen *et al.*, 2007). The yeast system bears the advantage that maturation of the precursor proteins can be assessed in mitochondria lacking individual mitochondrial proteases. Afg3l1 and Afg3l2 were overexpressed from a multicopy plasmid under the control of the *YTA10* promoter and initially expressed in Δ*yta10*Δ*yta12* cells deficient for the yeast *m*-AAA protease. Immunoblot analysis of mitochondria isolated from these strains showed that both mouse *m*-AAA protease subunits accumulated in the mature form (Figure 4C).

As Afg3l1 and Afg3l2 were found to cleave paraplegin upon import into mitochondria, it is conceivable that Afg3l1 and Afg3l2 mediate their maturation autocatalytically. To examine this possibility, we expressed proteolytically inactive variants of both proteins harboring mutations in the proteolytic sites (Afg3l1^{E567Q}, Afg3l2^{E574Q}) in Δ*yta10*Δ*yta12* cells. Strikingly, Afg3l1^{E567Q} accumulated in a larger form in these cells, which corresponded in size to the precursor form of Afg3l1 synthesized in a cell-free system in vitro (Figure 4C). Similarly, proteolytically inactive Afg3l2^{E574Q} was not converted into the mature form when expressed in Δ*yta10*Δ*yta12* cells (Figure 4C). Rather, an intermediate-sized form of Afg3l2^{E574Q} was formed that was slightly larger than the mature form of Afg3l2 (Figure 4C). We conclude from these experiments that both Afg3l1 and Afg3l2 can receive autocatalytic processing when expressed in yeast. Although Afg3l2 appears to be cleaved initially by another peptidase before autocatalytic processing, the precursor form of Afg3l1 is directly converted into the mature form.

Processing Sites in Afg3l1 and Afg3l2

We overexpressed Afg3l1 and Afg3l2 variants harboring C-terminal hexahistidine peptides in Δ*yta10*Δ*yta12* yeast cells. Both proteins accumulated in their mature form within mitochondria whose electrophoretic mobility was indistinguishable from Afg3l1 or Afg3l2 in liver mitochondria (Supplemental Figure S2B). After purification by metal chelating chromatography (Figure 3, B and C), N-terminal amino acid residues of mature Afg3l1 and Afg3l2 were determined. Edman sequencing identified a serine residue as the first amino acid of both mature Afg3l1 and Afg3l2 (Figure 3D). Amino acids following these serine residues, which correspond to position 71 within Afg3l1 and position 67 within Afg3l2 (Figure 3D), are highly conserved between both *m*-AAA protease subunits. Interestingly, an arginine residue is found at position −3 in both precursor proteins characteristic of MPP cleavage motifs (Figure 3D). Our in vitro experiments, however, did not provide any evidence for a role of MPP for Afg3l1 or Afg3l2 processing illustrating that recognition by MPP depends on additional properties of precursor proteins.

To determine N-terminal amino acids of the intermediate form of Afg3l2, proteolytically inactive Afg3l2^{E574Q} containing a C-terminal hexahistidine peptide was expressed in Δ*yta10*Δ*yta12* cells. The intermediate form of Afg3l2^{E574Q} was affinity purified and subjected to Edman sequencing (Supplemental Figure S3). Leucine 39 was identified as the N-terminal amino acid residue of the intermediate form of Afg3l2 (Figure 3D). Neighboring amino acid residues of this cleavage site are not conserved between Afg3l1 and Afg3l2, which are highly homologous and share 72% identical amino acids in their mature regions.

Autocatalytic Processing of Afg3l2 in Mammalian Mitochondria

The expression of Afg3l1 or Afg3l2 variants in yeast cells provided compelling evidence that *m*-AAA protease complexes have the ability to process their subunits. To examine whether *m*-AAA protease subunits are processed autocatalytically in mammalian mitochondria, we used RNAi in MEFs. Both Afg3l1 and Afg3l2 were down-regulated simultaneously, as depletion of only Afg3l1 or Afg3l2 did not impair processing of the other subunit in vivo (Figure 1A). Moreover, paraplegin-deficient MEFs derived from *Spg7*$^{-/-}$ mice were used in these experiments to exclude the assembly of remaining Afg3l1- and Afg3l2 subunits with paraplegin into heteromeric *m*-AAA protease isoforms. Mitochondria were isolated from cells lacking *m*-AAA protease subunits, and import experiments were performed using ^{35}S-labeled, proteolytically inactive Afg3l2^{E574Q} and, for control, paraplegin^{E575Q}. In this way, potential processing defects upon import into *m*-AAA protease-deficient mitochondria are not masked by the proteolytic activity of the newly imported subunits. Paraplegin^{E575Q} was converted into intermediate and mature forms upon mitochondrial import irrespective of the presence of Afg3l1 and Afg3l2 (Figure 5A). However, the ratio of both forms was shifted in a statistically significant manner toward the intermediate form upon down-regulation of Afg3l1 and Afg3l2. More than 70% of imported paraplegin accumulated in the intermediate form under these conditions compared with ~50% in control mitochondria (Figure 5B). These results are consistent with the observed accumulation of the intermediate form of endogenous paraplegin upon depletion of Afg3l1 and Afg3l2 (Figure 1A) and demonstrate that depletion of Afg3l1 and Afg3l2 is sufficiently efficient to detect processing defects of *m*-AAA protease subunits in these experiments.

