Erythroid differentiation and protoporphyrin IX down-regulate frataxin expression in Friend cells: characterization of frataxin expression compared to molecules involved in iron metabolism and hemoglobinization

Erika M. Becker, Judith M. Greer, Prem Ponka, and Des R. Richardson

Friedreich ataxia (FA) is an inherited neurodegenerative condition1-2 with an incidence of 1:30,000 in the European population.3 The gene FRDA that is defective in FA encodes a mitochondrial protein known as frataxin.4,5 In 97% of individuals with FA the defect in this gene is due to a GAA triplet repeat expansion in intron 1 (chromosome 9q13) that results in a marked decrease in gene expression.4,6 Over the last few years evidence has accumulated to suggest that frataxin plays a role in mitochondrial iron (Fe) metabolism.5,24 However, the function of mammalian frataxin remains unclear.24

Experiments using the yeast, Saccharomyces cerevisiae, led to a model that may explain the pathogenesis of FA.10,12 The yeast gene YFH1 is homologous to the human gene FRDA,12 and encodes a mitochondrial protein (Yfh1p) involved in Fe homeostasis and respiration.5,9,10,12 When the YFH1 gene was deleted, a marked accumulation of mitochondrial Fe occurred, resulting in loss of mitochondrial DNA and impaired respiration.5,6,7,12,13 Thus, this led to a hypothesis that the accumulated Fe resulted in the production of free radicals that damage biologic targets. When YFH1 was reintroduced into the yeast, mitochondrial Fe was exported back out into the cytosol as free non–heme-bound Fe.10,12 Therefore, Yfh1p appeared to regulate Fe export from mitochondria in yeast cells.10 However, Yfh1p may not be an Fe transporter, because it has no transmembrane sequences necessary for this role.10

Both the human and mouse frataxin genes (FRDA and Frda, respectively), are highly expressed in tissues that are rich in mitochondria, including heart, liver, and skeletal muscle.5,14 Consistent with the yeast knockout model, fibroblasts from FA patients were more sensitive than normal cells to oxidant stress,24 and contained higher levels of mitochondrial Fe.16,21 Further, Fe accumulation and reductions of mitochondrial DNA, complex I/II/III, and aconitate have been noted in the heart11 and central nervous system of patients with FA.5,23 Other studies in FA patients showing Fe deposits within heart myofibrils18,19 and defective mitochondrial respiration7,4 also suggest mitochondrial Fe overload. Considering these observations, it is significant that antioxidants have successfully treated the cardiomyopathy of FA.25

If frataxin plays a role in Fe metabolism, an understanding of the pathogenesis of FA will require the examination of mitochondrial Fe processing pathways. The best characterized function of mammalian mitochondria in Fe metabolism comes from studies in erythroid cells, where it is involved in the production of heme for...
hemoglobin synthesis.\textsuperscript{24,26} In these cells, the Fe-transport protein transferrin (Tf) binds to the transferrin receptor (TfR) and is endocytosed. Iron is released from Tf by a decrease in endosomal pH and traverses the membrane via the transporter, Nramp2 (also known as the divalent metal ion transporter 1).\textsuperscript{24} The Fe is then targeted to the mitochondrion where it is incorporated into protoporphyrin IX (PIX) to form heme (for reviews, see Becker and Richardson\textsuperscript{24} and Ponka\textsuperscript{26}). This molecule is then exported out of the mitochondrion to combine with globin chains to form hemoglobin. The rate-limiting step in the heme synthesis pathway is the acquisition of Fe from Tf.\textsuperscript{26} When the heme biosynthesis pathway is inhibited using isonicotinic acid hydrazide (an inhibitor of δ-aminolevulinic acid synthase)\textsuperscript{28} or succinylacetone (SA; a competitive inhibitor of δ-aminolevulinic acid dehydratase),\textsuperscript{29} Fe accumulates within the mitochondrion and to some degree the cytoplasm.\textsuperscript{26,27,30-33}

Only when PIX is added does Fe become incorporated into this molecule to form heme that is transported out of the mitochondrion.\textsuperscript{10,31}

The role of frataxin in Fe and heme metabolism remains unknown. It is possible to suggest a hypothesis that the decreased levels of frataxin in patients with FA may lead to some defect in either the process of Fe incorporation into PIX, or Fe influx or export, which results in mitochondrial Fe accumulation. Recently, it has been suggested that the yeast frataxin homologue (Yfh1p) may polymerize to store Fe-like ferritin.\textsuperscript{35} Another study suggested that an anionic surface patch on frataxin could oxidize Fe\textsuperscript{++} to Fe\textsuperscript{3+} when this molecule combines with itself or another ligand.\textsuperscript{35} In this way, frataxin may accumulate Fe similarly to ferritin.\textsuperscript{35} However, structural and functional studies of mammalian frataxin have shown that this molecule cannot bind Fe directly and does not polymerize to sequester Fe.\textsuperscript{36} Indeed, frataxin shares little homology with other known molecules involved in Fe or heme metabolism.\textsuperscript{36} The clustering of conserved residues onto one face of frataxin suggests an interaction with potential ligands and may indicate that frataxin acts indirectly on Fe metabolism.\textsuperscript{36}

