Maturation of wild-type and mutated frataxin by the mitochondrial processing peptidase

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Frataxin is a mitochondrial protein deficient in Friedreich ataxia (FRDA) and which is associated with abnormal intramitochondrial iron handling. We identified the mitochondrial processing peptidase β (MPPβ) as a frataxin protein partner using the yeast two-hybrid assay. In in vitro assays, MPPβ binds frataxin which is cleaved by the reconstituted MPP heterodimer. MPP cleavage of frataxin results in an intermediate form (amino acids 41–210) that is processed further to the mature form. In vitro and in vivo experiments suggest that two C-terminal missense mutations found in FRDA patients modulate interaction with MPPβ, resulting in a slower maturation process at the normal cleavage site. The slower processing rate of frataxin carrying such missense mutations may therefore contribute to frataxin deficiency, in addition to an impairment of its function.

INTRODUCTION

The gene responsible for Friedreich ataxia (FRDA), an autosomal recessive neurodegenerative disease (1), codes for a novel 18 kDa protein, frataxin (2,3), located at mitochondrial membranes (3–5). Most patients are homozygous for a large trinucleotide expansion in the first intron of the gene, that causes a severe reduction of the transcript and protein steady-state levels. Four per cent of patients are compound heterozygotes for an expansion mutation and a point mutation. Two missense mutations have been reported, both being relevant to the function of the protein (2,6).

Frataxin is conserved from yeast to man and has a distant homologue in purple bacteria that share a common phylogeny with the prokaryotic mitochondrial ancestor. The study of yeast frataxin null mutants revealed a growth defect on a non-fermentable carbon source, mitochondrial DNA instability, decreased activity in cytochrome c oxidase and high sensitivity to hydrogen peroxide, copper and iron (5,7,8). The most pronounced effect of yeast frataxin deficiency is an accumulation of iron in the mitochondria (5,8). In addition, iron deposits (9) and deficiency of iron–sulfur enzymes (10) were found in the heart of FRDA patients, the former suggesting that frataxin plays a role in iron handling and the latter suggesting involvement of oxidative stress.

We applied a yeast two-hybrid assay (11) to unravel frataxin function in mitochondria, and we identified mitochondrial processing peptidase β (MPPβ), a subunit of heterodimeric peptidase that is involved in proteolytic cleavage of N-terminal mitochondrial targeting sequences (12,13). Frataxin with C-terminal missense mutations found in FRDA patients showed decreased interaction in the yeast two-hybrid assay. Processing of wild-type and mutated frataxin was analysed in vitro with reconstituted MPP and in vivo by COS cell overexpression. The results suggest that the maturation efficiency of the missense mutants is reduced and may contribute to the pathogenicity of these mutations.

RESULTS

Identification of MPPβ as a frataxin-interacting protein

We searched for protein partners of frataxin by yeast two-hybrid screening. An expression vector that encodes mouse frataxin fused to the DNA-binding domain of the transcription factor LexA was used as a bait in a two-hybrid screen of an embryonic (E9.5–E12.5) cDNA library. Frataxin is expressed during mouse embryogenesis and, therefore, its putative partners are expected to be represented in such a library. From ~1.5 × 10^6 transformants, 11 independent positive clones were obtained as determined by activation of his and lacZ reporter genes. Seven clones code for known non-mitochondrial proteins and three code for proteins that are unlikely to be mitochondrial based on sequence similarity. A single mitochondrial protein, MPPβ, was identified. The specificity of interaction between MPPβ and frataxin was verified by retransformation into yeast cells. The MPPβ two-hybrid clone encodes a protein homologous to rat MPPβ from amino acids 40 to 489, and would therefore contain six additional N-terminal amino acids compared with the mature MPPβ protein (14).

In order to test whether the interaction between frataxin and MPPβ is part of the recognition process that removes its mitochondrial targeting peptide, we constructed a series of frataxin deletion and point mutants and tested them using the yeast two-hybrid assay. The C-terminal domain of frataxin used as a bait did not activate the his and lacZ reporter genes, while a strong activation was detected when the N-terminal domain was used (Fig. 1). The C-terminal missense mutations found in FRDA patients, corresponding to the G127V and I151F changes on the
Figure 1. Activation of the lacZ reporter gene by the interaction of MPPβ with various frataxin baits. Schematic diagram of LexA fusion constructs (black box, LexA; white box, frataxin) used to test for the specificity of interaction between MPPβ and frataxin. The construction of the plasmids pBTM-frataxin, pBTM-N, pBTM-C, pBTM-G127V and pBTM-I151F is described in Materials and Methods.

mouse sequence, surprisingly reduced the activation of the reporter genes. We observed a 50% decrease in the activation of the lacZ reporter gene with the corresponding mouse I151F mutant and a 90% decrease with the G127V mutant, assuming an identical expression of the wild-type and point mutation constructs. Indeed, we found that the level of expression of the wild-type and point mutation frataxin–LexA fusions are comparable by western blot analysis (data not shown).

