Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia

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Mutations in paraplegin, a putative mitochondrial metallopeptidase of the AAA family, cause an autosomal recessive form of hereditary spastic paraplegia (HSP). Here, we analyze the function of paraplegin at the cellular level and characterize the phenotypic defects of HSP patients’ cells lacking this protein. We demonstrate that paraplegin coassembles with a homologous protein, AFG3L2, in the mitochondrial inner membrane. These two proteins form a high molecular mass complex, which we show to be aberrant in HSP fibroblasts. The loss of this complex causes a reduced complex I activity in mitochondria and an increased sensitivity to oxidant stress, which can both be rescued by exogenous expression of wild-type paraplegin. Furthermore, complementation studies in yeast demonstrate functional conservation of the human paraplegin–AFG3L2 complex with the yeast m-AAA protease and assign proteolytic activity to this structure. These results shed new light on the molecular pathogenesis of HSP and functionally link AFG3L2 to this neurodegenerative disease.

Introduction

Both clinical and genetic heterogeneity apply to hereditary spastic paraplegia (HSP), a disabling disorder affecting 1 in 10,000 people in the Western world (Polo et al., 1993). The main phenotypic trait common to all forms of spastic paraplegia is a progressive lower limb weakness, spasticity, and decreased vibratory sense. Complicated forms show additional neurological signs such as retinopathy, optic neuropathies, ataxia, dementia, and mental retardation (Harding, 1983). Neuropathological studies on pure HSP patients describe specific alterations of the cortical spinal tract due to axonal degeneration of motor and sensory neurons without cell body loss (Schwarz and Liu, 1956).

A more precise classification of HSP forms originates from the inheritance pattern, which may be autosomal dominant, autosomal recessive, or X-linked recessive. 20 different loci have been associated with diverse forms of HSP (Crosby and Proukakis, 2002; Reid, 2003); however, causative mutations have been found in eight genes only. The most recent gene associated with HSP is the neuronal specific kinesin (KIF5A) gene SPG10, a member of the kinesin superfamily of molecular motors that transport cargoes along microtubules in an ATP-dependent manner. Mutations in the cell adhesion module L1, L1CAM (SPG1), and in the proteolipid protein SPG2 cause X-linked forms of HSP characterized by defects in the development of the corticospinal tract and in axonal–glial interactions, respectively. Little is known about the four known autosomal HSP genes encoding atlastin (SPG3A), spartin (SPG20), spastin (SPG4), and paraplegin (SPG7), whereas HSP60 is a well-characterized shock response protein (SPG13). Atlastin is a novel GTPase that has sequence homology to members of the dynamin family of large GTPases, particularly guanylate binding protein-1 (Zhao et al., 2001). Spartin is responsible for Troyer Syndrome, an autosomal recessive HSP, and is homologous to proteins L. Atorino and L. Silvestri contributed equally to this work.

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Abbreviations used in this paper: BN-PAGE, blue native PAGE; HSP, hereditary spastic paraplegia; ROS, reactive oxygen species.
involved in endosomal morphology and protein trafficking of late endosomal component (Patel et al., 2002). Both spastin and paraplegin are AAA proteins, which are ATPases associated with different cellular activities (Casari et al., 1998; Hazan et al., 1999; Ogura and Wilkinson, 2001). Members of this superfamily of ATPases exert chaperone-like activities and mediate assembly and disassembly of macromolecular structures involved in different cellular processes. Although a still unclear role at the nuclear level exists (Charvin et al., 2003), spastin interacts with microtubules and seems to modulate microtubule dynamics, which is an essential attainment for maintenance of long axons (Errico et al., 2002).

Apart from these mutant proteins that associate the HSP pathogenetic cascade to intracellular and axonal trafficking, another pathogenetic mechanism points to the mitochondrion. In fact, both paraplegin and HSP60 are nuclear-encoded mitochondrial proteins (Casari and Rugarli, 2001; Hansen et al., 2002).

Paraplegin is closely related to ATP-dependent metalloproteases (Langer, 2000). By database scanning, two additional mitochondrial AAA proteases are highly homologous to paraplegin that have been identified, namely AFG3L2 and YME1L1 (Banfi et al., 1999; Coppola et al., 2000). Yeast orthologues of these proteins are membrane-bound peptidases, which ensure the quality control of mitochondrial inner membrane proteins (Leonhard et al., 1996).

