Human frataxin maintains mitochondrial iron homeostasis in *Saccharomyces cerevisiae*

Patrizia Cavadini¹,², Cinzia Gellera², Pragna I. Patel³ and Grazia Isaya¹,*+

¹Departments of Pediatric and Adolescent Medicine and Biochemistry and Molecular Biology, Mayo Clinic and Foundation, 200 First Street SW, Rochester, MN 55905, USA, ²Division of Biochemistry and Genetics, Istituto Nazionale Neurologico ‘Carlo Besta’, Milan, Italy and ³Departments of Neurology and Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

Received 14 June 2000; Revised and Accepted 18 August 2000

Frataxin is a nuclear-encoded mitochondrial protein widely conserved among eukaryotes. Human frataxin (fxn) is severely reduced in Friedreich ataxia (FRDA), a frequent autosomal recessive neuro- and cardio-degenerative disease. Whereas the function of fxn is unknown, the yeast frataxin homolog (Yfh1p) has been shown to be involved in mitochondrial iron homeostasis and protection from free radical toxicity. Evidence of iron accumulation and oxidative damage in cardiac tissue from FRDA patients suggests that fxn may have a similar function, but whether yeast and human frataxin actually have interchangeable roles in mitochondrial iron homeostasis is unknown. We show that a wild-type FRDA cDNA can complement Yfh1p-deficient yeast (yfh1Δ) by preventing the mitochondrial iron accumulation and oxidative damage associated with loss of Yfh1p. We analyze the functional effects of two FRDA point mutations, G130V and W173G, associated with a mild and a severe clinical presentation, respectively. The G130V mutation affects protein stability and results in low levels of mature (m) fxn, which are nevertheless sufficient to rescue yfh1Δ yeast. The W173G mutation affects protein processing and stability and results in severe m-fxn deficiency. Expression of the FRDA (W173G) cDNA in yfh1Δ yeast leads to increased levels of mitochondrial iron which are not as elevated as in Yfh1p-deficient cells but are above the threshold for oxidative damage of mitochondrial DNA and iron–sulfur centers, causing a typical yfh1Δ phenotype. These results demonstrate that fxn functions like Yfh1p, providing experimental support to the hypothesis that FRDA is a disorder of mitochondrial iron homeostasis.

INTRODUCTION

Friedreich ataxia (FRDA), the most common of the inherited ataxias, is an autosomal recessive neuro- and cardio-degenerative disease primarily characterized by progressive gait and limb ataxia leading to wheelchair confinement and hypertrophic cardiomyopathy, which is the most frequent cause of premature death (1). The vast majority of patients are homozygous for GAA repeat expansions in the first intron of the FRDA locus (2) and show a reduction in the levels of fxn ranging between 6 and 30% of control levels (3). Approximately 4% of FRDA patients carry one GAA expansion and a truncating or missense mutation in the other allele (4). Most compound heterozygotes have a typical FRDA phenotype but certain point mutations have been associated with milder clinical presentations (4–6). The mechanism by which fxn defects result in the pathology of FRDA has not yet been elucidated.

Studies in *Saccharomyces cerevisiae* have shown that the yeast frataxin homolog (Yfh1p) is involved in mitochondrial iron efflux (7) and that genetic inactivation of Yfh1p (yfh1Δ) results in mitochondrial iron overload (8,9). It has also been proposed that Yfh1p plays a role in iron–sulfur cluster biosynthesis and that loss of this function is responsible for iron accumulation in yfh1Δ mitochondria (10). In either case, excess mitochondrial iron is thought to react with H₂O₂, a by-product of respiration, to generate highly reactive hydroxyl radicals via Fenton chemistry (8,9). The resulting oxidative damage of iron–sulfur centers and mitochondrial DNA (mtDNA) yields respiratory-deficient yeast cells with an increased sensitivity to exogenous H₂O₂ (8,9). We have recently reported that iron induces self-assembly of recombinant Yfh1p into higher order multimers that sequester iron in a soluble and available form (11). These findings together suggest that native Yfh1p serves to sequester uncomplexed iron, maintain it in a form competent for export to the cytoplasm or iron–sulfur cluster biosynthesis and prevent iron-induced oxidative damage. Several observations indicate that fxn may have a similar function. Iron deposits, multiple iron–sulfur enzyme deficiencies and reduced levels of mtDNA have been observed in cardiac tissue from FRDA patients (12–14). In addition, hypersensitivity to oxidative stress that responds to iron chelators has been observed in cultured FRDA fibroblasts (15). It is therefore probable that, like Yfh1p, fxn plays a role in mitochondrial iron homeostasis and protection from iron-induced oxidative damage. To test this hypothesis directly, we have expressed wild-type and mutant FRDA alleles in yfh1Δ yeast. We show that fxn can replace Yfh1p and that FRDA point mutations lead to yeast phenotypes that correlate with disease severity in