In the present study we have assessed the role of frataxin in Fe and heme metabolism by implementing a well-characterized model of erythroid differentiation, namely, Friend cells induced with dimethyl sulfoxide (DMSO).\textsuperscript{37-41} This model has previously been used to assess the role of other molecules involved in Fe and heme metabolism.\textsuperscript{37-41} We demonstrate that DMSO induction of erythroid differentiation and hemoglobinization results in a marked decrease in frataxin expression. In addition, incubation of uninduced Friend cells and other cell types with PIX resulted in down-regulation of frataxin protein levels, suggesting a possible regulatory role of frataxin in Fe and heme metabolism. Because decreased frataxin expression leads to mitochondrial Fe loading in FA, our data suggest the decrease in frataxin levels during erythroid differentiation favors mitochondrial Fe sequestration and heme synthesis. These results may explain why there are no marked effects of decreased frataxin expression in the erythroid cells of FA patients; that is, decreased frataxin expression may physiologically facilitate Fe uptake and heme synthesis.

Materials and methods

Cell treatments and reagents

Hemin, phenylmethylsulfonylfluoride (PMSF), PIX, and SA were obtained from Sigma Chemical (St Louis, MO). All experiments with PIX and heme were performed in tubes wrapped in aluminium foil to prevent the photodynamic generation of free radicals. Desferrioxamine (DFO) was obtained from Novartis (Basel, Switzerland). PIH was synthesized as described previously.\textsuperscript{42}

Preparation of \textsuperscript{59}Fe-transferrin

Aprotiferrin (Sigma Chemical) was labeled with \textsuperscript{59}Fe (Dupont NEN, MA) to produce \textsuperscript{59}Fe\textsubscript{2}-transferrin (\textsuperscript{59}Fe-Tf).\textsuperscript{43}

Cell culture

The mouse Friend erythroleukemia cell lines 707 and 745 as well as the human SK-N-MC neuroepithelioma cell line were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Friend cells were induced to differentiate into hemoglobin-producing cells by incubation with 1.5% DMSO.\textsuperscript{24,26}

The mouse fibroblast cell line, LMTK\textsuperscript{+}, and the mouse HepA1-6 hepatoma cell line were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, United Kingdom). The mouse M15 kidney cell line was obtained from Dr Veronica van Heyningen (Medical Research Council of Edinburgh, Edinburgh, United Kingdom). All cell lines above were grown in Eagle modified minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia), 1% (vol/vol) nonessential amino acids (Gibco BRL, Sydney, Australia), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin (Gibco), and 0.28 μg/mL Fungizone (amphotericin B; Squibb Pharmaceuticals, Montreal, Quebec, Canada). The RAW264.7 and J774 mouse macrophage cell lines were obtained from Dr David Cestor (Centre for Molecular and Cellular Biology, University of Queensland) and the ATCC, respectively. Both RAW 264.7 and J774 cells were grown in RPMI containing 10% Serum Supreme (Biowhittaker, Walkersville, MD), 1% (vol/vol) nonessential amino acids (Gibco BRL), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine, 100 μg/mL streptomycin (Gibco), 100 U/mL penicillin (Gibco), and 0.28 μg/mL Fungizone (Squibb Pharmaceuticals).

Assays to measure Fe uptake and Fe incorporation into heme

Friend cells were incubated in the presence or absence of DMSO for 24 to 120 hours. After this, aliquots of cells (1 \times 10\textsuperscript{7}) were placed in tubes and incubated with \textsuperscript{59}Fe-Tf (10 μM) for 1 to 120 minutes at 37°C.\textsuperscript{37} After appropriate incubation times the tubes were plunged into ice and the cells washed 3 times in a large excess of ice-cold phosphate-buffered saline.\textsuperscript{37} Incorporation of \textsuperscript{59}Fe into heme was performed by standard procedures.\textsuperscript{27} Radioactivity was measured using a γ-scintillation counter (LKB Wallace 1282 Compu gamma, Turku, Finland).