Here, we analyze the function of paraplegin and its homologous protein, AFG3L2. They assemble in the mitochondrial inner membrane into a high molecular mass complex, which is aberrant in HSP patients. Impaired complex formation in HSP cells causes a decreased activity of respiratory complex I and increases the sensitivity to oxidative stress. Heterologous expression of paraplegin and AFG3L2 in yeast cells and complementation studies establish the complex as a proteolytically active structure involved in maintenance of respiratory competence. Together, these findings provide novel insights into the pathogenic mechanism of neurodegeneration in HSP patients.

**Results**

**Paraplegin and AFG3L2 assemble in the inner mitochondrial membrane into a high molecular mass complex, which is aberrant in HSP patients**

Paraplegin is homologous to mitochondrial AAA metalloproteases that have been exclusively studied in fungi. Two additional human members of the AAA metalloprotease family, AFG3L2 and YME1L1, localize to mitochondria (Banfi et al., 1999; Coppola et al., 2000). Although YME1L1 is orthologous to the yeast protein Yme1p and forms a homooligomeric structure able to rescue the Δyme1 mutant yeast phenotype (Shah et al., 2000), the function of paraplegin and AFG3L2 is not known.

The presence of two hydrophobic regions, which have the characteristics of transmembrane domains (Casari et al., 1998; Banfi et al., 1999), suggests that both paraplegin and AFG3L2 are integral membrane proteins. To test this prediction, we determined their submitochondrial localization. Upon selective disruption of the mitochondrial outer membrane by digitonin, both proteins are found exclusively in the mitoplast fraction (Fig. 1 A, lane 1) as well as in HSP60, which is a matrix marker (Fig. 1 A, lane 1). We also monitored the presence of porin, as an outer membrane marker, and cytochrome c, as outer membrane and intermembrane fractional markers, respectively. Porin, which resides on the
outer membrane, but takes connections to the inner membrane, was found either in the outer membrane–intermembrane fraction (Fig. 1 A, lane 2) or in the mitoplast fraction as expected (Fig. 1 A, lane 1). Cytochrome c appears in the outer membrane/intermembrane fraction (Fig. 1 A, lane 2).

Isolated mitoplasts were subjected to alkaline extraction and divided into supernatant (containing matrix and peripheral membrane proteins) and membrane fractions (containing integral proteins of the inner membrane) by ultracentrifugation. Paraplegin and AFG3L2 were recovered from the membrane fraction, indicating that both are integral proteins of the mitochondrial inner membrane (Fig. 1 A, lane 3).

To explore a potential physical interaction between paraplegin and AFG3L2, we performed coimmunoprecipitation studies in HEK293. Mitochondria were solubilized and supplemented with either AFG3L2 or paraplegin antibodies. Immunoblotting of the precipitates revealed that paraplegin was isolated with AFG3L2 antibody and that, accordingly, AFG3L2 coprecipitated with paraplegin antibody (Fig. 1 B, lanes 1 and 3). Preimmune sera failed to precipitate any protein. We conclude from these experiments that paraplegin assembles with AFG3L2.

To substantiate this conclusion, we performed gel filtration experiments. Mitochondria isolated from HEK293 cells or from primary human control fibroblasts were solubilized and fractionated. Eluate fractions were analyzed by immunoblotting using AFG3L2 and paraplegin antibodies. Both proteins, which show a similar molecular mass of ~80 kD, eluted in a rather broad peak from the column with fractions corresponding to a molecular mass of ~800–900 kD (Fig. 1, C and D). Notably, the elution pattern of AFG3L2 was altered in mitochondria from primary fibroblasts of HSP patients lacking paraplegin (see Materials and methods, Cell cultures). In fact, AFG3L2 was present in a complex with a reduced native molecular mass of ~250 kD (Fig. 1 D).

These data are in agreement with the complex formation of paraplegin and AFG3L2, and they show that the paraplegin–AFG3L2 complex is aberrant in HSP cells lacking paraplegin. The latter also suggests the inability of AFG3L2 to homopolymerize or to assemble with other proteins to form the 900-kD complex.

**HSP cells show increased susceptibility to reactive oxygen species (ROS)**

Because mitochondria are sensitive targets for ROS, we examined the sensitivity of HSP patients’ cells towards hydrogen peroxide (H$_2$O$_2$) challenge (1 h after 100 μM H$_2$O$_2$ treatment). To assess the activity of mitochondria, the membrane potential was monitored by staining control and HSP fibroblasts with the potentiometric probes rhodamine 123 and JC-1.