*+To whom correspondence should be addressed. Tel: +1 507 266 0110; Fax: +1 507 284 1399; Email: isaya@mayo.edu
affected patients. Our results demonstrate that Yfh1p and fxn are functional homologs and that fxn defects affect mitochondrial iron homeostasis.

RESULTS

Expression of wild-type fxn in yfh1Δ yeast

Similar to most mitochondrial proteins, fxn is translated in the cytoplasm as a larger precursor protein. The fxn precursor is imported by mitochondria and processed to the mature form in two sequential steps by the mitochondrial processing peptidase (MPP) (16). The second step has been shown to be slow and inefficient in a variety of heterologous systems (16–18, unpublished data), suggesting that it may be difficult to express a functional form of fxn in yeast. In a previous study, a fxn–GFP fusion protein was targeted to mitochondria when expressed in yeast, but could not complement a yfh1Δ mutant lacking the endogenous Yfh1p (8). In another study, the C-terminal region of fxn fused to the N-terminus of Yfh1p rescued in part the ability of yfh1Δ cells to grow on non-fermentable carbon sources (19). We therefore sought to develop a strain that would express the full-length fxn precursor in sufficient amounts to yield levels of mature protein comparable to the physiologic levels of Yfh1p. To achieve this, we cloned the FRDA cDNA downstream of the constitutive yeast glycerolaldehyde-3 phosphate dehydrogenase promoter (20) in a low-copy plasmid by counterselection with 5-uracil-based YC-YFH1 plasmid (hyde-3 phosphate dehydrogenase promoter (20) in a low-copy YCp vector (21) (Fig. 1A, step 1). Transformants were cured of the URA3-based YC-YFH1 plasmid by counterselection with 5′-fluorouracil yielding strain yfh1Δ [YFH1] (Fig. 1A, step 2). Complete elimination of YFH1 from yfh1Δ [YFRDA] cells was confirmed by our inability to detect YFH1 by PCR amplification of total DNA using YFH1-specific primers. In addition, western analysis of yfh1Δ [YFRDA] cell extracts using anti-Yfh1p antibody failed to detect Yfh1p (Fig. 1B, top). Conversely, western analysis using an anti-fxn polyclonal antibody revealed two cross-reacting bands of 21 and 18 kDa (Fig. 1B, middle). Our group and others have shown previously that these products result from sequential cleavage of the fxn precursor by MPP (16,17). The 21 kDa product (i-fxn) is found at low levels in human tissues but tends to accumulate in excess of the 18 kDa product in heterologous systems due to inefficient processing by MPP (16–18, unpublished data). The 18 kDa product (m-fxn) corresponds to the mature form of frataxin as detected in human tissues (16,17, unpublished data). In agreement with these previous findings, western analysis showed that yeast-produced i-fxn and m-fxn correspond to the endogenous intermediate and mature form of frataxin in human lymphoblasts (see below). When calculated as a ratio with the levels of cytochrome c oxidase subunit IV (CoxIV), the m-fxn levels in yfh1Δ [YFRDA] cells were 1.3-fold higher than the levels of Yfh1p in yfh1Δ [YFH1] cells (Fig. 1B). Thus, the yfh1Δ [YFRDA] strain expresses close to physiologic levels of the mature form of human frataxin.

