Missense Mutations Linked to Friedreich Ataxia Have Different but Synergistic Effects on Mitochondrial Frataxin Isoforms

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Background: Missense mutations in frataxin contribute to Friedreich ataxia pathophysiology with undefined mechanisms.

Results: Clinically aggressive mutations variably affect stability, biogenesis, or catalytic activity of two mitochondrial frataxin isoforms.

Conclusion: A single point mutation may lead to destabilization or inactivation of multiple frataxin isoforms.

Significance: The clinical severity of frataxin mutations reflects complex deleterious effects that synergistically lead to global loss of functional frataxin.

Friedreich ataxia is an early-onset multisystemic disease linked to a variety of molecular defects in the nuclear gene FRDA. This gene normally encodes the iron-binding protein frataxin (FXN), which is critical for mitochondrial iron metabolism, global cellular iron homeostasis, and antioxidant protection. In most Friedreich ataxia patients, a large GAA-repeat expansion is present within the first intron of both FRDA alleles, that results in transcriptional silencing ultimately leading to insufficient levels of FXN protein in the mitochondrial matrix and probably other cellular compartments. The lack of FXN in turn impairs incorporation of iron into iron-sulfur cluster and heme cofactors, causing widespread enzymatic deficits and oxidative damage catalyzed by excess labile iron. In a minority of patients, a typical GAA expansion is present in only one FRDA allele, whereas a missense mutation is found in the other allele. Although it is known that the disease course for these patients can be as severe as for patients with two expanded FRDA alleles, the underlying pathophysiological mechanisms are not understood. Human cells normally contain two major mitochondrial isoforms of FXN (FXN42–210 and FXN81–210) that have different biochemical properties and functional roles. Using cell-free systems and different cellular models, we show that two of the most clinically severe FXN point mutations, I154F and W155R, have unique direct and indirect effects on the stability, biogenesis, or catalytic activity of FXN42–210 and FXN81–210 under physiological conditions. Our data indicate that frataxin point mutations have complex biochemical effects that synergistically contribute to the pathophysiology of Friedreich ataxia.

Friedreich ataxia is an autosomal recessive disease with an estimated incidence of 1:40,000 newborns. Patients are largely asymptomatic during the first 5–10 years of life but subsequently develop a progressive loss of movement coordination and other abnormalities such as cardiac disease, muscle weakness, skeletal deformities, vision and hearing impairment, and diabetes. The disease eventually leads to wheelchair confinement and inability to perform daily activities independently, with cardiac failure representing a frequent cause of death as early as the second decade of life (for review, see Refs. 1 and 2). The FRDA2 gene encodes the frataxin precursor (FXN1–210), which is primarily targeted to the mitochondrial matrix (3, 4), where it is processed to shorter products that collectively play a critical role in mitochondrial iron metabolism (Refs. 5–7; for review, see Ref. 8) and global cellular iron homeostasis (for review, see Ref. 9).

In most Friedreich ataxia patients, both FRDA alleles contain a GAA-trinucleotide repeat expansion in the first intron, which causes transcriptional silencing and ultimately a drastic reduction in the levels of FXN1–210 mRNA as compared with normal individuals (10–12). Some patients, however, have a GAA expansion in only one FRDA allele and otherwise carry a missense mutation in the other (13, 14). A number of different missense mutations have been identified, and although some are linked to mild clinical presentations, others are linked to clinical courses as severe as those presented by patients who have a GAA expansion in both FRDA alleles (13, 14).

Our understanding of how FRDA missense mutations affect the pathophysiology of Friedreich ataxia has been hindered by the complexity of FXN1–210 biogenesis. Upon import to the mitochondrial matrix, this precursor polypeptide is cleaved stepwise to different shorter products (5–7). The two most abundant of these products (FXN42–210 and FXN81–10 isoforms) have been shown to possess strikingly different func-

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This article contains supplemental Figs. S1—S3.

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functional consequences of such mutations have been studied in the pathophysiology of Friedreich ataxia. To date, however, the chemical effects that may contribute synergistically to the iron-sulfur cluster synthesis under oxidizing conditions. In addition, oligomeric FXN(42–210) and monomeric FXN(81–210) exhibit different binding affinities for other key proteins involved in iron-sulfur cluster synthesis, namely, the NFS1-ISD11 complex, which provides elemental sulfur, and the ISCU protein, which serves as a scaffold for [2Fe-2S] and [4Fe-4S] cluster assembly (for review, see Refs. 17 and 18). Whereas oligomeric FXN(42–210) forms stable complexes with NFS1-ISD11 in the absence or presence of ISCU (15), monomeric FXN(81–210) only binds to a preformed NFS1-ISD11-ISCU complex (15, 16, 19). Both isoforms are normally present in cultured cells and tissues and are thought to ensure incremental rates of iron-sulfur cluster synthesis depending on iron availability and metabolic requirements (8). Extra-mitochondrial isoforms of FXN have also been identified (20–22); they appear to originate from alternative splicing of the FRDA transcript and may participate in iron-sulfur cluster synthesis and/or antioxidant protection in the cytoplasm and the nucleus (21, 22).

Thus, as observed for other multifunctional proteins (23, 24), it is likely that FRDA missense mutations have complex biochemical effects that may contribute synergistically to the pathophysiology of Friedreich ataxia. To date, however, the functional consequences of such mutations have been studied almost exclusively in the context of FXN(81–210) (19, 25–27). Here we show that two of the most clinically severe mutations, I154F and W155R, have multiple distinct effects on the biogenesis, stability, and functions of FXN(42–210) and FXN(81–210). Our work has implications for the pathophysiology and treatment of Friedreich ataxia patients affected by FRDA missense mutations.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of the Proteins Used in This Study—* Expression constructs for the mutant FXN(81–210) or FXN(42–210) isoforms were made via PCR-mediated mutagenesis and verified by DNA sequencing. Monomeric and oligomeric FXN isoforms were made via PCR-mediated mutagenesis and verified by DNA sequencing. Monomeric and oligomeric FXN81–210 and monomeric FXN42–210 exhibit different binding affinities for other key proteins involved in iron-sulfur cluster synthesis, namely, the NFS1-ISD11 complex, which provides elemental sulfur, and the ISCU protein, which serves as a scaffold for [2Fe-2S] and [4Fe-4S] cluster assembly (for review, see Refs. 17 and 18). Whereas oligomeric FXN(42–210) forms stable complexes with NFS1-ISD11 in the absence or presence of ISCU (15), monomeric FXN(81–210) only binds to a preformed NFS1-ISD11-ISCU complex (15, 16, 19). Both isoforms are normally present in cultured cells and tissues and are thought to ensure incremental rates of iron-sulfur cluster synthesis depending on iron availability and metabolic requirements (8). Extra-mitochondrial isoforms of FXN have also been identified (20–22); they appear to originate from alternative splicing of the FRDA transcript and may participate in iron-sulfur cluster synthesis and/or antioxidant protection in the cytoplasm and the nucleus (21, 22).