Northern blot analysis

Northern blot analysis was performed as described previously.\textsuperscript{44} The Frda probe consisted of an 870-base pair (bp) fragment from the coding region of murine Frda cloned into the pT7T3pac plasmid (from Dr R. Williamson, Murdoch Institute, Melbourne, Australia). The TR probe of 800 bp was obtained from the coding region of the murine TR cloned into the vector pUC18 (from Dr G. Anderson, The Queensland Institute of Medical Research, Brisbane, Queensland). The Nramp2 probe consisted of 1.7 kb of coding sequence from murine Nramp2 cloned into the pMT2 plasmid (from Dr N. C. Andrews, Howard Hughes Medical Institute, Children’s Hospital, Boston, MA). The β-globin probe of 612 bp was excised from the pMG5 vector (from Dr P. Ponka). The β-actin probe consisted of a 1.4-kb fragment of β-actin complementary DNA (cDNA) cloned into pBluestrip SK (ATCC catalogue no. 37997).

Western blot analysis

All procedures used for Western blotting were the same as those described previously.\textsuperscript{45} The monoclonal antibody (MoAb) against frataxin (clone 1G2) was from Chemicon International (Temecula, CA), and the MoAb against β-actin (clone AC-15) was from Sigma. The TR MoAb (clone H68.4) was purchased from Zymed Laboratories (San Francisco, CA).
Statistics
Data were compared using the Student paired t test. Results were considered statistically significant when \( P < .05 \).

Results

\textit{Frda} (frataxin) expression is down-regulated during erythroid differentiation

Friend cells exposed to DMSO undergo erythroid differentiation resulting in an increase in Fe uptake from Tf, heme synthesis, and hemoglobinization.\textsuperscript{26,37-41} Our initial studies examined the effect of DMSO on \(^{59}\)Fe uptake from \(^{59}\)Fe-Tf (10 mM) and the incorporation of \(^{59}\)Fe into heme by the Friend 707 cell line (Figure 1). Cells were incubated with 1.5% DMSO or control media for incubation times of 24 to 120 hours and the effect on \(^{59}\)Fe uptake and incorporation into heme assessed by incubating cells with \(^{59}\)Fe-Tf for 1 to 120 minutes (Figure 1A,B). Our studies showed that after a 24-hour incubation with DMSO there was little effect on \(^{59}\)Fe uptake from \(^{59}\)Fe-Tf and incorporation of \(^{59}\)Fe into heme, as shown previously (Figure 1A).\textsuperscript{40} However, after a 96-hour incubation with DMSO, there was a significant (\( P < .001 \)) increase in \(^{59}\)Fe uptake and incorporation of \(^{59}\)Fe into heme when compared to control cells for time points from 30 to 120 minutes (Figure 1B). Similar results to those found in Figure 1B were also found after incubating cells with DMSO for 48, 72, and 120 hours (data not shown). These data examining Fe uptake are similar to those found by others using Friend cells.\textsuperscript{40,50}

To examine the possible role of frataxin in Fe or heme metabolism, experiments were designed to assess the effect on \textit{Frda} messenger RNA (mRNA) levels by inducing erythroid differentiation in Friend 707 cells (Figure 2). To further investigate the function of frataxin, we also assessed changes in expression of \(\beta\)-globin mRNA (Figures 2, 6, and 7), and \(\text{TIR} \) and \(\text{Nramp2} \) mRNAs (Figures 6 and 7). The expression of these genes was relevant to examine because \(\beta\)-globin is essential for hemoglobinization,\textsuperscript{26} whereas \(\text{TIR} \) and \(\text{Nramp2} \) are involved in Fe uptake.\textsuperscript{24}

A 120-hour incubation with DMSO resulted in a marked decrease in \textit{Frda} mRNA levels (Figure 2B) compared to cells incubated with medium alone. After normalization to \(\beta\)-actin, densitometric analysis demonstrated that compared to control cells, induction of erythroid differentiation using DMSO resulted in a significant (\( P < .006 \)) 6 ± 2-fold decrease in \textit{Frda} mRNA levels in 5 separate experiments (Figure 2B). From this point on, all reported changes in gene or protein expression quantitated below are normalized to the \(\beta\)-actin loading control. The \textit{Frda} transcript migrated as a single band at approximately 1.2 kb just below the 18S ribosomal RNA (rRNA) band (Figure 2B), as reported previously.\textsuperscript{14} In some experiments, 2 \textit{Frda} bands were observed, the main 1.2-kb band and a less intense band that may correspond to an alternative \textit{Frda} transcript (Figure 6B). As a positive control for erythroid differentiation \(\beta\)-globin mRNA levels were assessed, and increased markedly after incubation with DMSO (Figure 2C).\textsuperscript{37,38}

In agreement with the decrease in \textit{Frda} mRNA levels (Figure 2B), Western blot analysis also demonstrated that frataxin protein levels were decreased after induction of erythroid differentiation with DMSO in Friend 707 cells (Figure 3). In all experiments a band at approximately 18 kDa was identified that was in good agreement with the relative molecular mass of frataxin reported previously.\textsuperscript{6} The decrease in frataxin protein levels began after 24 hours of induction with DMSO, with the greatest decrease becoming clear between 72 and 120 hours (Figure 3). Further, the effect of erythroid differentiation on decreasing frataxin protein levels was not specific for the Friend cell clone, with similar results being observed in the 707 and 745 cell lines (Figure 4). All subsequent experiments described were performed using the Friend 707 cell line.