Wild-type fxn complements the yfh1Δ phenotype

Loss of Yfh1p leads to mitochondrial iron accumulation and iron-induced oxidative damage of iron–sulfur centers and mtDNA (8,9,13) yielding yeast cells (yfh1Δ) that are respiratory-deficient and exhibit an increased sensitivity to exogenous H2O2 (8,9) (see yfh1Δ in Fig. 2A and B). To test whether fxn can functionally replace Yfh1p, a series of parameters were assessed. Analysis of mitochondrial iron content revealed an 8-fold reduction in isolated yfh1Δ [YFRDA] mitochondria relative to yfh1Δ mitochondria (Table 1). In addition, yfh1Δ [YFRDA] cells grew at the same rate as yfh1Δ [YFH1] cells on solid and liquid media containing fermentable (dextrose;YPD) or non-fermentable (ethanol and glycerol; YPEG) carbon sources at 30 and 37°C (Fig. 2A). Moreover, when the yfh1Δ [YFRDA] strain was crossed with a ρ0 tester (lacking mtDNA), >90% of the diploid progeny grew on YPEG, indicating that yfh1Δ [YFRDA] cells do not contain ρ0 mutations that otherwise develop at high frequency in yfh1Δ cells (8). These results
Table 1. Iron concentration and aconitase activity in fxn-expressing strains

<table>
<thead>
<tr>
<th></th>
<th>Mitochondrial iron&lt;sup&gt;a&lt;/sup&gt; (nmol/mg protein)</th>
<th>Aconitase&lt;sup&gt;b&lt;/sup&gt; (nmol/min/mg)</th>
<th>Citrate synthase&lt;sup&gt;b&lt;/sup&gt; (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>yfh1∆ [YFH1]</td>
<td>0.74 ± 0.05</td>
<td>13.1 ± 0.4</td>
<td>132 ± 8</td>
</tr>
<tr>
<td>yfh1∆ [FRDA]</td>
<td>1.30 ± 0.27</td>
<td>10.7 ± 1.4</td>
<td>142 ± 5</td>
</tr>
<tr>
<td>yfh1∆ [G130V]</td>
<td>1.34 ± 0.39</td>
<td>12.6 ± 0.6</td>
<td>143 ± 3</td>
</tr>
<tr>
<td>yfh1∆ [W173G]</td>
<td>4.97 ± 0.26</td>
<td>ND</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>yfh1∆</td>
<td>10.30 ± 2.43</td>
<td>ND</td>
<td>59 ± 3</td>
</tr>
</tbody>
</table>

ND, activity not detected.
Values are expressed as means ± SD.
<sup>a</sup>n = 6.
<sup>b</sup>n = 3.

show that fxn can prevent mitochondrial iron accumulation in yfh1∆ cells thereby preserving their respiratory competence and mtDNA integrity. The iron concentration in yfh1∆ [FRDA] mitochondria fell within the range of values reported previously for wild-type yeast mitochondria (8,9,21) but was twice that in yfh1∆ [YFH1] mitochondria (Table 1). We therefore analyzed whether yfh1∆ [FRDA] cells showed signs of increased oxidative damage as compared with yfh1∆ [YFH1] cells. Iron–sulfur proteins are particularly sensitive to iron-induced oxidative damage and as such they are severely affected in yfh1∆ yeast (10,13) (Table 1). However, the activity of the iron–sulfur enzyme aconitase in isolated yfh1∆ [FRDA] mitochondria was similar to that in yfh1∆ [YFH1] mitochondria (Table 1). In addition, the iron–sulfur protein of the cytochrome bc<sub>1</sub> complex (Fe/S), which is undetectable in yfh1∆ cells (16), was present in yfh1∆ [FRDA] cell extracts at levels comparable to those in yfh1∆ [YFH1] cells (Fig. 1B). In agreement with these data, the sensitivity to exogenous H<sub>2</sub>O<sub>2</sub> was not increased in yfh1∆ [FRDA] relative to yfh1∆ [YFH1] cells (Fig. 2B). These results indicate that fxn can protect yeast mitochondria from iron-induced oxidative damage by fulfilling the role of Yfh1p in mitochondrial iron homeostasis.