Upon rhodamine staining, mitochondria in HSP fibroblasts subjected to ROS challenge displayed an increased punctuate and diffuse pattern when compared with control cells (Fig. 2 A). By using JC-1, the hyperpolarized orange-staining mitochondria of HSP cells shift to greenish upon H$_2$O$_2$ treatment faster than mitochondria of control cells (Fig. 2 B). These results point to an increased sensitivity of HSP fibroblasts towards oxidative damage, which is accompanied by depolarization of mitochondria.

The increased sensitivity towards ROS of HSP mitochondria lacking the paraplegin–AFG3L2 complex was confirmed by measurements of mitochondrial ATP synthesis (Fig. 3 A). ATP synthesis after H$_2$O$_2$ challenge (at 400 μM for 4 h) was remarkably reduced in HSP fibroblasts compared with controls (from 27.7 ± 4 nmol ATP/mg protein in untreated cells to 4.9 ± 2.8 nmol ATP/mg protein in H$_2$O$_2$-treated cells; P < 0.0005).

Cell vitality ascertained by MTT reduction assay confirmed the dose-dependent vulnerability of HSP cells to ROS (upon exposure to 50 μM H$_2$O$_2$ for 8 h, 35.1 ± 4.9% HSP cells vs. 79.7 ± 2.5% controls; at 200 μM, 30.0 ± 2.6% vs. 67.0 ± 6.2%, P < 0.000001; at 400 μM, 16.3 ± 4.7% vs. 52.3 ± 7.7%, P < 0.004; Fig 3 B). Catalase administration showed a protective action both in HSP and control cells exposed to H$_2$O$_2$ (unpublished data).

To examine whether the increased sensitivity of HSP fibroblasts towards oxidative stress is directly caused by a lack of paraplegin, we set up paraplegin cDNA transfection experiments. HSP and control cells were transfected with constructs harboring the full-length paraplegin cDNA or the empty vector, and then were exposed to different concentrations of H$_2$O$_2$. Cell damage was evaluated by assessing the MTT metabolism (Fig. 3 C). The increased sensitivity of HSP cells to oxidative stress was almost completely reversed.
to control values by exogenous expression of wild-type paraplegin. Trypan blue exclusion assays revealed identical results (unpublished data). Together, these results demonstrate an increased susceptibility to ROS of HSP patients’ fibroblasts lacking paraplegin, which can be reversed by transfecting cells with paraplegin.

**Impaired respiratory complex I activity in HSP fibroblasts**

The decrease of ATP levels and mitochondrial transmembrane potential, together with the increased vulnerability of HSP cells under oxidative stress, prompted us to explore specific impairments of mitochondrial respiratory chain complexes in HSP fibroblasts under standard growth conditions. When compared with control cells, the growth rate of HSP cells was strongly reduced in galactose medium (Fig. 4 A). This is characteristic of cells with defective mitochondrial metabolism (Robinson et al., 1992) and, therefore, indicates an impaired respiratory activity in HSP cells even in the absence of an induced oxidative stress.

To dissect the contribution of the different respiratory chain complexes to mitochondrial ATP synthesis, we quantified ATP production in permeabilized fibroblasts in the presence of different substrates and inhibitors of respiratory complexes. The basal activity of the respiratory chain in control and HSP cells is similar (Fig. 4 B, group 1). By providing pyruvate/malate or glutamate/malate (Fig. 4 B, groups 2 and 3), we stimulated ATP synthesis that is dependent on complexes I, II, III, IV, and V. Using a complex I inhibitor, rotenone, and succinate, we measured ATP production that is dependent on complexes II, III, IV, and V; 5, antimycin, ascorbate, and TMPD (complexes IV and V). *, P < 0.05; **, P < 0.001; ***, P < 0.0001. Bars represent ±SEM.
To confirm this data, we used blue native PAGE (BN-PAGE) to isolate enzymatically active membrane proteins from mitochondria. In agreement with previous estimates (Schagger et al., 1994; Klement et al., 1995), complex I of both HSP and control cells exhibited a molecular mass of ~750 kD (Fig. 5 A). By combining BN-PAGE with an in situ activity staining reaction that specifically detects enzyme activity of complex I, we confirmed a decrease in complex I activity in HSP cells (Fig. 5 B, HSP) when compared with control fibroblasts (Fig. 5 B, control). Transfection of HSP cells with paraplegin partially recovered the activity of complex I (Fig. 5 C, HSP/pSPG7), pointing to a direct role of paraplegin in the regulation of this respiratory complex.