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after which sulfide concentration was calculated using a standard curve. To measure the effects of wild type and mutant FXN isoforms on NFS1 activity, the NFS1-ISD11 complex was incubated with 3 mol eq of ISCU in the presence or absence of 3 mol eq of the indicated FXN isoform at 37 °C for 30 min before adding l-cysteine to the reaction mixture. An Fe-S cluster assembly assay were performed as described in detail previously (15, 30).

**Ni-NTA Pulldown Assays—**Detergent-soluble supernatants were prepared from COS-7 cells as described above, and total protein concentration was measured and adjusted to 1 mg/ml. Pulldown assays were performed with Ni-NTA-agarose beads (40 μl slurry) with or without bound HisNFS1-ISD11, WT-HisFXN42–210, or W155R-HisFXN42–210. Beads were added to 1 ml (final volume) of the cell lysate described above in the presence of 20 mM imidazole. The final concentration of HisNFS1-ISD11, WT-HisFXN42–210, or W155R-HisFXN42–210 was 1.6 μM. Binding reactions were incubated and analyzed by SDS-PAGE and Western blotting as described previously (31).

**RESULTS**

Residues Ile-154 and Trp-155 Are Predicted to Play Different Structural Roles in Monomeric and Oligomeric Frataxin—Frataxins across species share a typical α/β sandwich fold with two α helices packed against a five-strand β sheet (for review, see Ref. 32) (Fig. 1A). Residues Ile-154 and Trp-155 are highly conserved and are located on the five-strand β sheet, with Ile-154 oriented toward the hydrophobic core of the protein and Trp-155 exposed on the protein surface (Fig. 1A). It has been proposed that Trp-155 creates a binding site on the surface of the FXN81–210 monomer for the NFS1-ISD11-ISCU complex and that this site is disrupted directly by the W155R mutation and indirectly by the I154F mutation via secondary effects on Trp-155 and neighboring residues (26, 27). On the other hand, the crystal structure of the yeast frataxin trimer shows that this highly conserved residue (Trp-131 in the yeast protein) mediates direct contacts between the β sheet of one subunit and the N-terminal region of a neighboring subunit (33, 34) (Fig. 1B). We, therefore, hypothesized that the W155R and the I154F mutations might have as yet uncharacterized effects on oligomeric FXN42–210. Moreover, early studies had shown that missense mutations in other conserved residues within the β sheet altered processing of FXN42–210 to smaller isoforms (5, 35). We, therefore, postulated that the W155R and I154F mutations might also impair processing of FXN42–210 and thereby indirectly affect the biogenesis of FXN81–210.

In E. coli and in Vitro, the I154F Mutation Severely Affects the Stability of Monomeric and Oligomeric FXN42–210 but Has No Obvious Effects on the Stability of Monomeric FXN81–210—We initially analyzed the effects of the I154F mutation on the solubility of FXN42–210 versus FXN81–210 upon overexpression of each individual protein in E. coli at different temperatures. Bacterial cells were first grown at 37 °C, as this is the physiological temperature for human cells, i.e. the temperature at which FXN isoforms would need to be folded to be functional. At this temperature the presence of the I154F mutation severely compromised the solubility of FXN42–210 (Fig. 2, A and B) but had no obvious effect on the solubility of FXN81–210 (Fig. 2, C and D), as compared with the respective wild type proteins. Co-expression of FXN42–210 with the molecular chaperone GroEL and its co-chaperone GroES (36) could not improve the loss of solubility associated with the I154F mutation at 37 °C (data not shown). In the presence of I154F, the solubility of FXN42–210 remained significantly compromised even when E. coli cells were grown at 30 °C (Fig. 2, A and B). However, when the growth temperature was further lowered to 15 °C, the solubility of the mutant FXN42–210 was rescued to essentially wild type levels (Fig. 2, A and B). The solubility of WT-FXN42–210 was slightly reduced at 15 °C compared with 37 or 30 °C (Fig. 2B), possibly reflecting susceptibility to cold denaturation, which has been reported at temperature above 0 °C for yeast frataxin (37). Because the solubility of the mutant I154F-FXN42–210 protein at 15 °C was similar to that of the wild type protein (Fig. 2B), the I154F mutation did not appear to contribute to this particular effect.

As reported previously, purification of wild type FXN42–210 from E. coli yielded both monomeric FXN42–210 and oligomeric FXN42–210, whereas purification of wild type FXN81–210 yielded monomeric protein only (15). The same was true in the presence of the I154F mutation as long as overexpression was car-
**Synergistic Effects of Frataxin Point Mutations**

The stability of the mutant FXN<sup>81–210</sup> was consistent with a previous report in which the I154F mutation had not altered the fold of a monomeric FXN protein slightly shorter than FXN<sup>81–210</sup> (i.e. FXN<sup>91–210</sup>) (25).