Collectively, these data above indicate that the induction of erythroid differentiation results in a decrease in frataxin expression at both the mRNA and protein level. This decrease is inversely related to hemoglobinization (as judged by cell pellet color) and the expression of \(\beta\)-globin mRNA (Figure 2C).

\textbf{DMSO does not nonspecifically down-regulate \textit{Frda} gene expression}

Control experiments were performed to ensure that the effect of DMSO on \textit{Frda} expression was not due to nonspecific down-regulation of gene expression (Figure 5). In these studies, nonerythroid LMTK\textsuperscript{-} fibroblast or RAW264.7 macrophage cell lines were incubated in the presence or absence of DMSO for 120 hours and then \textit{Frda} mRNA levels assessed (Figure 5). Incubation with DMSO had no effect on \textit{Frda} mRNA expression when compared to cells exposed to control medium (Figure 5). These results indicate that \textit{Frda} expression was not nonspecifically down-regulated by DMSO.

\textbf{Kinetic analysis of the effects of DMSO on the expression of \textit{Frda}, \textit{Nramp2}, \textit{TIR}, \(\beta\)-\textit{globin}, and \(\beta\)-\textit{actin}}

To examine the kinetics of the effect of DMSO on gene expression, Friend cells were incubated in the presence or absence of this agent for 24 to 120 hours and the mRNA isolated (Figure 6). The levels of \textit{Frda} mRNA decreased markedly in the presence of DMSO as the incubation time increased to 120 hours (Figure 6B). For example, in the presence of DMSO, densitometric analysis demonstrated that
normalized Frda mRNA expression significantly (P < .001) decreased 14 ± 4-fold (3 experiments) comparing levels at time 0 and 120 hours (Figure 6B). In contrast, in the absence of DMSO, normalized Frda mRNA levels increased and peaked at 72 hours, and then decreased somewhat at later time points (Figure 6B). The normalized levels of Frda mRNA in the presence of DMSO were greater than those observed in the presence of this agent for all time points more than 24 hours (Figure 6B).

In contrast to Frda mRNA expression, normalized TfR mRNA levels significantly (P < .0001) increased up to 25-fold (3 experiments) in the presence of DMSO between 24 and 48 hours compared to initial levels (time 0; Figure 6C). The levels of TfR mRNA then markedly decreased as the incubation was continued up to 120 hours (Figure 6C). In contrast, in the absence of DMSO, normalized TfR mRNA levels significantly (P < .001) decreased more than 6-fold (3 experiments) as the incubation increased from 0 to 120 hours (Figure 6C).

From Figure 6D, it is clear that there are 2 Nramp2 transcripts at 2.3 and 3.1 kb in Friend cells, as observed in previous studies for murine tissues and cell lines. Although the precise identification of these 2 transcripts is uncertain, they may correspond to the Nramp2 splice variants with and without the iron-responsive element (IRE; 2.3 and 3.0 kb, GenBank accession codes AF029758 and L33415, respectively). The change in expression of the Nramp2 transcripts after incubation with DMSO was generally similar (Figure 6D). In the presence or absence of DMSO, densitometric analysis showed there was a slight increase in the normalized expression of both Nramp2 transcripts in the first 24 hours. In the presence of DMSO, this initial increase in Nramp2 mRNA expression was then followed by a significant (P < .01) decrease in 3 experiments (Figure 6D). In one typical experiment (representative of 3) in the presence of DMSO, the 2.3-kb and 3.1-kb Nramp2 transcripts decreased 3.7-fold and 2.5-fold, respectively, comparing initial levels and those found after a 120-hour incubation (Figure 6D). In the absence of DMSO, the levels of both the 3.1-kb and 2.3-kb transcripts peaked at 72 hours and then decreased to initial levels or slightly below initial levels, respectively, at 120 hours (Figure 6D). The expression of Nramp2 and TfR mRNA was important to assess because their protein products act as partners involved in Fe uptake from Tf, which is increased in erythroid differentiation. 40,50

In accordance with previous studies, we found that the expression of β-globin mRNA increased markedly and significantly (P < .00005) during the incubation with DMSO (3 experiments), whereas it remained very low in the absence of this inducing agent (Figure 6E). In the absence of DMSO, the normalized level of β-globin mRNA expression was 85-fold greater in induced compared to uninduced Friend cells at the 120-hour time point (Figure 6E). Collectively, the results above clearly indicate that the expression of Frda mRNA is markedly different from that of TfR mRNA and β-globin mRNA.