The reduced activity of complex I in HSP fibroblasts could reflect either a reduced specific activity of the enzymatic complex or a smaller number of complex I units per mitochondrion. To distinguish between these possibilities, we performed BN-PAGE followed by immunoblotting with an antibody directed against the 39-kD subunit of complex I. This experiment revealed a decreased amount of the assembled complex I in HSP cells (Fig. 5 D, HSP), which can be rescued to a normal level upon transfection with paraplegin cDNA (Fig. 5 E, HSP/pSPG7).

The reduced amount of complex I may have different causes: a defective expression, or import of nuclear-encoded mitochondrial preproteins, or an inefficient synthesis of mitochondrially encoded proteins. We addressed these points by examining the mitochondrial import of the 39-kD protein in control and HSP mitochondria (p, precursor; m, mature). (B) Western blot analysis of the 39-kD protein in control and HSP mitoplasts. (C) Mitochondrial protein synthesis in control and HSP cells (COI, cytochrome c oxidase subunit I; ND2, NADH dehydrogenase subunit 2; COIII, cytochrome c oxidase subunit III; COII, cytochrome c oxidase subunit II; A6, ATPase subunit 6; A8, ATPase subunit 8).

Figure 5. Blue native gel electrophoresis of mitochondrial complexes. (A) Coomassie brilliant blue staining of mitoplast preparations. (B) Complex I in situ activity staining by nitro blue tetrazolium reduction assay. (C) Recovery of complex I in situ activity in HSP cells after pSPG7 transfection. (D and E) Western blot analysis of complex I revealed by anti–39-kD antibody. Immunoblotting with HSP60 antibody was used to verify equal loading. Activity and protein amount of complex I were quantified by densitometric analysis. *, P < 0.05; **, P < 0.001. Bars represent ±SEM.

Figure 6. Mitochondrial import and protein synthesis in HSP and control fibroblasts. (A) Mitochondrial protein import efficiency of the 39-kD protein in control and HSP mitochondria (p, precursor; m, mature). (B) Western blot analysis of the 39-kD protein in control and HSP mitoplasts. (C) Mitochondrial protein synthesis in control and HSP cells (COI, cytochrome c oxidase subunit I; ND2, NADH dehydrogenase subunit 2; COIII, cytochrome c oxidase subunit III; COII, cytochrome c oxidase subunit II; A6, ATPase subunit 6; A8, ATPase subunit 8).
proteins are synthesized under these conditions (Fig. 6 C), indicating that the decreased activity of complex I is not due to an impaired synthesis of mitochondrially encoded subunits. Therefore, we conclude from these experiments that the paraplegin–AFG3L2 complex is required for the efficient assembly of complex I.

Because the chaperone-like and the proteolytic activities of the yeast mitochondrial metalloprotease complex are both essential for the respiratory competence (Arlt et al., 1998), we tested whether the proteolytic domain of paraplegin is responsible for the complex I rescue obtained on paraplegin mutant fibroblasts. Therefore, we mutagenized the glutamic acid residue in position 575, which is conserved in the yeast and human metal binding site, and when changed to glutamine (E575Q; Fig. 7 A), is known to neutralize the proteolytic activity (Arlt et al., 1996). As shown in Fig. 7 B, by expressing the mutagenized construct pSPG7<sup>Q575</sup>, we were able to revert the reduced activity of complex I of HSP fibroblasts in a comparable manner to the construct expressing wild-type paraplegin. Again, in our system, the complex I activity is directly correlated to the assembled complex I amount (Fig. 7 C). Hence, the integrity of the proteolytic domain of paraplegin is not indispensable for the correct assembly of complex I. The same pattern of results was obtained when we analyzed the sensitivity to ROS. The mutagenized construct pSPG7<sup>Q575</sup> rescued the increased sensitivity to oxidant stress of HSP cells comparably to the wild-type construct (unpublished data).