We performed Far-UV circular dichroism spectroscopy (CD) and thermal denaturation analysis on the mutant FXN<sup>81–210</sup> and FXN<sup>42–210</sup> proteins and the corresponding wild type proteins. Because the I154F-FXN<sup>42–210</sup> protein was unstable at physiological conditions (i.e. pH 7.4 and 37 °C), we initially performed these studies at pH 8.9. We chose this pH because another group had reported previously that wild type FXN<sup>91–210</sup> exhibited considerable stability at this relatively high pH value (25). Pre-thermal Far-UV CD at 4 °C demonstrated that all four proteins were folded (supplemental Fig. S2, B, D, F, and H). Thermal denaturation from 4 to 90 °C showed that both wild type and mutant FXN<sup>81–210</sup> were considerably stable and that unfolding of both proteins was close to 100% reversible (supplemental Fig. S2, A and C). The melting temperature (T<sub>m</sub>) was ~68 °C for wild type FXN<sup>81–210</sup> and ~12 °C lower for I154F-FXN<sup>81–210</sup>, although still well above the physiological temperature of 37 °C, which was similar to the T<sub>m</sub> values reported previously for FXN<sup>91–210</sup> and I154F-FXN<sup>91–210</sup> (25). In contrast, wild type and mutant FXN<sup>42–210</sup> were less stable, and unfolding of both proteins was only minimally reversible (<10%) (supplemental Fig. S2, E and G). Furthermore, the T<sub>m</sub> was ~55 °C for WT-FXN<sup>42–210</sup> and ~12 °C lower for I154F-FXN<sup>42–210</sup>, corresponding to a T<sub>m</sub> of ~43 °C, close to the physiological temperature of 37 °C. We repeated this analysis with the WT-FXN<sup>42–210</sup> and I154F-FXN<sup>42–210</sup> proteins at pH 7.4. Pre-thermal Far-UV CD at 4 °C demonstrated that both proteins were folded (not shown). Thermal denaturation analysis showed a T<sub>m</sub> of ~56 °C for WT-FXN<sup>42–210</sup> and ~43 °C for I154F-FXN<sup>42–210</sup>, similar to the values obtained at pH 8.9, indicating that the thermal stability of these proteins was not significantly influenced within the pH range analyzed. The data together suggested that wild type FXN<sup>42–210</sup> was less stable than wild type FXN<sup>81–210</sup>; thus, even though the I154F mutation reduced the T<sub>m</sub> of both isoforms by the same extent (~12 °C), the reduction could be much more detrimental to the stability of the FXN<sup>42–210</sup> isoform at the physiological temperature of 37 °C.

We tested the ability of wild type and mutant FXN<sup>81–210</sup> to stimulate the cysteine desulferase activity of NFS1 in vitro. As reported previously by another group (27), the presence of the I154F mutation resulted in ~50% reduced ability to activate NFS1 as compared with wild type FXN<sup>81–210</sup> (Fig. 3A). The I154F mutation had an overall mild effect on the ability of FXN<sup>81–210</sup> to stimulate Fe-S cluster synthesis, although the initial reaction rate was slower for the mutant protein compared with wild type (Fig. 3B), as also reported previously (27). Monomeric and oligomeric FXN<sup>42–210</sup> were not stable at 37 °C and could not be reliably analyzed.

**I154F Causes Aggregation of FXN<sup>42–210</sup> in Yeast, Which Hinders Processing of FXN<sup>42–210</sup> to FXN<sup>81–210</sup>**—The I154F mutation had overall modest effects on the stability of monomeric FXN<sup>81–210</sup> (Ref. 25; Fig. 2, C and D) as well as its ability to

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**FIGURE 2.** I154F alters the solubility of FXN<sup>42–210</sup>, not FXN<sup>81–210</sup>, in E. coli. A and C, each of the indicated FXN isoforms was expressed in E. coli at the indicated temperatures. Upon lysis of bacterial cells at 4 °C, solubility was determined from the amounts of FXN isoform present in the total cell lysate (T) and the soluble fraction (S) after centrifugation of total cell lysate as described under “Experimental Procedures.” B and D, protein bands were quantified by densitometry. In each case, shown are the mean ± S.D. of two independent experiments; the asterisk denotes p ≤ 0.008 as determined by Student’s t test.

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ried out at 15 °C and protein purification at 4 °C (data not shown). This offered the opportunity to separately assess the effects of the I154F mutation on monomeric FXN<sup>42–210</sup> and oligomeric FXN<sup>42–210</sup>, which had not been possible in the experiment described above where monomers and oligomers had been analyzed together in whole E. coli cell lysate. Thus, wild type and mutant proteins were over-expressed at 15 °C, purified at 4 °C, and tested by shifting each protein from 4 °C to 30 or 37 °C for different periods of time (supplemental Fig. S1). In the presence of the I154F mutation, monomeric FXN<sup>42–210</sup> became progressively less soluble with increasing temperatures and incubation times. The stability of oligomeric FXN<sup>42–210</sup> was similarly affected as expected (data not shown). In addition, the I154F mutation compromised the solubility of oligomeric FXN<sup>82–210</sup> upon freezing at −80 °C followed by thawing at 4 °C (supplemental Fig. S1). Monomeric FXN<sup>81–210</sup> was otherwise stable under all conditions tested regardless of the presence of the I154F mutation (supplemental Fig. S1 and data not shown).
stimulate NFS1 activity and Fe-S cluster synthesis (Ref. 27 and Fig. 3, A and B). As noted previously by another group (25), this scenario did not correlate well with the severe phenotypes associated with the I154F mutation in experimental cell lines or FRDA patients (13, 38). On the other hand, the detrimental effect of the I154F mutation on FXN42–210 stability at 37 °C, which is the physiological temperature for mammalian cells, appeared potentially relevant to the pathogenic mechanism of this mutation. To investigate this possibility further, we created *Saccharomyces cerevisiae* yeast strains lacking the endogenous yeast frataxin homologue (via *yfh1Δ::HIS3* gene knock out) and expressing either the wild type (WT) or the mutant (I154F) FXN precursor (denoted *yfh1Δ[WT-FXN1–210]* or *yfh1Δ[I154F-FXN1–210]* strain, respectively). We showed previously that the mitochondrial import and processing of the human FXN1–210 precursor involves the same steps in yeast as in human cells and that FXN42–210 and FXN81–210 maintain their functional properties in yeast mitochondria (15). Therefore, because the yeast *S. cerevisiae* can grow robustly both at 30 and 37 °C, the *yfh1Δ[WT-FXN1–210]* and *yfh1Δ[I154F-FXN1–210]* strains were suitable to test the temperature-dependent effects of the I154F mutation *in vivo*. Under non-stressful conditions, *yfh1Δ[I154F-FXN1–210]* cells exhibited no obvious growth or survival deficits relative to *yfh1Δ[WT-FXN1–210]* cells at 30 or 37 °C, even on a non-fermentable carbon source that could only be utilized through mitochondrial oxidative phosphorylation (Fig. 4A). We examined the levels of FXN isoforms in mitochondria isolated from each of the two strains after growth at 30 or 37 °C. Regardless of the growth temperature, both WT-FXN81–210 and WT-FXN81–210 were readily detected, with WT-FXN81–210 representing the most abundant isoform (Fig. 4B, lanes 1 and 3). In contrast, I154F-FXN42–210 was consistently more abundant than I154F-FXN81–210 (Fig. 4B, lanes 2 and 4). In particular, after growth at 37 °C there was accumulation of I154F-FXN42–210 but barely detectable levels of I154F-FXN81–210 (Fig. 4B, lane 4). At either 30 or 37 °C, there was no accumulation of I154F-FXN1–210 precursor (Fig. 4B, lanes 2 and 4). Together these data indicated that cleavage of I154F-FXN1–210 to I154F-FXN42–210 occurred normally, whereas cleavage of I154F-FXN42–210 to I154F-FXN81–210 was progressively impaired in a temperature-dependent manner. The mature forms of two additional mitochondrial matrix proteins, Nfs1p (the yeast homologue of human NFS1) and Aco1p (yeast mitochondrial aconitase), were present at very similar levels in both strains regardless of the growth conditions (Fig. 4B), indicating that the processing defect was unique to the mutant I154F-FXN42–210.