Expression of fxn-G130V and fxn-W173G in yfh1∆ yeast

To obtain a yeast model of FRDA, we expressed two mutant FRDA alleles in yfh1∆ yeast. We chose the FRDA (G130V) and FRDA (W173G) alleles which in heterozygosity with expanded FRDA alleles, lead to mild and severe clinical presentation, respectively (4,5). Strain yfh1∆ [YFH1] was transformed with a low-copy YC-FRDA plasmid carrying the G130V or W173G mutation and strains yfh1∆ [G130V] and yfh1∆ [W173G] were derived by counterselection, as outlined in Figure 1A. The expression levels of G130V- and W173G-fxn were initially analyzed during 8 h of exponential growth in rich medium containing galactose (YPGal), a fermentable carbon source that does not repress yeast mitochondria biogenesis. When compared with yfh1∆ [FRDA] cells, both i-fxn and m-fxn were reduced ~3.5-fold in yfh1∆ [G130V] cells (Fig. 3). On the other hand, i-fxn was reduced ~4-fold while m-fxn was undetectable in yfh1∆ [W173G] cells (Fig. 3) although trace amounts
of m-fxn could be seen in overexposed blots. These results were confirmed through western analysis of several independent isolates of the \( \Delta yfh1 \) [G130V] and \( \Delta yfh1 \) [W173G] strains (data not shown). Semi-quantitative PCR analysis showed that \( yfh1 \) [G130V], \( yfh1 \) [W173G] and \( yfh1 \) [FRDA] cells contained similar amounts of FRDA cDNA and RT–PCR revealed that they also expressed similar levels of FRDA mRNA (data not shown). Thus, the reduction in fxn levels observed in \( yfh1 \) [G130V] and \( yfh1 \) [W173G] cells must arise from post-translational effects of the G130V and W173G mutations. We noted that the G→V change decreases the mobility of fxn in SDS–PAGE. We observed this abnormal mobility also when the fxn-G130V precursor was translated in a rabbit reticulocyte lysate and processed \textit{in vitro} by purified MPP; N-terminal radiosequencing, however, showed that the N-terminus of i-fxn-G130V corresponds to that of wild-type i-fxn (unpublished data).

**The G130V mutation affects fxn stability whereas the W173G mutation impairs fxn maturation and stability**

To elucidate the origin of m-fxn deficiency in \( \Delta yfh1 \) [G130V] and \( \Delta yfh1 \) [W173G] cells, wild-type and mutant fxn precursor polypeptides were translated and analyzed \textit{in vitro}. Our group as well as others have shown under different experimental conditions that the fxn precursor (p) is converted to i-fxn in excess of the rate at which i-fxn can be processed to m-fxn, leading to i-fxn accumulation (16,17, unpublished data). Accordingly, analysis of protein import into isolated yeast mitochondria showed that >90% of wild-type p-fxn was imported and processed to i-fxn (~80%) and to much lower levels of m-fxn (~10%) in 60 min (Fig. 4). Import and processing of p-fxn-G130V was slightly less efficient, with ~75% of the mutant precursor being converted to i-fxn (~70%) and m-fxn (~5%) in 60 min (Fig. 4). In contrast, only ~35% of p-fxn-W173G was imported and processed to i-fxn and there was no detectable conversion from the intermediate to the mature form (Fig. 4). To assess whether the G130V and W173G mutations have any effects on fxn stability, the turn-over rates of intermediate species accumulated in isolated yeast mitochondria were measured during a 2 h chase. Both i-fxn-G130V and i-fxn-W173G were degraded more rapidly (\( t = 45 \) min) than wild-type i-fxn (\( t = 90 \) min) (Fig. 4). These findings explain the results obtained by western analysis of the fxn-expressing strains (Fig. 3). The main effect of the G130V mutation is a significant reduction in protein stability with only a modest effect on mitochondrial import and processing. This causes a global reduction in the steady state levels of both the intermediate and mature forms of the protein. The W173G mutation results in a dramatic decrease in the rate at which the fxn precursor is imported and processed to the mature form, and at the same time affects protein stability. These two effects, in combination, cause a reduction in the steady-state levels of the intermediate form and an even more drastic deficiency of m-fxn. Thus, the \( yfh1 \) [G130V] and \( yfh1 \) [W173G] strains represent suitable models to study the effects of fxn defects on mitochondrial iron homeostasis.