Coexpression of paraplegin and AFG3L2 restores the respiratory competence of yta10yta12 mutant yeast cells

Paraplegin shares high sequence identity with subunits of the m-AAA protease in the yeast Saccharomyces cerevisiae, Yta10 (Afg3) and Yta12 (Rca1; Casari et al., 1998). To examine functional conservation, we performed a complementation analysis in yeast. Mitochondrial targeting of paraplegin and AFG3L2 in the heterologous organism was ensured by replacing their putative mitochondrial targeting sequences (amino acid residues 1–58 and amino acid residues 1–35, respectively) with the sorting signal of Yta10 (amino acid residues 1–63). The resulting hybrid proteins were expressed in yeast cells lacking Yta10, Yta12, or both. Expression and mitochondrial localization were confirmed by immunoblotting of cell extracts (unpublished data). Yeast cells lacking the m-AAA protease are respiratory deficient and cannot use nonfermentable carbon sources like glycerol (Tauer et al., 1994; Tzagoloff et al., 1994; Arlt et al., 1998). Expression of paraplegin or AFG3L2 did not restore the respiratory competence of Δyta10Δyta12 cells (Fig. 8 A) nor of Δyta10 or Δyta12 (unpublished data). However, the respiratory deficiency of Δyta10Δyta12 cells was suppressed upon coexpression of both paraplegin and AFG3L2 (Fig. 8 A). Thus, in agreement with the experiments in the human system, paraplegin and AFG3L2 functionally interact in yeast mitochondria. Complex formation of both proteins in yeast was subsequently observed by coimmunoprecipitation experiments (unpublished data) and BN-PAGE (Fig. 8 B), confirming that paraplegin and AFG3L2 act in a cooperative manner in a 900-kD complex, and, thereby, can substitute for the m-AAA protease in yeast.

These results demonstrate functional conservation of the human paraplegin–AFG3L2 complex and the yeast m-AAA protease. They also provide first evidence for a proteolytic activity of paraplegin and AFG3L2 because maintenance of the respiratory competence of yeast cells requires proteolysis by the m-AAA protease (Arlt et al., 1998).

To substantiate this conclusion, point mutations were introduced into the predicted proteolytic center of both paraplegin and AFG3L2. Glutamic acid residues 575 in the consensus metal binding sites of both proteins were replaced by glutamine residues by site-directed mutagenesis (Fig. 7 A). The mutant proteins (fused to the mitochondrial targeting sequence of Yta10) were expressed in yeast lacking Yta10, Yta12, or both. Expression and mitochondrial localization were confirmed by immunoblotting of cell extracts (unpublished data) and BN-PAGE (Fig. 8 B), confirming that paraplegin and AFG3L2 act in a cooperative manner in a 900-kD complex, and, thereby, can substitute for the m-AAA protease in yeast.
respiratory competence (Fig. 8 C). Thus, in contrast to the yeast m-AAA protease (Arlt et al., 1998), the catalytic activity of both subunits of the human paraplegin–AFG3L2 complex is required for proteolytic activity.

Discussion
Mutations in paraplegin are known to cause neurodegeneration in an autosomal recessive form of HSP that approximately accounts for the 12% of recessive cases (unpublished data). However, the pathogenetic mechanism of this disorder, particularly the role of mitochondria, is presently not understood.

Here, we have identified paraplegin and its interactor, the homologous protein AFG3L2, as part of a high molecular mass complex in the inner membrane of mitochondria. They form a complex of about 900 kD, which is aberrant in HSP patients’ cells lacking paraplegin, where a smaller complex of 250 kD is present. Our data identify the paraplegin–AFG3L2 complex as a functionally active structure whose absence plays a key role in the establishment of a cellular phenotype in HSP fibroblasts. We observed a remarkable reduction of complex I (NADH–ubiquinone oxidoreductase) activity in HSP cells compared with control cells. Furthermore, blue native gels confirmed this result and showed that the reduction of complex I activity is due to stoichiometric limitation of the enzymatic complex.

Our results reveal the first functional role of the paraplegin–AFG3L2 complex in the biogenesis of the respiratory chain. The deficiency is apparently caused by neither an impaired import of nuclear-encoded proteins nor an inefficient synthesis of mitochondrially encoded subunits, suggesting a role for the paraplegin–AFG3L2 complex in effectively assembling complex I. This defect was restored upon transfection of HSP patients’ cells with a paraplegin-expressing vector.

A complementation study in yeast revealed, for the first time, the functional conservation of mitochondrial m-AAA proteases between distantly related species such as human and yeast. In yeast cells, inactivation of m-AAA protease by deleting one or both Yta10 and Yta12 causes pleiotropic phenotypes because maintenance of the respiratory competence requires proteolysis by the yeast m-AAA protease (Arlt et al., 1998). The respiratory deficiency of Δyta10Δyta12 cells (Langer, 2000) was suppressed by coexpression of paraplegin and AFG3L2, providing the first evidence for their proteolytic activity. This result was substantiated by the observation that the integrity of the proteolytic center of both paraplegin and AFG3L2 is required for functional complementation. Thus, the complementation experiments in yeast unambiguously demonstrate the proteolytic activity of the paraplegin–AFG3L2 complex.