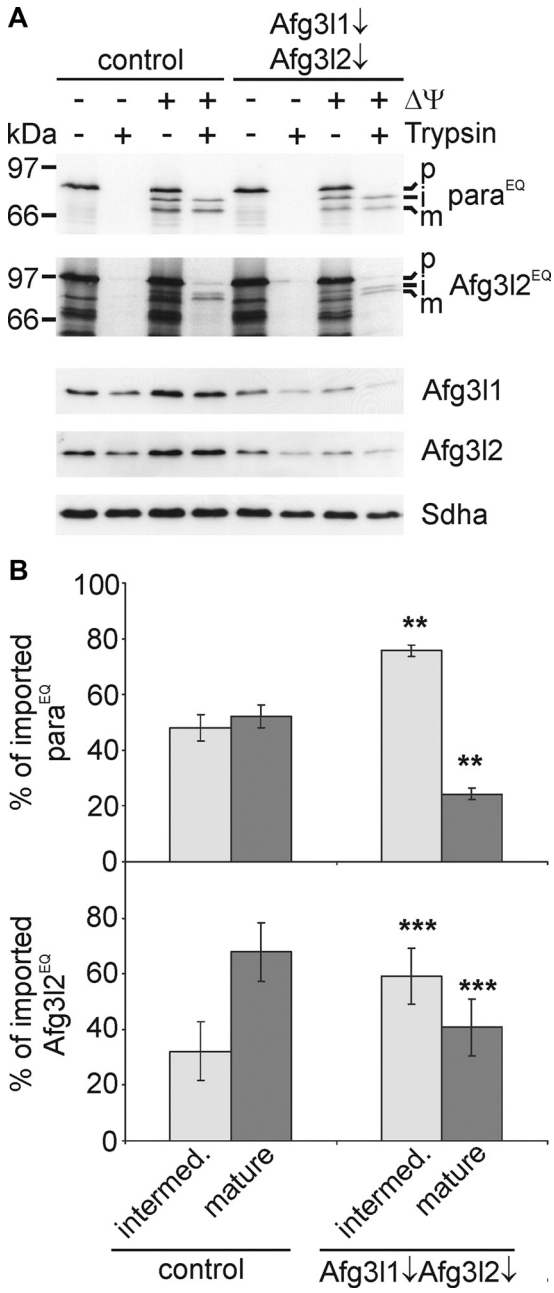

Figure 5. Autocatalytic processing of Afg3l2 in MEFs. (A) Down-regulation of *m*-AAA protease subunits impairs processing of newly imported paraplegin and Afg3l2. Paraplegin-deficient *Spg7*$^{-/-}$ MEFs were transfected twice with scrambled siRNA (control) or siRNAs directed against Afg3l1 and Afg3l2. Mitochondria were isolated after ~2.5 d and ^{35}S-labeled paraplegin^{E575Q} (para^{EQ}) or Afg3l2^{E574Q} (Afg3l2^{EQ}) containing a mutation in the proteolytic center were imported as described in Figure 1B. Import reactions were analyzed by SDS-PAGE and autoradiography. Down-regulation of Afg3l1 and Afg3l2 was monitored by immunoblotting using Afg3l1- and Afg3l2-specific antibodies. Equal loading of the gel was assessed by immunoblotting with antibodies directed against the 70 kDa-subunit of succinate dehydrogenase (Sdha). p, precursor; i, intermediate form; m, mature form; ΔΨ, mitochondrial membrane potential. (B) Quantification of import reactions shown in A. Imported (trypsin-protected) paraplegin^{E575Q} and Afg3l2^{E574Q} were quantified by phosphorimaging in control mitochondria and Afg3l1/Afg3l2-depleted mitochondria and are given as percent of the total imported protein. The average of three to four independent experiments (± SD) is shown. ** $p < 0.01$; *** $p < 0.001$.