![Figure 4](image)

The mutant fxn-G130V protein complements the \( yfh1 \) \( \Delta \) phenotype

The iron content of isolated \( yfh1 \) [G130V] mitochondria was very similar to that of \( yfh1 \) [FRDA] mitochondria (Table 1). Accordingly, \( yfh1 \) [G130V] cells grew as well as \( yfh1 \) [FRDA] or \( yfh1 \) [YFH1] cells on non-fermentable carbon sources (Fig. 2A) and >90% of the diploid progeny of a cross between \( yfh1 \) [G130V] cells and a \( \rho^{0} \) strain was respiratory-
Deficiency of the mature form of fxn-W173G is associated with a yfh1Δ-like phenotype

The yfh1Δ [W173G] and the yfh1Δ strain behaved similarly under a variety of experimental conditions. The iron concentration in yfh1Δ [W173G] mitochondria was increased 7- and 4-fold relative to yfh1Δ [YFH1] and yfh1Δ [FRDA] mitochondria, respectively, and corresponded to ~50% of the iron concentration in yfh1Δ mitochondria (Table 1). As expected, yfh1Δ [W173G] cells did not grow on non-fermentable carbon sources (Fig. 2A) and <1% of the diploid progeny of a cross between yfh1Δ [W173G] cells did not grow on non-fermentable carbon sources (data not shown). In addition, the YC-G130V plasmid was retrieved from several independent yfh1Δ [G130V] isolates (22) and the presence of the G130V mutation was confirmed by DNA sequencing. Thus, the essentially normal phenotype of yfh1Δ [G130V] yeast is specifically associated with expression of the FRDA [G130V] cDNA. This implies that relatively low levels of fxn are sufficient to maintain iron homeostasis in yeast mitochondria and that the G130V mutation per se does not impair the function of fxn, at least not to a degree that can be revealed in the yeast experimental model.

Severe m-fxn deficiency in GAA/G130V and GAA/W173G compound heterozygotes

Western analysis of cultured cells from two GAA/G130V and two GAA/W173G compound heterozygous patients showed almost undetectable levels of m-fxn in all cases (Fig. 5). A GAA/G130V patient with undetectable fxn in muscle was previously reported (3). The lack of fxn detection in this patient was attributed to reduced cross-reactivity of the fxn-G130V protein with the monoclonal 1G2 antibody, which is directed against an epitope that includes the G130 residue (3). Given that a polyclonal antibody against the full-length fxn precursor was used in our study, we conclude that the essentially complete lack of m-fxn detection in GAA/G130V cells reflects an actual deficiency of the fxn-G130V protein. Likewise, the nearly complete absence of m-fxn in GAA/W173G cells must reflect a deficiency of the fxn-W173G protein. There are two possible mechanisms that could lead to fxn-G130V or fxn-W173G deficiency in these cell lines. The G130V and W173G mutations could cause mRNA instability or they could have post translational effects similar to those we have observed in yeast. The former mechanism seems unlikely given that: (i) RNAse protection studies previously showed that the levels of FRDA mRNA in a carrier of the G130V mutation were similar to those of controls (5); and (ii) our RT–PCR revealed that yfh1Δ [G130V], yfh1Δ [W173G] and yfh1Δ [FRDA] cells contained similar levels of FRDA mRNA. Therefore, m-fxn deficiency in the GAA/G130V and GAA/W173G cell lines may originate from impaired expression of the expanded allele and reduced processing and/or stability of the mutant fxn-G130V and fxn-W173G proteins.