Next, we isolated mitochondria from the two yeast strains analyzed above after growth at 30 or 37 °C and assessed the solubility of FXN isoforms in mitochondrial extracts as described previously (29). At 30 °C, both FXN42–210 and FXN81–210 were mostly detected in the soluble mitochondrial protein fraction regardless of the presence of I154F (Fig. 4C, lanes 1 and 2 and lanes 4 and 5). However, at 37 °C, I154F-FXN42–210 was once again accumulated, whereas I154F-FXN81–210 was barely detectable, and the accumulated I154F-FXN42–210 was mostly recovered in the insoluble fraction (Fig. 4C, lanes 7–9 versus 10–12). Thus, it appeared that the insolubility of I154F-FXN42–210 was responsible for its impaired processing to I154F-FXN81–210. The mature form of Nfs1p was largely soluble under all conditions tested except that some of this protein was recovered in the insoluble fraction of *yfh1Δ[I154F-FXN1–210]* mitochondria at 37 °C (Fig. 4C, lane 6 versus 12), suggesting that sequestration of Nfs1p with insoluble I154F-FXN42–210 might be an indirect effect of the I154F mutation in yeast mitochondria. Mitochondria isolated after growth at 37 °C were also treated with Na₂CO₃ or Triton X-100, which normally solubilizes proteins that are peripherally or integrally associated with mitochondrial membranes, respectively. Under both conditions, the mutant
I154F-FXN\textsubscript{42–210} protein was again recovered almost exclusively in the insoluble fraction, and only when mitochondria were treated with SDS, could soluble protein be observed (data not shown). These results confirmed that I154F-FXN\textsubscript{42–210} was largely aggregated within mitochondria, which prevented us from establishing its membrane topology.

### Aggregation of FXN\textsubscript{42–210} Impairs Yeast Ability to Detoxify Mitochondrial Iron

—The absence of obvious growth defects in yfh1\textsubscript{Δ}[I154F-FXN\textsubscript{41–210}] relative to yfh1\textsubscript{Δ}[WT-FXN\textsubscript{1–210}] yeast at 37 °C (Fig. 4A) suggested that under non-stressful conditions low levels of I154F-FXN\textsubscript{81–210} were sufficient to support mitochondrial function, at least at a rate not limiting for yeast growth. This interpretation was consistent with previous studies in which unremarkable growth phenotypes had been observed in yeast strains with very low levels of yeast frataxin under non-stressful conditions (39, 40). We, therefore, challenged our strains with growth conditions known to lead to a rapid increase in mitochondrial iron uptake, which is an established approach to assess yeast oxidative stress tolerance (29).

Cultures of the two strains were synchronized at 30 °C in minimal medium, diluted into fresh minimal medium with or without iron supplementation, and shifted to 37 °C for 24 h. Under these conditions, in the absence of iron supplementation, mitochondrial iron remains stationary at low nanomolar concentrations; however, if the culture medium is supplemented with iron, mitochondrial iron rapidly reaches micromolar concentrations (29). The two strains showed similar cell viability under all conditions tested (Fig. 4D, solid bars); however, with iron supplementation, the yfh1\textsubscript{Δ}[I154F-FXN\textsubscript{210–210}] strain accumulated a much higher portion of respiration-deficient cells (Fig. 4D; red bars). Similarly, expression of I154F-FXN\textsubscript{1–210} in mouse fibroblasts lacking endogenous mouse frataxin had been associated with reduced Fe-S enzyme activities, mitochondrial iron accumulation, and increased sensitivity to oxidative stress (38). Moreover, a more recent study had shown that in the absence of frataxin mouse heart mitochondria contained aggregates of iron, phosphorus, and sulfur (41). These data together suggested that the I154F mutation compromised the ability to detoxify mitochondrial iron because aggregation of monomeric FXN\textsubscript{42–210} impaired both its assembly into oligomeric FXN\textsubscript{42–210} and its cleavage to FXN\textsubscript{81–210} and its cleavage to FXN\textsubscript{81–210}. A previous study had shown that FXN\textsubscript{56–210} may serve as an alternative processing intermediate for the formation of FXN\textsubscript{81–210} (7); however, in our yeast strains the levels of FXN\textsubscript{56–210} remained undetectable under all conditions tested (Fig. 4, B and C), indicating that this potentially compensatory mechanism was not operational in our yeast model.