We expressed the proteolytic site variant of paraplegin in paraplegin-deficient fibroblasts, and observed both a rescue of complex I activity and an increased resistance towards oxidative stress. This is reminiscent of findings in the yeast system where the expression of a proteolytic site variant of one homologous subunit of the m-AAA protease restores respiratory competence (Arlt et al., 1998). The control experiment demonstrating the inability of the mutant paraplegin–AFG3L2 complex (i.e., coexpression of proteolytic site variants of both paraplegin and AFG3L2) to rescue the described HSP phenotypes is not feasible in human cells because AFG3L2-deficient fibroblasts are currently not available. However, we envision the following scenario explaining these findings in the two systems. Maintenance of respiration in yeast requires only very low specific activity of the m-AAA protease; hence, a single proteolytic site mutant of a yeast subunit is sufficient for complementation. Although we see complementation of the yeast double mutant by the human paraplegin–AFG3L2 complex, the res-
cule of the respiratory competence is at the lower limit. In fact, a further decrease of the specific activity of the protease complex (for instance by introducing a point mutation into the proteolytic site of one subunit) abolishes complementation, explaining the effect of a single proteolytic site mutation of the human protease in yeast. However, paraplegin–AFG3L2 complex is in the presence of the natural mitochondrial substrates in the autologous human system. Lowering the proteolytic activity by introducing a point mutation in the proteolytic site of paraplegin does not completely inactivate the protease (as it happens in yeast, when autologous elements are involved. This is due to the presence of the wild-type copy of AFG3L2; therefore, we still observe complementation of an assembly defect (as in yeast; Arlt et al., 1998).

In view of the observed functional conservation, we propose to term the paraplegin–AFG3L2 complex the human m-AAA protease, which combines proteolytic and chaperone-like activities. The detected complex I deficiency in HSP cells is very likely due to an inefficient folding process in the absence of a physiological paraplegin–AFG3L2 complex. Impaired complex I activity is frequently accompanied by enhanced ROS production and lipid peroxidation (Barrientos and Moraes, 1999; Li et al., 2003). Indeed, an intimate link between ROS and defects in the electron transport function leading to a further increase in ROS production plays an important role in the development and progression of neurodegenerative diseases (Schapira et al., 1989; Wong et al., 2002; Taylor et al., 2003). Therefore, we analyzed the sensitivity of HSP patients’ cells towards oxidative stress, and observed a significantly increased vulnerability of these cells by ROS. This effect was restored, again, upon exogenous expression of paraplegin.

In HSP, complex I deficiency could directly contribute to neurodegeneration via a free radical mechanism by direct ROS production and by a decreased ATP synthesis leading to energy failure. In particular, mitochondrial alterations have been documented in neuronal aging (Beal, 1996), and excessive production of ROS has been associated with different forms of neurodegeneration including: Parkinson’s disease (Jenner and Olanow, 1996); Alzheimer’s disease (Behl, 1999); amyotrophic lateral sclerosis (Rosen et al., 1993; Aguirre et al., 1998); Friedreich’s ataxia (Wong et al., 1999); and, more recently, neurodegeneration related to prion infection (Milhavet et al., 2000).

Neurons are particularly susceptible to oxidative stress due to the high rate of oxidative metabolic activity, low level of antioxidant enzymes, catalase and glutathione peroxidase, extended axonal morphology prone to peripheral injury, and impairment of the respiratory chain enzymes. In particular, complex I is considered to be one of the most severely affected by age-related increases in oxidative stress (Wong et al., 2002). In synaptic mitochondria, this effect is even worse (Davey et al., 1998). In addition, the effects of ROS on mitochondrial respiratory activity limits the ability of neuronal cells to respond appropriately to increase of ATP demand (Sims et al., 2000; Mattson and Liu, 2002). These findings, together with the evidence of a different complex I distribution among types of neurons (Pettus et al., 2000), may account for the selective vulnerability of a restricted subset of neurons, and applies, in particular, to the retrograde axonopathy typical of HSP.