DISCUSSION

Frataxin is a nuclear-encoded mitochondrial protein widely conserved among eukaryotes (23). The human protein, fxn, is severely reduced in FRDA, the most frequent of the inherited ataxias (1 in 50 000 live births) (3). Although the function of fxn is unknown, studies in *S. cerevisiae* have shown that the yeast frataxin homolog, Yfh1p, is required for mitochondrial...
iron efflux (7) and protection from iron-induced oxidative damage of iron–sulfur centers and mtDNA (8–10). We have shown that in the presence of ferrous-iron recombinant Yfh1p assemblies into a high molecular weight multimer that can sequester >3000 atoms of iron in an available and non-toxic form (11, unpublished data). These data together indicate that Yfh1p plays a central role in mitochondrial iron homeostasis. Evidence of iron deposits and increased oxidative damage in cardiac tissue (12–14) and cultured fibroblasts (15) from FRDA patients suggests that fxn may play a similar role. To test this hypothesis, we have expressed the wild-type and two mutant forms of fxn in a yfh1Δ yeast knock-out mutant lacking endogenous Yfh1p. The yfh1Δ [FRDA] strain expressed levels of mature fxn close to the steady-state levels of Yfh1p and sufficient to prevent mitochondrial iron overload and loss of iron–sulfur proteins and mtDNA. These results provide direct evidence that fxn and Yfh1p are functionally interchangeable and that fxn is involved in mitochondrial iron homeostasis.

To obtain a yeast model of FRDA, we expressed mutant forms of fxn carrying the G130V or W173G point mutation. Approximately 4% of FRDA patients are compound heterozygotes for an intrinsic GAA expansion and a point mutation (4). The G130V mutation is among the most frequent of the known FRDA point mutations and is generally associated with an atypically mild clinical presentation (5), whereas the W173G mutation most commonly leads to a more severe FRDA phenotype (4). The yfh1Δ [G130V] strain was phenotypically indistinguishable from the yfh1Δ [FRDA] strain. The only obvious effect of the G130V mutation was an increase in the protein turnover rate with a reduction in the steady-state levels of i-fxn. The yfh1Δ [FRDA] strain expressed levels of mature fxn close to the steady-state levels of Yfh1p and sufficient to prevent mitochondrial iron overload and loss of iron–sulfur proteins and mtDNA. These results provide direct evidence that fxn and Yfh1p are functionally interchangeable and that fxn is involved in mitochondrial iron homeostasis. Thus, the G130V mutation does not affect the function of fxn, at least not to a degree detectable by our experimental system. Protein instability, however, may play a more important role in compound heterozygotes who carry an expanded FRDA allele in addition to the FRDA (G130V) allele. RNase protection studies previously showed that the levels of frataxin mRNA in a carrier of the G130V mutation were similar to that of controls (5). This and the severe m-fxn deficiency we observed in lymphoblasts from GAA/G130V heterozygotes suggest that the disease in these patients is caused by impaired expression of the expanded allele and reduced stability of the fxn-G130V protein. There are three possible explanations for the inability of the G130V allele to complement human cells as fully as it does complement yeast cells: (i) yeast mitochondria may require levels of frataxin lower than those needed by human mitochondria; (ii) the fxn-G130V protein may be even more unstable in human than in yeast mitochondria; and/or (iii) the G130V mutation may cause subtle mitochondrial abnormalities that cannot be revealed in rapidly dividing yeast cells but may chronically affect non-dividing neuronal cells, leading to the delayed clinical presentation of GAA/G130V heterozygotes (5).