### Aggregation of FXN\textsubscript{42–210} and Lack of Processing to FXN\textsubscript{81–210} Occurs in Cultured Mammalian Cells at 37 °C

—In yeast, the instability of monomorphic I154F-FXN\textsubscript{42–210} at 37 °C appeared to be a critical direct effect of the I154F mutation. We, therefore, asked if this mutation would cause a similar effect in mammalian cells. We made COS-7 cells transiently overexpressing wild type or mutant FXN\textsubscript{1–210} precursor as described by other groups (6, 7). In COS-7 cells overexpressing wild type precursor, WT-FXN\textsubscript{81–210} was the most abundant isoform and was largely detected in the soluble fraction along with less abundant

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**FIGURE 4.** I154F alters FXN\textsubscript{42–210} solubility in \textit{S. cerevisiae}. The wild type (WT) or mutant (I154F) FXN\textsubscript{1–210} precursors were expressed in yfh1\textsubscript{Δ} yeast lacking endogenous yeast frataxin to give the yfh1\textsubscript{Δ}[WT-FXN] and yfh1\textsubscript{Δ}[I154F-FXN] strains. A, growth phenotype of the indicated strains at different temperatures on rich medium supplemented with a fermentable (YP-Dextrose) or non-fermentable (YP-Ethanol) carbon source. Liquid cultures were started from freshly streaked frozen glycerol stocks, synchronized to late logarithmic phase in minimal medium supplemented with dextrose at 30 °C, diluted with fresh medium to 100,000 cells/ml, and shifted to 37 °C for another 24 h. Aliquots of the cultures were treated with SDS, and soluble protein was observed. B, mitochondrial import and/or processing is impaired (7) as seen in Fig. 6 from the fact that this is the form of the protein that accumulates in cells when mitochondria were started from freshly streaked frozen glycerol stocks, synchronized to late logarithmic phase in minimal medium supplemented with dextrose at 30 °C for 12 h, diluted into fresh medium in the absence or presence of 100 μM FeCl\textsubscript{3}, and shifted to 37 °C for another 24 h. Aliquots of the cultures were then plated on YP-Dextrose or YP-Glycerol plates, and colonies were counted after 5 days at 30 °C. C, total (T), soluble (S), and insoluble (P) mitochondrial fractions were prepared from isolated mitochondria and analyzed by Western blotting. D, yeast cultures were started from freshly streaked frozen glycerol stocks, synchronized to late logarithmic phase in minimal medium supplemented with dextrose at 30 °C for 12 h, diluted into fresh medium in the absence or presence of 100 μM FeCl\textsubscript{3}, and shifted to 37 °C for another 24 h. Aliquots of the cultures were then plated on YP-Dextrose or YP-Glycerol plates, and colonies were counted after 5 days at 30 °C. The black and gray columns show the total number of viable cells, and the red columns show the total number of non-respiring colonies in each culture (expressed per A\textsubscript{600} = 1). Shown are the means ± S.D. of three cultures; the asterisk denotes p = 0.002 as determined by Student’s t-test.
isoforms including WT-FXN92–210 (Fig. 5, lane 2 versus 5). In striking contrast, in COS-7 cells overexpressing the mutant precursor, I154F-FXN92–210 was the most abundant isoform and was largely recovered in the insoluble fraction; moreover, only very low levels of I154F-FXN91–210 were detected in these cells (Fig. 5, lane 3 versus 6). Therefore, the I154F mutation compromised FXN92–210 stability and its conversion to FXN91–210 in mammalian cells as observed in yeast. Low levels of FXN96–210 were produced from the wild type but not the mutant precursor (Fig. 5, lanes 2 and 5 versus lanes 3 and 6), once again indicating that production of FXN96–210 was not induced in response to FXN92–210 aggregation.

The W155R Mutation Does Not Affect the Stability of FXN92–210 or FXN91–210 in E. coli and in Vitro—Unlike the I154F mutation, the W155R mutation did not have any obvious effects on the solubility of FXN92–210 versus FXN91–210 in whole bacterial cell lysate upon overexpression of each individual protein in E. coli at 37 °C (supplemental Fig. S3). However, the presence of W155R led to extensive degradation of FXN92–210 during our standard protein purification procedure, such that only limited amounts of this protein could be obtained in purified form. The addition of a six-histidine tag to the N terminus of W155R-FXN92–210 enabled faster purification by affinity chromatography, which limited protein degradation. In a previous study monomeric FXN91–210 was shown to contain partially destabilized regions that made this protein more susceptible to proteolytic degradation in vitro (25). Together, these data suggested that the W155R mutation destabilized FXN92–210 and FXN91–210 to different extents and that FXN92–210 was more susceptible to degradation during purification from E. coli. However, in purified form, monomeric or oligomeric W155R-FXN92–210 showed no increased tendency toward degradation and was as soluble as the corresponding WT proteins (data not shown).

In yfh1Δ Yeast the W155R Mutation Blocks Mitochondrial Import of the Human FXN92–210 Precursor—To investigate potential effects of the W155R mutation on the biogenesis of FXN isoforms, we created yeast strains lacking the endogenous yeast frataxin to give the indicated strains. A, total protein extracts were prepared from the four strains by rapid trichloroacetic acid treatment, and FXN isoforms and other proteins were analyzed by Western blotting. B, growth phenotype of the indicated strains on rich medium supplemented with a fermentable (YP-Dextrose) or non-fermentable (YP-Ethanol) carbon source at 30 °C is shown. Experimental procedures were as described in the legend of Fig. 4B. C, lymphoblastoid cells from one control (designated Control; GM07521, female, 19 years old, normal GAA repeats) and one FRDA patient (designated FRDA; GM16197, male, 14 years old, 760 and 830 GAA repeats) were analyzed by Western blotting before or 48 h after transfection with pcDNA4 vectors containing the W155R-FXN91–210 or W155R-FXN92–210 precursor. Detergent-soluble extracts were prepared as described under “Experimental Procedures”; 20 μg of total protein was analyzed in lanes 1 and 2 and lanes 5 show 6 and 5 and 10 μg in lanes 3 and 4, respectively. Virtually identical results were obtained in two independent experiments, one of which is shown. NT, non-transfected.