MPPβ from mammals is structurally related to, but functionally distinct from, the core I protein of the respiratory chain complex III (15). We tested, therefore, whether the interaction of frataxin is specific for MPPβ or also applies to the complex III core I protein. We found only a very weak interaction between frataxin and core I protein in vitro, by GST pull-down assay. In addition, no interaction was detected in vivo using the yeast two-hybrid assay and mature core I protein (amino acids 35–472) as a prey (data not shown).

In vitro and in vivo processing of frataxin

In order to test whether frataxin is indeed processed by MPP, we have reconstructed MPP in vitro by co-expression of the α and β subunits in E.coli. Total bacterial protein extract was assayed for processing activity using mouse wild-type and I151F and G127V mutant frataxins tagged at their C-terminus by five [35S]methionine residues. The β subunit of ATPase was used as a positive control (data not shown), and non-recombinant bacterial protein extract served as a negative control for non-specific degradation. Both wild-type and mutant frataxins are cleaved by MPP. About 25% of wild-type frataxin is cleaved by MPP after 1 h incubation, while cleavage appeared slower for I151F frataxin (~15% cleavage over the same time period; Fig. 3A) and for G127V (~16%, data not shown).

We then compared the cleavage site of MPP with the different forms observed during frataxin in vivo maturation. The maturation process can be followed in COS cells overexpressing human frataxin. The cells use the endogenous mitochondrial import system to target overexpressed frataxin to the mitochondria, and different maturation intermediates are obtained at different time points. Therefore, samples of in vitro processed frataxin were compared with overexpressed maturation product in COS cells and with the endogenous frataxin from muscle tissues by western blot analysis, using the 1G2 anti-frataxin antibody (Fig. 3B). The MPP processed form indeed corresponds to a major intermediate (~20 kDa), and one additional cleavage of frataxin results in the mature form.

We found a putative MPP recognition motif at the N-terminus of frataxin, predicted from a consensus sequence derived from the comparison of mitochondrial precursor protein sequences [RXhXX/S,T,G], where h is a hydrophobic residue and cleavage occurs before the hydrophobic residue (16)]. The corresponding mouse frataxin sequence RGLHVT would predict the cleavage to
Frataxin is a mitochondrial protein whose precise function is unknown. In order to unravel this function, we searched for frataxin protein partners using the yeast two-hybrid assay. We identified 11 positive clones, only one of which codes for a mitochondrial protein, MPPβ, that was characterized further.

MPPβ is a subunit of the heterodimeric MPP that cleaves the maturation sequence of nuclearly encoded mitochondrial proteins. MPP acts at the N-terminal α-helix amphiphilic structure of its substrate, and we demonstrated that MPPβ specifically interacts with amino acids 4–87 of frataxin using the yeast two-hybrid assay. The interaction between frataxin and MPPβ was confirmed independently by in vitro affinity binding, and the specificity of this interaction was demonstrated by the absence of interaction with the complex III core I protein which is highly homologous to MPPβ (55% identity).

MPP activity reconstituted in vitro was used to test the cleavage of wild-type frataxin. Frataxin cleavage was found to result in a protein of ∼20 kDa. The comparison of the in vitro MPP processed frataxin and N-terminally truncated frataxin with maturation products from frataxin overexpression in COS cells indicates that MPP cleaves frataxin to an intermediate form corresponding to amino acids 41–210. This intermediate form is then cleaved into the mature form. A two-step cleavage process is known to occur in the case of proteins that are targeted either to the mitochondrial matrix (17) or to the inner membrane and intermembrane space (17–19). The second cleavage might thus be associated with subsequent re-routing to the inner membrane or the intermembrane space, since frataxin was found by immunoelectron microscopy to reside at the mitochondrial intermembranes (3), while it did not co-localize with porin, an outer mitochondrial membrane protein in yeast (5).