**The effect of iron chelators, iron donors, and the heme precursor, PIX, on the expression of Frda, TfR, Nramp2, β-globin, and β-actin**

To further assess the possible role of frataxin in Fe or heme metabolism, we examined the effect on gene expression of
chelators, which deplete cellular Fe, compounds which donate Fe to cells, and the heme precursor, PIX (Figure 7). Two chelators were used: pyridoxal isonicotinoyl hydrazone (PIH), which depletes cytoplasmic and mitochondrial Fe pools,51,52 and DFO, which chelates cytoplasmic Fe.53-55 Because either or both Fe pools could be involved in regulating Frda expression, it was necessary to use both chelators so that Fe levels in each pool could be depleted. Two Fe donors, hemin and ferric ammonium citrate (FAC), were also used. These donate Fe to cells by different mechanisms,56-58 resulting in down-regulation of the TfR59 and up-regulation of ferritin expression.60 The heme precursor PIX was used because it acts as a substrate for heme synthesis.27,31

Friend cells were incubated for 96 hours with control medium or medium containing 1.5% DMSO and then exposed for a further 20 hours to either control medium, DFO (100 µg/mL), PIH (50 µM), FAC (100 µg/mL), or PIH (50 µM) in the presence or absence of 1.5% DMSO. Levels of Frda mRNA (Figure 7B) and Nrmrp2 mRNA (Figure 7D) were significantly (P < .003) greater under all conditions in cells not induced with DMSO in 4 experiments. Densitometric analysis (not shown) demonstrated that none of the modulatory agents above had any significant effect on normalized Frda mRNA levels when compared to the control (Figure 7B). In contrast, DFO increased TfR mRNA levels in uninduced cells, whereas it had less effect on TfR mRNA expression in cells incubated with DMSO (Figure 7C). A similar but less marked effect was also seen for PIH. The Fe donors, FAC and hemin, had a greater effect at reducing TfR mRNA in uninduced than induced Friend cells (Figure 7C). The fact that DFO, FAC, and hemin affected TfR expression provided a positive control that showed these agents acted in accordance with their reported effects to remove or donate Fe to cells.51,44,47,54-56,60

Studies on the effect of DFO and FAC on the expression of Frda mRNA compared to TfR mRNA were also examined in 5 nonerythroid cell lines available in our laboratory (LMTK− fibroblasts, HepAl-6 hepatoma cells, M15 kidney cells, and J774 and RAW264.7 macrophages). In each case, Frda mRNA levels did not change significantly, in contrast to TfR mRNA that was up-regulated by DFO and down-regulated by FAC (data not shown).

Figure 3. Induction of erythroid differentiation in Friend 707 cells by DMSO decreases frataxin protein levels. Western blot analysis was performed using proteins extracted from cells incubated in the presence (+) or absence (−) of 1.5% DMSO for 24 to 120 hours at 37°C. The membrane was sequentially probed with antifrataxin and anti-β-actin antibodies (see “Materials and methods” for details). Densitometric analysis of the results normalized to the β-actin loading control are shown below the blot. The result illustrated is a typical experiment from 4 experiments performed.

Figure 4. The effect of DMSO on decreasing frataxin protein levels is not Friend cell clone specific. Western blot analysis was performed using proteins extracted from the Friend 707 and 745 cell lines incubated in the presence (+) or absence (−) of 1.5% DMSO for 120 hours at 37°C. The membrane was sequentially probed with antifrataxin and anti-β-actin antibodies (see “Materials and methods” for details). Densitometric analysis of the results normalized to the β-actin loading control are shown below the blot. The result illustrated is a typical experiment from 4 experiments performed.