Import of Afg3l2^{E574Q} occurred with similar efficiency into control and Afg3l1/Afg3l2-depleted mitochondria (Figure 5A). Intermediate and mature Afg3l2^{E574Q} were formed upon membrane potential-dependent import. However, down-regulation of Afg3l1 and Afg3l2 significantly reduced the amount of mature Afg3l2^{E574Q} detected in mitochondria (Figure 5B). Only ~40% of newly imported Afg3l2^{E574Q} accumulated in the mature form in Afg3l1- and Afg3l2-deficient mitochondria, whereas ~70% of the imported molecules were matured in control mitochondria (Figure 5B). These findings are in agreement with the observed processing defects of Afg3l2 in *m*-AAA protease-deficient yeast cells and point to a crucial role of preexisting Afg3l1 and Afg3l2 for proteolytic cleavage of the intermediate form of newly imported Afg3l2 molecules. Together with our experiments in yeast, we conclude that maturation of Afg3l2 upon import into mitochondria occurs in an autocatalytic manner.

DISCUSSION

We report here on an unexpected role of Afg3l1 and Afg3l2 for processing of paraplegin and their own maturation upon import into mitochondria. These findings provide direct evidence that mammalian *m*-AAA proteases can act as processing enzymes in mitochondria in vivo and raise the possibility that paraplegin dysfunction might be a common factor in the pathogenesis of two distinct neurodegenerative diseases, which are caused by mutations in paraplegin and Afg3l2, respectively.

Although *m*-AAA protease subunits are highly conserved in their mature region, their processing upon import into mitochondria varies considerably. We used knockdown experiments in MEFs, heterologous expression in yeast, and in vitro processing assays to define cleavage events converting precursor into mature forms. Two processing steps are required to generate mature paraplegin: after formation of an intermediate form by MPP, Afg3l1, and/or Afg3l2 mediate maturation of paraplegin. Similarly, the precursor form of Afg3l2 is converted first into an intermediate form before autocatalytic maturation by Afg3l1 and/or Afg3l2 occurs. However, we did not obtain any evidence for an involvement of MPP in the formation of the intermediate form, despite the presence of a putative MPP cleavage site in the presequence of Afg3l2. It remains to be determined whether this reflects different substrate specificities of mouse and yeast MPP used in our experiments, or whether another, yet to be identified peptidase is involved in Afg3l2 processing. The rhomboid protease PARL has been discussed as a candidate processing enzyme for *m*-AAA protease subunits (Pellegrini and Scorrano, 2007). However, mitochondrial import experiments in vitro and experiments in PARL-deficient MEFs did not provide any evidence for a requirement of PARL for maturation, regardless of the presence of Afg3l1 and Afg3l2 (Supplemental Figure S4). In contrast to paraplegin and Afg3l2, we did not detect an intermediate form of Afg3l1. Rather, autocatalytic cleavage of the Afg3l1 precursor form generates directly mature Afg3l1.

The two-step processing of paraplegin and Afg3l2 is reminiscent of other mitochondrial inner membrane and intermembrane space proteins, whose intramitochondrial sorting is ensured by bipartite presequences (Neupert and Herrmann, 2007). Accordingly, stepwise cleavage may coordinate the sorting and insertion of paraplegin and Afg3l2 into the mitochondrial inner membrane. However, deletion of amino acid residues of paraplegin that are cleaved off by Afg3l1/Afg3l2 did not interfere with the import or the assembly of newly imported paraplegin molecules. Moreover, both intermediate and mature forms of paraplegin were detected in *m*-AAA protease complexes, indicating that maturation is not required for assembly after completion of import. Similarly, assembly of newly imported Afg3l1 subunits with Afg3l2 does not depend on their maturation. We therefore favor the possibility that maturation occurs in an assembled state and might be accompanied with the activation of the newly imported subunit. This scenario is reminiscent of the autocatalytic removal of a proregion from subunits of the ATP-dependent LON/PIM1 protease in the matrix space (Wagner *et al.,* 1997) and may explain why maturation of paraplegin upon import occurred with slow kinetics. Interestingly, maturation did not depend on the proteolytic activity of paraplegin or Afg3l2 subunits themselves, demonstrating that cleavage can occur in *trans* within assembled complexes. However, we cannot rule out the possibility that preexisting *m*-AAA protease complexes bind newly imported subunits as proteolytic substrates and mediate their maturation before assembly into high-molecular-weight complexes occurs.