The rare missense point mutations found in FRDA patients are all located within the C-terminal conserved domain of the protein. Vassarotti et al. reported that changes in the mature domain of the substrate may affect the precursor cleavability, probably by modifying the primary sequence in such a way that the accessibility of the targeting signal to the mitochondrial import apparatus is somehow restricted (20). We therefore constructed two mouse missense mutants corresponding to mutations reported in patients, and found that they cause reduced interaction with MPPβ in the yeast two-hybrid assay, suggesting that the maturation of the mutated frataxins may be defective in FRDA.

In vitro cleavage of the I151F mutant with reconstituted MPP occurred at the same position as in wild-type frataxin but with lower efficiency. The overall processing of the mutant frataxin in vivo in transfected COS cells also appeared less efficient, with a lower mature to precursor ratio for the same incubation period when compared with normal frataxin. However, the different sites of cleavage appeared unaltered between normal and mutant frataxin, indicating that no aberrant form of frataxin is produced as a consequence of the missense mutation. The lower efficiency
of processing of the mutant frataxin at the intermediate MPPβ cleavage site might contribute to the pathogenicity of the missense mutations in addition to the likely functional alteration caused by the modification of an amino acid highly conserved during evolution.

MATERIALS AND METHODS

**Bait constructions and mutagenesis**

Mouse frataxin cDNA was PCR amplified using primers UT56, 5′-TACATAGAATTCCGGAGTCGCGCAGCC-3′; and UT60, 5′-CAGTTCGGATCCTCAAGTGCCTTTCCAC-3′ (EcoRI and BamHI sites are indicated in italics), and fused in-frame into the EcoRI–BamHI sites of the LexA DNA-binding domain of the yeast two-hybrid vector pBTM116 (a gift of Dr S. Hollenberg, Seattle, WA) generating pBTM-frataxin. The N-terminal construct yeast two-hybrid vector pBTM116 (a gift of Dr S. Hollenberg, RI–EcoHI sites are indicated in italics), and fused in-frame into the BamHI site of the pGex4T3 vector for expression of a GST–mature MPPβ fusion protein (amino acids 46–489).

Overexpression of GST fusion protein was performed in the *E.coli* BL21 strain, and proteins were purified as described by the manufacturer (Pharmacia Biotech).

**GST pull-down assay**

GST pull-down assay was performed as follows. Glutathione–Sepharose beads were washed three times in GST buffer [50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.3 mM dithiothreitol (DTT), 5% glycerol, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated with 1 mg of purified GST fusion protein or GST at 4°C for 2 h. Unbound protein was washed off with GST buffer three times. Protein–protein interaction was assayed using an *in vitro* translated frataxin (or its mutant) in GST buffer in a final volume of 100 µl with 13.3 µl of the in *vitro* translation reaction (TNT7 Quick Promega) for 1 h at 4°C on a rocking table. Beads were washed with GST buffer three times, and as much supernatant as possible was removed after the last wash. Bound proteins were released in 2× Laemmli buffer by boiling and analysed by western blot.

**Construction of eukaryotic expression vectors and *in vitro* translation**

The complete coding sequence of mouse frataxin and of the I151F mutant was fused to a 5× methionine tag at the 3′ end and cloned in the pTL1 eukaryotic expression vector, resulting in pTLfrataxin-5×M and pTL1I151F-5×M. The human I154F mutant was constructed by overlap extension mutagenesis of the human frataxin pTL1 clone (3). The N-terminally truncated human frataxin clone was constructed by PCR (primers XP22, 5′-GGGCGAATTCATGCTGCGCAGCAGACAC-3′ and XP23, 5′-GGGCGAATTCATGCTGCGCAGCAGACAC-3′) and cloned into the EcoRI–KpnI sites of the pTL1 vector. Overexpression of the truncated protein was performed in COS cells as described in Campuzano et al. (3). *In vitro* translation was performed using TNT7 quick system as described by the manufacturer (Promega).

**In vitro processing and western blot analysis**

*In vitro* processing was performed using the pGEMMahiny plasmid coding for the MPPα and MPPβ subunits (a gift of Dr J. Adamec, Prague) as described in Striebel et al. (25). The β subunit of ATPase (a gift of Dr J. Adamec, Prague) served as a positive control for the *in vitro* processing. Western blot analysis was performed as described in Campuzano et al. (3).

**Protein expression in *E.coli***

The original pASV3-MPPβ clone was modified by PCR at its N-terminus (primers VU155, 5′-TAAGGTACCCGGATCC-CAGGCTGCCCCACAGG-3′ and VU1156, 5′-GCTCTGGGCGCAGAATCT-3′) and cloned into the BamHI site of the pGex4T3 vector for expression of a GST–mature MPPβ fusion protein (amino acids 46–489).

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