In yfh1Δ Yeast the W155R Mutation Blocks Mitochondrial Import of the Human FXN92–210 Precursor—To investigate potential effects of the W155R mutation on the biogenesis of FXN isoforms, we created yeast strains lacking the endogenous yeast frataxin homologue (via yfh1Δ; HIS3 gene knock-out) and was largely recovered in the insoluble fraction; moreover, only very low levels of I154F-FXN91–210 were detected in these cells (Fig. 5, lane 3 versus 6). Therefore, the I154F mutation compromised FXN92–210 stability and its conversion to FXN91–210 in mammalian cells as observed in yeast. Low levels of FXN96–210 were produced from the wild type but not the mutant precursor (Fig. 5, lanes 2 and 5 versus lanes 3 and 6), once again indicating that production of FXN96–210 was not induced in response to FXN92–210 aggregation.

The W155R Mutation Does Not Affect the Stability of FXN92–210 or FXN91–210 in E. coli and in Vitro—Unlike the I154F mutation, the W155R mutation did not have any obvious effects on the solubility of FXN92–210 versus FXN91–210 in whole bacterial cell lysate upon overexpression of each individual protein in E. coli at 37 °C (supplemental Fig. S3). However, the presence of W155R led to extensive degradation of FXN92–210 during our standard protein purification procedure, such that only limited amounts of this protein could be obtained in purified form. The addition of a six-histidine tag to the N terminus of W155R-FXN92–210 enabled faster purification by affinity chromatography, which limited protein degradation. In a previous study monomeric FXN91–210 was shown to contain partially destabilized regions that made this protein more susceptible to proteolytic degradation in vitro (25). Together, these data suggested that the W155R mutation destabilized FXN92–210 and FXN91–210 to different extents and that FXN92–210 was more susceptible to degradation during purification from E. coli. However, in purified form, monomeric or oligomeric W155R-FXN92–210 showed no increased tendency toward degradation and was as soluble as the corresponding WT proteins (data not shown).
Synergistic Effects of Frataxin Point Mutations

The yfh1Δ[W155R-FXN^{1–210}] strain, the levels of Isu1p (the yeast homologue of human ISCU) were increased, and the levels of Ac01p (yeast aconitase, a [4Fe-4S] enzyme that becomes inactive and unstable in the presence of defects in Fe-S cluster synthesis) were undetectable, features that are typical of frataxin-depleted yeast (42, 43) (Fig. 6A, lanes 4 versus 3). Accordingly, the yfh1Δ[W155R-FXN] strain was unable to grow on non-fermentable carbon sources (Fig. 6B, YP-Ethanol). Thus, the yfh1Δ phenotype led to lack of FXN isoforms and a phenotype virtually indistinguishable from the yfh1Δ phenotype (4).

We repeated this analysis in the presence of endogenous yeast frataxin (strains denoted YFH1[WT-FXN^{1–210}] and YFH1[W155R-FXN^{1–210}]). Under these conditions the levels of FXN^{1–210} precursor protein and its processing to shorter isoforms were essentially identical in the absence or presence of the W155R mutation (Fig. 6A, lanes 1 and 2). These results suggested that the W155R mutation had a negative effect on the mitochondrial import and processing of FXN^{1–210} precursor, which became evident only in the absence of endogenous wild type frataxin.

In FRDA Lymphoblasts, Residual Levels of Wild Type FXN Are Sufficient to Maintain Normal Import and Processing of W155R-FXN^{1–210} Precursor—Friedreich ataxia patients compound heterozygotes for a GAA expansion and a W155R mutation express residual levels of wild type FXN^{1–210} from the expanded allele (13). As cell lines from these patients were not available to us, we expressed the W155R-FXN^{1–210} precursor (with and without a C-terminal FLAG tag) in lymphoblastoid cells from a patient homozygous for GAA expansions and a normal control. Before transfection, patient cells contained significantly lower levels of FXN^{42–210} and FXN^{81–210} compared with the control as reported previously (15) (Fig. 6C, lane 1 versus 2). Upon transfection with the untagged W155R-FXN^{1–210} precursor, the total levels of FXN^{81–210} increased severalfold in both patient and control cells (Fig. 6C, lane 5 versus 6). In addition, upon transfection with the W155R-FXN^{1–210} FLAG precursor, it was possible to distinguish the endogenous wild type FXN^{81–210} protein from the exogenous W155R-FXN^{1–210} FLAG protein, which demonstrated significant production of the latter protein in both cell lines (Fig. 6C, lane 2 versus 3). These results indicated that the W155R mutation did not impair import and processing of FXN^{1–210} precursor in the presence of normal or even very low levels of wild type frataxin.

W155R Blocks Interactions of FXN^{81–210} with NFS1 but Does Not Affect FXN^{42–210} Interactions—In a previous study, Trp-155 was shown to be required for the interaction of FXN^{81–210} with the NFS1-ISD11-ISCU complex (27). Here, we investigated potential effects of the W155R mutation on FXN^{42–210} oligomerization and the ability to form stable complexes with NFS1 (15). Total cell extracts were prepared from COS-7 cells overexpressing the normal, WT-FXN^{1–210}, or mutant, W155R-FXN^{1–210} FLAG precursor. Each extract was fractionated by size exclusion chromatography + Western blotting as described under "Experimental Procedures." Aliquots of each cell extract (Input) and the total protein bound to Ni-NTA-agarose beads as described under "Experimental Procedures." Aliquots of each cell extract (Input) and the total protein bound to Ni-NTA-agarose beads as described under "Experimental Procedures."
Synergistic Effects of Frataxin Point Mutations

exclusion chromatography, and fractions were analyzed by Western blotting, a procedure developed previously for the analysis of endogenous frataxin in human lymphoblastoid cells (15). Essentially identical protein distributions were observed in the absence or presence of the W155R mutation, and therefore, only the data for the mutant FXN protein are shown in Fig. 7A. The fractionation revealed a low molecular weight pool and a high molecular weight pool of W155R-FXN⁴²–⁵⁰ and a low molecular weight pool for W155R-FXN⁸¹–¹⁰ (Fig. 7A). The bulk of endogenous NFS1 was eluted in one high molecular weight pool together with the high molecular weight pool of W155R-FXN⁴²–⁵⁰ (Fig. 7A). ISCU was eluted in two pools, a high and a low molecular weight pool, with a distribution closely paralleling that of W155R-FXN⁴²–⁵⁰ (Fig. 7A). The elution profile of W155R-FXN⁸¹–¹⁰ did not match the elution profiles of NFS1 or ISCU (Fig. 7A). These data were consistent with the distributions we had observed previously with endogenous FXN, NFS1, and ISCU proteins in normal human lymphoblastoid cells (15).