The W173G mutation inhibited precursor import and processing and increased the protein turnover rate, which together resulted in a severe m-fxn deficiency. This condition led to increased levels of mitochondrial iron that were not as elevated as in yfh1Δ cells but reached the threshold for oxidative damage of mtDNA and iron–sulfur proteins, ultimately causing a typical yfh1Δ phenotype. The iron concentration in yfh1Δ [W173G] mitochondria was consistently lower (50%) than in yfh1Δ mitochondria, however, indicating that mitochondrial iron homeostasis is not completely abolished in yfh1Δ [W173G] yeast. Trace amounts of m-fxn-W173G may be sufficient to maintain some degree of mitochondrial iron efflux thereby preventing iron from accumulating as much as in yfh1Δ mitochondria. This observation is important as it indicates that the phenotype of yfh1Δ [W173G] cells does not simply arise by default, but actually reflects the consequences of fxn deficiency in yeast mitochondria. The fact that some residual fxn-W173G mutant frataxin was detected in two GAA/W173G cell lines (Fig. 5) suggests that the effects of the W173G mutation may have been exacerbated in our yeast expression system. Indeed, processing of i-fxn to m-fxn is known to be inefficient in heterologous systems (16–18; unpublished data) and the W173G mutation could make it even more inefficient in the context of yeast mitochondria. In any case, the dramatic reduction in m-fxn levels in GAA/W173G lymphoblasts as compared with normal controls clearly indicates that the W173G mutation leads to frataxin deficiency in yeast and human mitochondria as well. The iron accumulation linked to m-fxn-W173G deficiency in yeast may therefore explain the typical FRDA phenotype of GAA/W173G patients (4).

In summary, we have shown that fxn can substitute for Yfh1p and that FRDA point mutations affect yeast cells in a manner that correlates with disease severity in compound heterozygotes. These findings indicate that fxn and Yfh1p share similar mechanisms of function and bring experimental support to the hypothesis that FRDA is a disorder of mitochondrial iron homeostasis.

MATERIALS AND METHODS

Plasmids

The 5′ and 3′ untranslated regions (UTRs) of the FRDA cDNA were modified by PCR using a forward primer (5′-cgccgcatcgg- tagaagtggcaactcgggagc-3′), that introduces a BamHI site and the ribosome-binding sequence of the YFH1 gene transcript immediately upstream of the initiator ATG, and a reverse primer (5′-cgccgcatctcacatagcatcgttccgagta-3′) that introduces two ATG upstream and a BamHI site downstream of the stop codon. The PCR product was cloned into the BamHI site of vector pG-3 (20) downstream of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter and a 3 kb PstI fragment, including the promoter and the FRDA cDNA, was excised and cloned in a YCplac22 vector carrying the TRP1 selectable marker (24) to yield the YC-FRDA plasmid. PCR-mediated site-directed mutagenesis was used to introduce two FRDA point mutations in YC-FRDA, yielding YC-FRDA (G130V) and YC-FRDA (W173G) vectors. A 647 bp BamHI fragment was excised from YC-FRDA (G130V) or YC-FRDA (W173G) and cloned into pGEM-3Zf (+) (Promega, Madison, WI) vector to yield pGEM-G130V and pGEM-W173G, that were used in transcription–translation reactions. All constructs were confirmed by DNA sequencing.
Yeast strains and media

Strains yfh1Δ and yfh1Δ [YFH1] were as described (21). The pΔ tester strain CB11 was generously provided by Alexander Tzagoloff (Department of Biological Sciences, Columbia University, New York, NY). Strains yfh1Δ [FRDA], yfh1Δ [G130V] and yfh1Δ [W173G] were obtained by transformation of strain yfh1Δ [YFH1] with vectors YC-FRDA, YC-FRDA (G130V) or YC-FRDA (W173G), followed by counterselection with 5′-fluoroorotic acid (25). For analysis of mtDNA integrity, these strains were grown on YPD plates for 10 h and mated with CB11. Mating patches were streaked on synthetic dextrose medium (SD) plates without amino acids to select for diploids. These cultures were resuspended in water and plated to ~100 colony-forming units on YPD plates. The percentage of respiratory-competent pΔ colonies was determined by replica-plating on YPEG plates. The YPD, YPGal, YPEG and SD media were as previously described (21).