Our findings shed new light on the molecular pathogenesis of HSP. We showed how paraplegin mutations suppress the human m-AAA protease formation and generate complex I deficiency in HSP cells, which may lead to additional production of ROS causing oxidative stress in the particular subset of neurons affected by HSP. Therefore, the initial pathogenic mechanism is likely to stem from the combined effects of misfolded peptide accumulation and the lack of chaperone activity on complex I assembling. Specifically, our results link this mitochondriopathy to the increasing group of misfolding diseases such as Alzheimer’s disease, Parkinson’s disease, prion encephalopathies, and expanded polyglutamine disease (Dobson, 1999; Temussi et al., 2003). HSP commonly has a typical progressive course and onset in the third or fourth decade of life. This implies that the phenotypic expression of this disease may involve two factors, a predisposing mutation and an age-related factor that causes a decline in mitochondrial function, which manifests the inherited defect.

Materials and methods

Reagents

Cell culture media and chemicals were purchased from Life Technologies (Invitrogen) and Sigma-Aldrich, respectively.

Cell cultures

Primary fibroblasts were obtained from skin biopsies of two controls and two HSP patients belonging to the original SPG7 family, homozygous for a 9.5-kb deletion in the SPG7 gene (patients no. IV-9 and IV-11; De Michele et al., 1998). In addition, we obtained fibroblasts from a healthy heterozygous individual belonging to the same family, which was used as an internal control (patient no. IV-3). Primary fibroblasts and HEK293 were cultured in a minimal essential medium supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin, and 20% FBS at 37°C in 95% humidified air and 5% CO2.

Transfections

The paraplegin expression plasmid (pSPG7) was prepared by cloning the full-length SPG7 cDNA (GenBank/EMBL/DDB accession no. Y16610) in a modified pMT21 vector. The resulting recombinant plasmid was transiently transfected by the calcium phosphate (Gualberto et al., 1996) and metafectene (Biontex Laboratories) methods into cultured primary fibroblasts. pSPG7 was mutagenized to obtain pSPG79745 as described later for the analogous yeast constructs (see Yeast expression constructs section).

Antibodies

To generate rabbit polyclonal antiserum directed against paraplegin and AFG3L2, we generated GST fusion proteins (pGEX2T; Amersham Biosciences) with protein fragments spanning amino acids 243–844 of SPG7 gene product (GenBank/EMBL/DDB accession no. AAH07692) and amino acids 413–828 of AFG3L2 gene product (GenBank/EMBL/DDB) accession no. Q9Y4W6). After expression in Escherichia coli, proteins were purified according to standard procedures and used to immunize rabbits. Anti-Hsp60 mAb was from StressGen Biotechnologies; anti-OxPhos complex I 39-KD subunit and mouse antiparvin monoclonal antibodies from Molecular Probes were used. Anti-cytochrome c mAb was from Promega.

Isolation of mitochondria and mitoplasts

Mitochondria were isolated by differential centrifugation of HEK293 or fibroblast cell homogenates (Smith, 1967). In brief, cells were washed in PBS, resuspended in an appropriate isotonic buffer (0.25 M sucrose, 5 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF) and homogenized using a glass teflon homogenizer. Unbroken cells and nuclei were pelleted by centrifu-
Mitoplasts were obtained by incubating mitochondria to a final protein concentration of 1.0 mg/ml in PBS with 2.7 mg/ml digitonin for 20 min on ice. The sample was centrifuged at 10,000 g for 10 min, and the final pellet (mitoplast fraction) was washed twice with PBS.

Isolation of integral proteins of the inner mitochondrial membrane

Isolation of integral proteins of the inner mitochondrial membrane was performed by incubating mitoplasts in 100 mM NaCl, 50 mM MgCl2, 0.4 g/liter BSA fatty acid free, 7.5 mM DTT, 15 μM luciferin, and 10 μg/ml luciferase. Cells were lysed with the ATP lysis buffer (0.2 M NaOH and 0.5 mM EDTA), and an aliquot of the obtained extract was diluted with the ATP dilution buffer (0.1 M NaOH and 0.5 mM EDTA). In the luminometer, 20 μl of this mixture was added to 100 μl of the assay solution, and the ATP content was measured. Data was expressed as nanomoles of ATP per milligram of protein (Bradford, 1976).