Binding of wild type and mutant FXN isoforms to NFS1 was analyzed in whole extracts from COS-7 cells overexpressing the WT-FXN⁴¹–²¹⁰ or W155R-FXN⁴¹–²¹⁰ precursor by use of pull-down assays with a six-histidine-tagged version of the NFS1-ISD11 complex (denoted HisNFS1-ISD11) (15). Equivalent amounts of WT- and W155R-FXN⁴²–⁵⁰ proteins were present in the respective cell extracts (Fig. 7B, lane 1 versus lane 2), and equivalent amounts of WT- and W155R-FXN⁴²–⁵⁰ were pulled down with HisNFS1-ISD11 (Fig. 7B, lane 4 versus lane 6). In contrast, although equivalent amounts of WT-FXN⁸¹–¹⁰ and W155R-FXN⁸¹–¹⁰ proteins were present in the respective cell extract (Fig. 7B, lane 1 versus lane 2), only WT-FXN⁸¹–¹⁰ was pulled down in discrete amounts with HisNFS1-ISD11, whereas W155R-FXN⁸¹–¹⁰ was barely detected in the pulled down fraction (Fig. 7B, lane 4 versus lane 6). Endogenous ISCU was efficiently pulled down with HisNFS1-ISD11 in both cases (Fig. 7B, lanes 4 and 6), an important control as FXN⁸¹–¹⁰ is known to interact with NFS1-ISD11 only in the presence of ISCU (15, 16, 19). In additional independent experiments, only trace amounts of W155R-FXN⁴²–⁵⁰ were consistently detected in the pulled down fraction; on the other hand, W155R-FXN⁴²–⁵⁰ was consistently pulled down with HisNFS1-ISD11, although the amounts varied somewhat relative to WT-FXN⁴²–⁵⁰. We also carried out a reciprocal experiment using purified recombinant oligomeric WT-FXN⁴²–⁵⁰ or W155R-FXN⁴²–⁵⁰ (each with an N-terminal six-histidine tag) to pull down endogenous NFS1 from non-transfected COS-7 cell extracts. Both the WT- and W155R-HISFXN⁴²–⁵⁰ oligomers were able to form a stable interaction with endogenous NFS1 (Fig. 7C, lanes 3 and 4). In this case there was no detectable interaction with ISCU (Fig. 7C), perhaps because the N-terminal tag on HisFXN⁴²–⁵⁰ prevented a stable complex with ISCU from being formed. The absence of ISCU from the pulled-down complexes was not surprising because we had shown previously that oligomeric FXN⁴²–⁵⁰ can form stable contacts with NFS1-ISD11 in the absence of ISCU (15).

The W155R Mutation Affects the Ability of Both FXN⁴²–⁵⁰ and FXN⁸¹–¹⁰ to Promote Fe-S Cluster Synthesis—We tested the ability of wild type and mutant FXN isoforms to stimulate the cysteine desulfurase activity of NFS1 and to catalyze Fe-S cluster synthesis in vitro. Cysteine desulfurase activity (A) and Fe-S cluster synthesis (B) were measured as described in the legend of Fig. 3 with the indicated proteins. In A, each bar shows the mean ± S.D. of six independent measurements with at least two different FXN protein preparations. The *asterisk denotes p = 3.4 × 10⁻⁷ as determined by Student’s t test. In B, each plot shows the mean ± S.D. of two independent measurements with two different protein preparations except for W155R-FXN⁴²–⁵⁰, which was analyzed only one time, as the amounts of this protein were very limited due to its susceptibility to proteolytic degradation during purification from E. coli.

FIGURE 8. W155R has dramatic effects on the ability of FXN⁸¹–¹⁰ and FXN⁴²–⁵⁰ to activate NFS1 and catalyze Fe-S cluster synthesis in vitro. Cysteine desulfurase activity (A) and Fe-S cluster synthesis (B) were measured as described in the legend of Fig. 3 with the indicated proteins. In A, each plot shows the mean ± S.D. of six independent measurements with at least two different FXN protein preparations. The *asterisk denotes p = 3.4 × 10⁻⁷ as determined by Student’s t test. In B, each plot shows the mean ± S.D. of two independent measurements with two different protein preparations except for W155R-FXN⁴²–⁵⁰, which was analyzed only one time, as the amounts of this protein were very limited due to its susceptibility to proteolytic degradation during purification from E. coli.

DISCUSSION

The major product of the FRDA gene is the frataxin precursor polypeptide, a protein of 210 amino acids that is initially translated in the cytoplasm and subsequently transported to
the mitochondrial matrix where it is processed to at least four shorter products (5–7, 10). These products have different biochemical properties and are thought to represent functionally different isoforms of human frataxin (15). In most Friedreich ataxia patients, large intronic GAA-repeat expansions silence FRDA expression (11, 12) resulting in depletion of all mitochondrial FXN isoforms (15). In some patients there is only one expanded FRDA allele, whereas a single missense mutation is present on the other allele. As individuals carrying one GAA expansion were phenotypically normal, whereas compound heterozygotes for one GAA expansion and one missense mutation were affected with Friedreich ataxia, it was established early on that single missense mutations in FRDA could have severe clinical consequences (10); however, the underlying mechanisms have thus far remained elusive. Two of the most aggressive missense mutations, I154F and W155R, were previously characterized in the context of the FXN81–210 isoform or a shorter variant, FXN91–210 (19, 25–27). Here, we have compared the effects of the I154F and W155R mutations on the two most abundant mitochondrial FXN isoforms, FXN42–210 and FXN81–210. We have found that each mutation has specific primary and secondary effects on each isoform. Our findings imply that I154F and W155R and probably other FRDA missense mutations may have complex biochemical outcomes that synergistically influence the pathophysiology and clinical severity of Friedreich ataxia.