Our findings substantiate the recently discovered role of *m*-AAA proteases as processing enzymes and identify paraplegin, Afg3l1, and Afg3l2 as novel substrates of mammalian *m*-AAA isoenzymes. Notably, the yeast *m*-AAA protease subunits Yta10 and Yta12 do not undergo autocatalytic processing. Known proteolytic substrates of mammalian *m*-AAA proteases include MrpL32, a subunit of mitochondrial ribosomes, and the dynamin-like GTPase OPA1, an essential component of the mitochondrial fusion machinery (Nolden *et al.,* 2005; Ishihara *et al.,* 2006; Duvezin-Caubet *et al.,* 2007). Reconstitution experiments in yeast have demonstrated that both proteins are processed by mammalian *m*-AAA proteases (Nolden *et al.,* 2005; Duvezin-Caubet *et al.,* 2007; Koppen *et al.,* 2007). However, although a defective processing of MrpL32 has been observed in paraplegin-deficient *Spg7*$^{-/-}$ mice (Nolden *et al.,* 2005), in vivo evidence for a requirement of the *m*-AAA protease for OPA1 processing is still awaited. Our experiments demonstrate an essential role of Afg3l1 and Afg3l2 for maturation of *m*-AAA protease subunits in vivo as well as after heterologous expression in yeast. Notably, Afg3l1 and Afg3l2 can substitute for each other in these cleavage events providing direct evidence for an overlapping substrate specificity of both *m*-AAA protease subunits in vivo. These findings are in agreement with our previous experiments, which revealed that homomeric *m*-AAA isoenzymes build up of only Afg3l1 or Afg3l2 subunits are equally able to cleave OPA1 and MrpL32 in yeast (Duvezin-Caubet *et al.,* 2007; Koppen *et al.,* 2007).

The function of *m*-AAA proteases as both processing and quality control enzymes raises the question why some substrates are only processed rather than completely degraded. *m*-AAA protease cleavage sites in paraplegin, Afg3l1, and Afg3l2 do not show apparent sequence similarities among each other nor with cleavage sites in MrpL32 and OPA1. Thus, sequence-specific recognition of processing sites appears unlikely. In general, ATP-dependent proteases exhibit degenerate cleavage specificity and degrade their substrates in a processive manner to peptides (Tian *et al.,* 2005; Piwko and Jentsch, 2006). Processing by eukaryotic 26S proteasomes and bacterial Clp proteases is dictated by tightly folded domains which cannot be unfolded and transferred into the internal cavity of these proteolytic machines. Similarly, a tightly folded domain may interrupt processive degradation by *m*-AAA proteases initiated from the N-terminus resulting in the release of the matured protein. If newly imported *m*-AAA protease subunits are inserted into the

inner membrane or even assembled into oligomeric complexes, sterical hindrance may contribute to limit degradation and to determine the number of amino acid residues cleaved off by the *m*-AAA protease.

The identification of paraplegin as a substrate of Afg3l2 is also of relevance for the understanding of the pathogenesis of two distinct neurodegenerative disorders, HSP and SCA28, associated with mutations in these *m*-AAA protease subunits (Casari *et al.*, 1998; Cagnoli *et al.*, 2008; DiBella *et al.*, 2008). In human mitochondria lacking Afg3l1 (Kremmidiotis *et al.*, 2001), paraplegin function depends on its assembly with Afg3l2 into heteromeric *m*-AAA protease complexes (Koppen *et al.*, 2007). Accordingly, the complete loss of Afg3l2 is accompanied by the loss of paraplegin activity. Conversely, reduced levels of Afg3l2 in mouse have recently been observed to enhance the axonopathy in paraplegin-deficient *Spg7*$^{-/-}$ mice and to lead to cerebellar degeneration (Martinelli *et al.*, 2009). The requirement of the proteolytic activity of Afg3l2 for paraplegin maturation observed here suggests that even missense mutations in Afg3l2 might affect paraplegin function. Interestingly, missense mutations in human AFG3L2 have been described recently to be responsible for type 28 of SCA (Cagnoli *et al.*, 2008; DiBella *et al.*, 2008). Our results suggest that SCA type 28–causing mutations in human *AFG3L2* could lead to an impaired processing of paraplegin which thereby contributes to disease pathogenesis.

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