BN-PAGE and complex I in situ activity staining

Mitoplasts were solubilized at a protein concentration of 5 mg/ml by dodecyl maltoside (2% final concentration) and centrifuged, and supernatants were loaded on a linear 5–13% gradient polyacrylamide gel. Gels were stained with coomassie brilliant blue or transblotted onto PVDF membrane and immunodecorated with anti–39-kD antibody (Schägger et al., 1994). Complex I in situ activity staining was performed by incubation of the gel in 0.1 M Tris-HCl, 0.14 mM NADH, and 1 mg/ml nitro blue tetrazolium, pH 7.4; the reaction was carried out at RT and stopped after 30 min by 45% methanol and 10% acetic acid (Jung et al., 2000).

Band quantifications relative to in situ activity staining gels and Western blots have been performed by densitometric analysis (densitometer, Epson Twain Pro); a t test was used for significant calculation.

Import of 39-kD subunit of respiratory complex I in mitochondria isolated from control and HSP fibroblasts

Mitochondria from control and HSP fibroblasts were isolated as described previously, and import assay was performed (Yano et al., 2000). Precursor protein 39 kD was synthesized in vitro and translated on a nitrocellular brilliant blue or transblotted onto PVDF membrane and immunodecorated with anti–39-kD antibody (Schägger et al., 1994). The mitochondrial import assay was performed as follows: 660 μg of mitochondria was resuspended in 35 μl of mannitol buffer (225 mM mannitol, 25 mM sucrose, 10 mM Tris-HCl, pH 7.8, and 0.1 mM EDTA) and incubated with 70 μl of translate, 0.6 μl of 180 mM malate, and 1.1 μl of 1 M pyruvate. After 15, 45, and 135 min, 20-μl aliquots were removed, transferred to a fresh tube, incubated with trypsin, and centrifuged at 13,000 g for 5 min at 4°C. Pellets were washed once with mannitol buffer and resuspended in sample buffer for electrophoretic analysis in 12% SDS-PAGE. The imported protein was analyzed by fluorography.

Mitochondrial protein synthesis analysis

Primary fibroblasts were labeled with 35Smethionine (0,000 Ci/mmol and 100 μCi/ml) for 2 h in presence of 200 μg/ml cycloeximide as described previously (Chomyn, 1996). After labeling, the cells were washed and directly lysed in a sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% (v/vol) 2-mercaptoethanol, 1% (wt/vol) SDS, and 0.01% bromophenol blue. The mitochondrial translation products were analyzed by 15% SDS-PAGE and autoradiography.

Respiratory chain activity

Tests for respiratory chain defects were essentially performed as described by Robinson (1996). In brief, digitonin-permeabilized cells or isolated mitochondria were incubated at 37°C for 30 min in a respiratory buffer (0.25 M sucrose, 20 mM MOPS, 1 mM EDTA, 5 mM inorganic phosphate, 0.1% BSA fatty acid free, and 1 mM ADP, pH 7.4) containing specific substrates and inhibitors of the respiratory chain complexes. By providing pyruvate/malate (5 mM and 1 mM, respectively) and glutamate/malate (5 mM and 1 mM, respectively), we stimulated ATP synthesis dependent on complexes II, III, IV, and V. Finally, incubating cells with 20 μg/ml antimycin A, an inhibitor of complex III, and TmPDP/ascorbate (0.1 mM and 2 mM, respectively), we evaluated the ATP level due to the activity of complexes IV and V. ATP production was measured by luminometric assay. A t test was applied for significance calculation.

Luminometric assay of ATP

ATP concentration was determined with the Luciferin-Luciferase method as described previously (Runnor et al., 1999); in brief, the assay solution was prepared as was follows: 250 mM glycylglycine, 2 mM EGTA, 2 mM MgCl2, 0.4 g/liter BSA fatty acid free, 7.5 mM DTT, 15 μM luciferin, and 10 μg/ml luciferase. Cells were lysed with the ATP lysis buffer (0.2 M NaOH and 0.5 mM EDTA), and an aliquot of the obtained extract was diluted with the ATP dilution buffer (0.1 M NaOH and 0.5 mM EDTA). In the luminometer, 20 μl of this mixture was added to 100 μl of the assay solution, and the ATP content was measured. Data was expressed as nanomoles of ATP per milligram of protein (Bradford, 1976).

Immunoprecipitation

Mitochondrial membrane potential

Mitochondrial membrane potential (orange signal, high polarized mitochondria), whereas active mitochondria stained as described in BN-PAGE and Western blotting using AFG3L2- or paraplegin-specific antisera.
human paraplegin–AFG3L2 complex was revealed by the anti-c-Myc (9E10; 10 μg/ml).

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