Figure 5. The effect of incubation with DMSO on the mRNA levels of frataxin (Frda) and β-actin (loading control) in mouse LMTK− fibroblasts and the RAW264.7 macrophage cell line. Northern blot analysis was performed as described in Figure 2 using total mRNA extracted from cells after a 120-hour incubation at 37°C in the presence (+) or absence (−) of 1.5% DMSO. (A) Ethidium bromide staining of the agarose gel, (B) Frda, and (C) β-actin. The result illustrated is a typical experiment from 3 experiments performed.
None of the Fe chelators, Fe donors, or PIX, had any significant effect on normalized Nramp2 mRNA levels compared to the control in 4 separate experiments (Figure 7D). Certainly, the level of Nramp2 mRNA was not regulated in concert with TfR mRNA (Figure 7C). This observation is of interest because like TfR mRNA, Nramp2 mRNA contains an IRE in its 3′ untranslated region. These results are similar to those reported in our previous studies examining the effects of intracellular Fe levels on Nramp2 expression in nonerythroid cell types.44,47 Collectively, the results above suggest that Frda mRNA was not regulated by Fe in a similar way to TfR mRNA. To confirm this, we also incubated cells with the heme synthesis inhibitor, SA, which causes accumulation of mitochondrial Fe and, to a lesser extent, cytoplasmic Fe.26,27,30-33 In these studies, Friend cells were incubated for 96 hours with control medium or medium containing DMSO and then exposed for a further 20 hours with either control medium or medium containing DFO (100 μM), FAC (100 μg/ml), PIH (50 μM), hemin (50 μM), PIX (50 μM), or SA (2 mM) in the presence or absence of DMSO (Figure 8A). As shown in Figure 3, induction of erythroid differentiation with DMSO resulted in a marked decrease in frataxin protein levels (Figure 8A). Of all compounds tested, only incubation with PIX consistently decreased the level of frataxin protein in uninduced Friend cells over 8 independent experiments (Figure 8A). After normalization to β-actin, PIX significantly (P < .003) decreased the level of frataxin protein 2.2 ± 0.7-fold compared to the relevant control in 8 experiments (Figure 8A). For induced cells, PIX reduced frataxin expression (Figure 8A) but this was not significant (P > .05) in 8 experiments. All other agents tested in induced cells also had no significant effect on frataxin expression.

Similar to uninduced Friend cells, PIX also reduced frataxin expression in mouse LMTK fibroblasts and human SK-N-MC neuroepithelioma cells (Figure 8B). These results further suggest that frataxin plays a regulatory role in Fe or heme metabolism.

**PIX down-regulates frataxin protein levels in uninduced Friend cells and other cell types**

Western blot analysis was performed to assess whether DFO, FAC, PIH, hemin, PIX, or SA had any regulatory effects on frataxin protein levels. Friend cells were incubated for 96 hours with control medium or medium containing DMSO and then exposed for a further 20 hours with either control medium or medium containing DFO (100 μM), FAC (100 μg/ml), PIH (50 μM), hemin (50 μM), PIX (50 μM), or SA (2 mM) in the presence or absence of DMSO (Figure 8A). After normalization to β-actin, PIX significantly (P < .003) decreased the level of frataxin protein 2.2 ± 0.7-fold compared to the relevant control in 8 experiments (Figure 8A). For induced cells, PIX reduced frataxin expression (Figure 8A) but this was not significant (P > .05) in 8 experiments. All other agents tested in induced cells also had no significant effect on frataxin expression.

Similar to uninduced Friend cells, PIX also reduced frataxin expression in mouse LMTK fibroblasts and human SK-N-MC neuroepithelioma cells (Figure 8B). These results further suggest that frataxin plays a regulatory role in Fe or heme metabolism.
To assess if the above treatments had appropriately influenced cellular Fe metabolism, their effects on TfR expression were also assessed by Western blotting (Figure 9). As expected from previous studies, DFO increased TfR expression in both uninduced and induced Friend cells, but this was more pronounced in uninduced cells (Figure 9). In contrast, FAC and hemin reduced TfR levels in uninduced cells more than induced cells. In 5 experiments, densitometric analysis demonstrated that PIH, PIX, and SA had no consistent nor significant effect on TfR protein levels in induced or uninduced cells (Figure 9). The failure of PIH to increase TfR levels is of interest and may relate to its different site of action to DFO. Previous studies have also noted that in contrast to DFO, PIH...
did not increase iron regulatory protein-IRE–binding activity.62 Interestingly, SA did not decrease Tfr protein levels of induced cells (Figure 9), and this was in contrast to results at the mRNA level (see text above).