Biochemical determinations

For mitochondrial iron measurements, yeast were grown in 500 ml of YPGal at 30°C to an OD600 of 4–6. Mitochondria were isolated and resuspended in metal determination buffer (21) to a final protein concentration of 5 mg/ml. Triplicate samples (150 µl each) were analyzed by inductively coupled plasma emission spectroscopy (ICP) on a Perkin Elmer Optima ICP (Norwalk, CT) (26). Previously described methods were used for measurements of acountase and citrate synthase activity in isolated mitochondria (27,28) and western analysis of cell protein extracts (21). In mitochondrial protein import assays, precursor polypeptides were synthesized in vitro in the presence of [35S]methionine using TNT Quick Coupled Transcription/Translation System (Promega). Translation mixture (15 µl) was incubated with isolated yeast mitochondria (total protein = 400 µg) in import buffer (0.6 M mannitol, 20 mM HEPES–KOH pH 7.4, 1 mM ATP, 1 mM MgCl₂, 40 mM KCl, 5 mM methionine, 3 mg/ml bovine serum albumin, 20 mM phosphocreatine and 200 µg/ml phosphocreatine kinase) at 27°C as previously described (16). In chase experiments, radiolabeled precursors (9 µl) were incubated with mitochondria (240 µg) at 27°C for 60 min. Each import reaction was treated with proteinase K (250 µg/ml for 30 min at 0°C) and then separated into mitochondrial pellet and post-mitochondrial supernatant by centrifugation at 14,000 g for 5 min at 4°C in the presence of protease inhibitors. The mitochondrial pellet was washed twice with 0.6 M mannitol, 20 mM HEPES–KOH pH 7.4, resuspended in the same buffer (30 µl) and incubated at 27°C for an additional 120 min. Polyclonal antibodies against the yeast Yfh1p, CoxIV and Fe/S proteins were as previously described (16,21); a GST–SDH fusion protein was used to generate polyclonal antibodies in rabbits against yeast Yfh1p, CoxIV and Fe/S proteins. Polyclonal antibodies against a bacterially expressed GST–fxn fusion protein were a generous gift of Franco Taroni (Instituto Nazionale Neurologico ‘Carlo Besta’, Milan, Italy).

Semi-quantitative PCR and RT–PCR

Total DNA and total RNA were isolated from yeast cells as previously described (21). cDNA was synthesized from 1 µg of total RNA using Invitrogen cDNA Cycle kit (Carlsbad, CA). Total DNA (100 ng) or cDNA (5 µl) were used in PCR reactions to amplify a 480 bp fragment of FRDA (15). A 638 bp fragment of RNA polymerase II subunit 2 (RPO22) (primers: 5′-ggctggagggaggtctcgcatc-3′ and 5′-aaacacctgctgagcatc-3′) was simultaneously amplified in each PCR reaction as an internal control. Optimal conditions for quantitative analysis of PCR products were selected as described by Wong et al. (15). Agarose gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) and DNA levels determined on a STORM 840 with the Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA) and expressed as a percentage of the internal control.

Cell lines

PAT75 and PAT78 (GAA 840–875/G130V) are lymphoblastoid cell lines from two siblings of a previously described family (5). P131 (GAA 600/W173G) and P614 (GAA 760/W173G) are lymphoblastoid cell lines from two unrelated patients diagnosed with classic FRDA (C. Gellera, unpublished data). The indicated expansion sizes are those found in fresh blood samples and were not verified in the lymphoblastoid cell lines.

ACKNOWLEDGEMENTS

We thank Franco Taroni for anti-fxn antibodies, Cynthia McMurray for critical reading of the manuscript and Connie Lesnick for technical assistance. This work was supported by grants from the Muscular Dystrophy Association (P.I.P. and G.I.) and grant AG15709 from the National Institute on Aging (G.I.). P.C. is supported by a fellowship from the Pierfranco and Luisa Mariani Foundation, Milan, Italy.

REFERENCES


Human Molecular Genetics, 2000, Vol. 9, No. 17 1259