A previous study demonstrated that the I154F mutation resulted in partial destabilization of FXN91–210 during chemical or thermal denaturation but did not affect the fold of the protein at physiological temperature and pH (25). In agreement with this study, we have shown here that the I154F mutation does not alter the solubility of FXN81–210 under physiological conditions in E. coli, S. cerevisiae, COS-7 cells, or in cell-free systems. However, under the same physiological conditions, the I154F mutation severely compromises the solubility of FXN42–210. In both S. cerevisiae and COS-7 cells, destabilization of FXN42–1210 at 37 °C leads to aggregation of this protein, which in turn blocks its proteolytic processing to FXN81–210. The modest effects of the I154F mutation on monomeric FXN81–210 stability and function (Refs. 25 and 27 and this study) does not explain the enhanced susceptibility to iron toxicity that we and others have observed in yeast and murine cells expressing the I154F-FXN42–210 precursor (38) or the aggressive clinical course of compound heterozygotes with the I154F mutation (13). On the other hand, it is likely that the detrimental effect of I154F on the stability of FXN42–210 at 37 °C is the primary pathogenic event ensuing from this particular mutation. Thus, in compound heterozygotes, the association of one GAA expansion with a I154F mutation results in a state of frataxin deficiency via a combination of FRDA gene silencing and abnormal protein biogenesis. This may explain why these patients have a clinical course as severe as those suffered by patients homozygous for a GAA expansion on both FRDA alleles (13, 14).

A previous study demonstrated that the W155R mutation resulted in partial destabilization of FXN91–210 during chemical or thermal denaturation (25). Our data suggest that the W155R mutation destabilizes FXN42–210 to a greater extent as indicated by the fact that W155R-FXN42–210 is susceptible to degradation during purification from E. coli, whereas W155-FXN81–210 is obviously not obviously altered. However, in purified form, monomeric W155R-FXN42–210 shows no increased tendency toward degradation. Moreover, it is as soluble and able to oligomerize as WT-FXN42–210 in yeast and mammalian cells. Last, oligomeric W155R-FXN42–210 forms stable complexes with NFS1 in the presence or absence of ISCU, like the wild type protein. These and previous results (27) indicate that the W155R mutation does not affect the core fold of W155R-FXN42–210 as reported for W155R-FXN91–210 (25).

Interestingly, in S. cerevisiae depleted of endogenous yeast frataxin, the W155R mutation nearly completely blocks mitochondrial import of FXN42–210 precursor, causing FXN42–210 and FXN81–210 depletion and a phenotype indistinguishable from the phenotype of yeast frataxin knock-out mutants. This precursor-specific effect of the W155R mutation may be explained by the fact that mitochondrial precursor proteins must be in an unfolded conformation to be translocated across the two mitochondrial membranes (44). Therefore, it is possible that the partially destabilizing effect of the W155R mutation (as observed for FXN91–210 in vitro (25) and for FXN42–210 in E. coli (this study)) is enhanced in the context of the unfolded FXN42–210 precursor in yeast. Notably, this effect is evident in yeast only in the complete absence of endogenous yeast frataxin, and it is not evident in patient cells with only residual levels of wild type frataxin. This indicates that pre-existing wild type frataxin in the mitochondrial matrix can help to overcome the import defect associated with the W155R mutation, possibly by maintaining mitochondrial ATP production at a rate sufficient to keep the mutant precursor in an import-competent state (44). Thus, the loss of mitochondrial import we have observed in yfh1Δ yeast for the W155R-FXN42–210 precursor is unlikely to play a significant role in compound heterozygotes with the W155R mutation, as these patients produce low levels of functionally normal frataxin from their expanded FRDA allele. However, the loss of mitochondrial import most likely explains why murine cells expressing the W155R-FXN42–210 precursor lost viability upon deletion of the endogenous mouse frataxin gene (19).

As observed by others in a cell-free system (27), we have found that W155R abolishes binding of FXN81–210 to NFS1-ISD11 in total cell extract. Accordingly, in the presence of W155R, there is no appreciable activation of NFS1 by FXN81–210 and no appreciable Fe-S cluster synthesis (Ref. 27 and our study). Surprisingly, we have shown that the W155R mutation impairs the activity of oligomeric W155R-FXN42–210 as well even though this protein appears to bind normally to NFS1-ISD11-ISCU in pulldown assays. This result suggests that in the context of oligomeric FXN42–210, Trp-155 may play a role that is different from, or not limited to providing a binding site for its protein partners. Thus, the W155R mutation has different detrimental effects on FXN81–210 and FXN42–210. Consequently, the association of a GAA expansion with a W155R mutation results in a state of frataxin deficiency via a combination of reduced protein expression and a globally compromised ability to synthesize Fe-S clusters. This is consistent with the aggressive clinical course of compound heterozygous
patients with the W155R mutation, very similar to the clinical courses presented by patients homozygous for very large GAA expansions (13, 14).

We have not addressed the potential effects of I154F and W155R on the other less abundant mitochondrial FXN isoforms, FXN56–210 and FXN78–210 (7, 15), or two recently identified extra-mitochondrial isoforms, FXN II and FXN III (22). However, there is a high degree of amino acid sequence identity among FXN78–210 or FXN II and FXN81–210 and among FXN56–210 or FXN III and FXN42–210. Therefore, it is reasonable to predict that each mutation will have a spectrum of detrimental effects on all of these isoforms, similar to those we have observed for FXN81–210 and FXN42–210.

Our results have implications for Friedreich ataxia therapies. Although interventions that increase frataxin levels are suitable to reduce the gene silencing effect of a GAA expansion (11), compounds that specifically target stability, biogenesis, or catalytic function of FXN isoforms will be required to overcome the negative effects of I154F and W155R and probably other missense mutations as well. In general, our work emphasizes the potentially complex consequences of mutations in a multifunctional protein-frataxin being an excellent example among a growing number of such proteins (23). Understanding the intricate pathophysiology of defects in multifunctional proteins is relevant to our ability to make valid genotype-phenotype correlations and to develop effective treatments.

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