Discussion

The role of frataxin in mitochondrial Fe homeostasis is critical to understand because the decrease in expression of this molecule leads to mitochondrial Fe accumulation that may contribute to FA.2,24 Furthermore, understanding the function of frataxin may result in new strategies for the treatment of this disorder.24,62 We examined the role of frataxin in Fe and heme metabolism by using a well-characterized model of erythroid differentiation, namely, Friend cells induced with DMSO.26,40,56

To our knowledge this is the first report showing down-regulation of frataxin expression on erythroid differentiation. In contrast, and as expected,26,37,38,41,56 expression of Tfr and β-globin mRNA increased markedly after incubation of Friend cells with DMSO (Figure 6). Because there was a marked decrease in frataxin expression on induction of heme and hemoglobin synthesis, it is unlikely that frataxin plays a direct role in these processes. In addition, we demonstrate that modulation of intracellular Fe levels or inhibition of heme synthesis does not regulate the expression of Frda mRNA in a way similar to Tfr mRNA in Friend cells. The effect of Fe and heme on frataxin levels was important to investigate because the expression of many molecules involved in Fe and heme synthesis is regulated by the concentration of intracellular Fe or heme.26,60 Furthermore, although frataxin levels decreased during differentiation, there is increased expression of a variety of molecules involved in heme synthesis and other functions in the cytoplasm or mitochondrion.26,61,65 Indeed, there are only a few specific markers that are known to be down-regulated by DMSO treatment of Friend cells. In this respect, the decreases in Frda and Nramp2 gene expression are interesting (Figure 6), but they are not the only molecules that decrease during erythroid differentiation (eg, ribosomal proteins).

On erythroid differentiation there is a marked up-regulation of proteins involved in controlling Fe uptake (eg, Tfr),41,61 heme synthesis,26,40,56,63 and hemoglobinization (eg, β-globin).37,38 The marked up-regulation of Tfr expression results in a significant increase in Fe uptake (Figure 1B) as demonstrated by others.26,40,50,56,64 This Fe is used for heme synthesis within the mitochondrion (Figure 1B).26,40,50,56 The decrease in frataxin protein levels began after 24 hours of induction with DMSO, with the greatest decrease becoming obvious between 72 and 120 hours (Figure 3). The decreased frataxin expression is correlated with increased Fe uptake by the mitochondrion and the induction of heme biosynthesis.40 Considering this, using Friend cells, the incorporation of 59Fe and 14C-aminolevulinic acid into heme as well as the heme concentration becomes pronounced between 72 and 120 hours of incubation with DMSO.40 In comparison, δ-aminolevulinic acid synthetase activity begins to slowly increase after 24 hours of incubation with DMSO, with its activity becoming increasingly marked after 3 days of exposure to this agent.40 For both δ-aminolevulinic acid dehydratase and uroporphyrinogen-I synthetase, their activities increase between 24 and 48 hours of incubation with DMSO and then plateau after 72 hours.40

The finding that erythroid differentiation resulted in down-regulation of frataxin expression provides insight into the function of this protein. Indeed, it is likely that the Fe loading and oxidative damage of the mitochondrion in FA patients is due to a decrease in frataxin levels.6,11 Considering this, and in conjunction with our data, 2 possible roles of frataxin could be suggested that lead to mitochondrial Fe sequestration: (1) down-regulation of frataxin expression may increase mitochondrial Fe import or decrease Fe export by regulating metal transporters, leading to Fe accumulation for heme synthesis, or (2) frataxin could act as a metabolic switch, resulting in the diversion of Fe from one metabolic pathway (eg, Fe incorporation into ferritin or the synthesis of Fe-S clusters) to another, namely, Fe incorporation into heme.

For both of the above hypotheses, increased retention of mitochondrial Fe would occur that could favor heme synthesis.26 In support of the first hypothesis, it has been reported that Yfh1p plays a role in the release of nonheme mitochondrial Fe into the cytoplasm.10 Thus, the decrease in frataxin expression in erythroid cells may prevent mitochondrial Fe release and its use for heme synthesis rather than cytosolic Fe metabolism. Alternatively, or in combination with a decrease in mitochondrial Fe efflux, down-regulation of frataxin expression may increase mitochondrial Fe uptake. In this latter mechanism, frataxin could act as a negative regulator of a mitochondrial Fe import process. Considering this, a yeast gene known as CCC1 has been identified that inhibits mitochondrial Fe uptake.66 We suggest a similar molecule could be regulated by frataxin in mammalian cells. At this point it should be discussed that the regulation of cellular Fe metabolism is complex. The net flux of Fe coming into the cell via Nramp2 or into the mitochondrion as possibly regulated by frataxin, may be dependent on integrating the activity of multiple proteins. Thus, the observation that frataxin expression decreases as Fe uptake into heme increases does not prove direct causality.
In accordance with a role of frataxin in the regulation of Fe and heme metabolism, our studies demonstrated that incubation of uninduced Friend cells with PIX resulted in a significant decrease in frataxin protein levels (Figure 8A). Because PIX is the immediate precursor of heme, an increase in the intracellular PIX concentration may act as a signal to coordinate heme synthesis with mitochondrial Fe uptake. For instance, an elevation of cellular PIX could signal a decrease in frataxin expression that leads to mitochondrial Fe sequestration for heme biosynthesis. It is of interest to note that although PIX reduced frataxin protein expression (Figure 8A), it had no significant effect on the mRNA levels of this molecule (Figure 7B).

Considering the recent discussion of frataxin acting as a mitochondrial ferritin, it is relevant to note that incubation of Friend cells with the Fe donor, FAC, or the Fe chelator, DFO, did not significantly increase or decrease the level of frataxin, respectively (Figure 8A). This was in marked contrast to the known stimulatory and inhibitory effects of FAC and DFO, respectively, on ferritin protein levels. These data suggest that frataxin is not regulated by Fe in a manner similar to ferritin. It could be that a vital Fe storage molecule would respond to increased intracellular Fe levels. Hence, the lack of response of frataxin in our experiments is not consistent with an Fe storage role. In erythroid cells, excess mitochondrial Fe may be stored in the recently identified mitochondrial ferritin.

Interestingly, depletion of frataxin in FA patients does not result in marked defects in heme synthesis. Because a reduction in frataxin expression occurs concurrently with an increase in Fe uptake, heme synthesis, and TRβ- and β-globin mRNA expression (Figures 1B, 2, and 6), this decrease in frataxin levels may facilitate these latter processes. Indeed, it is well known in erythroid cells that the limiting factor for heme synthesis is Fe acquisition. Hence, in erythroid cells, the reduction in frataxin expression appears coupled to the marked increase in heme synthesis that is exported from the mitochondrion preventing toxic Fe accumulation. In contrast, in the nonerythroid cells affected in FA, there is a decrease in frataxin expression that results in mitochondrial Fe loading. However, because nonerythroid cells have very low rates of heme production, the increased Fe uptake is not matched by a similar rise in heme synthesis that is necessary for Fe incorporation into PIX and its transport out of the mitochondrion as heme. Under these circumstances, Fe will accumulate and cause toxicity via free radical production.

It is relevant to discuss that mitochondrial Fe must be in the Fe²⁺ state to contribute to heme synthesis. This is exemplified by sideroblastic anemia, where ferric Fe accumulates and cannot be used for heme synthesis via ferrochelatase. In this study we used a well-characterized model of erythroid differentiation where incubation of Friend cells with DMSO results in initiation of heme biosynthesis, a significant increase in Fe uptake from Tf, and incorporation of Fe²⁺ into PIX to form heme (Figure 1B). Hence, our experiments have focused on the role of frataxin during a physiologic response rather than a pathologic condition.

Previous studies by Cossee and associates showed that homozygous deletions of frataxin cause lethality in a homozygous mouse knockout model without apparent Fe accumulation. In the case of a developing embryo, an inappropriate increase in mitochondrial Fe levels may not need to be marked to cause apoptosis and death. Moreover, in this latter study, there was no attempt to quantitatively examine mitochondrial Fe concentrations by isolating mitochondria, and it is difficult to assess if the methods used (Perl staining and electron microscopic investigation of tissues) were sensitive enough to determine a small but critical increase in Fe. Nonetheless, considering the work of Cossee and others, it cannot be excluded that frataxin deficiency triggers apoptosis by a mechanism independent of Fe accumulation. It is notable that no significant mitochondrial Fe deposition takes place in sideroblastic anemia at day 11.5 during the embryonic life of the erythroid 5-aminolevulinate synthase knockout mouse. Relevant to this, Cossee and colleagues reported that frataxin knockout mice die soon after implantation. Thus, it is likely that these authors may have missed the Fe accumulation that occurs later in development.

In summary, we demonstrate that erythroid differentiation in Friend cells results in down-regulation of Frda mRNA levels and frataxin protein levels. Further, the down-regulation of frataxin expression during erythroid differentiation may favor the marked mitochondrial Fe uptake, heme synthesis, and hemoglobinization that occur. Indeed, the fact that incubation with PIX decreased frataxin protein levels suggests a role for this protein in the coordination of mitochondrial Fe and heme metabolism. Further studies on cell lines where overexpression or underexpression of frataxin is controlled by inducible promoters may establish the precise role of frataxin in Fe metabolism.

Acknowledgments

Professor Roger Dean, Dr Anna Baoutina, and members of our laboratory are kindly thanked for their detailed comments on the manuscript prior to submission. We gratefully acknowledge the expert assistance of Mr Ralph Watts in preparing the figures. Dr Jin Gao is also thanked for his help with Western blot analysis.

References


Erythroid differentiation and protoporphyrin IX down-regulate frataxin expression in Friend cells: characterization of frataxin expression compared to molecules involved in iron metabolism and hemoglobinization

Erika M. Becker, Judith M. Greer, Prem Ponka and Des R. Richardson