Iron–sulfur protein maturation in human cells: evidence for a function of frataxin

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The maturation of iron–sulfur (Fe/S) proteins in eukaryotes has been intensively studied in yeast. Hardly anything is known so far about the process in higher eukaryotes, even though the high conservation of the yeast maturation components in most Eukarya suggests similar mechanisms. Here, we developed a cell culture model in which the RNA interference (RNAi) technology was used to deplete a potential component of Fe/S protein maturation, frataxin, in human HeLa cells. This protein is lowered in humans with the neuromuscular disorder Friedreich’s ataxia (FRDA). Upon frataxin depletion by RNAi, the enzyme activities of the mitochondrial Fe/S proteins, aconitase and succinate dehydrogenase, were decreased, while the activities of non-Fe/S proteins remained constant. Moreover, Fe/S cluster association with the cytosolic iron-regulatory protein 1 was diminished. In contrast, no alterations in cellular iron uptake, iron content and heme formation were found, and no mitochondrial iron deposits were observed upon frataxin depletion. Hence, iron accumulation in FRDA mitochondria appears to be a late consequence of frataxin deficiency. These results demonstrate (i) that frataxin is a component of the human Fe/S cluster assembly machinery and (ii) that it plays a role in the maturation of both mitochondrial and cytosolic Fe/S proteins.

INTRODUCTION

Iron–sulfur clusters (ISC) are crucial cofactors of numerous proteins involved in enzyme catalysis, electron transport and regulation of gene expression. The synthesis of these ancient cofactors in the cell requires a complex set of proteins (1–3). In bacteria and yeast mitochondria, homologous ISC assembly machineries consisting of some 10 proteins have been shown to be essential for maturation of cellular iron–sulfur (Fe/S) proteins. In yeast, this ISC machinery co-operates with additional mitochondrial components and two recently identified cytosolic proteins for maturation of cytosolic and nuclear Fe/S protein (2). All these components are well conserved in higher eukaryotes such as mouse and man (4), and therefore it seems reasonable to expect similar mechanisms of Fe/S protein maturation in these organisms as those defined in yeast. However, direct functional studies in higher eukaryotes have not been reported due to the lack of an efficient experimental system to investigate the role of specific components in Fe/S protein assembly.

The development of the RNA interference (RNAi) technology to specifically deplete gene products (5,6) and the availability of several assays to measure the activity of Fe/S proteins in both mitochondria and the cytosol now offers the possibility to analyse the requirements for Fe/S protein maturation in human cell culture. In the present study, frataxin was chosen as an initial component to be examined, because this protein is deficient in the neurodegenerative disorder Friedreich’s ataxia (FRDA) (7,8). Frataxin is a highly conserved mitochondrial matrix protein with homologs in prokaryotes and eukaryotes (9–13). Its three dimensional structure has been elucidated (14–16) but its precise function is still under debate. Mainly on the basis of studies in yeast, this protein was suggested to be involved in the formation of Fe/S proteins (17–19). Alternatively, or in addition, the protein might play a role in the storage of mitochondrial iron as suggested by experiments performed in vitro (20,21). Moreover, deletion of frataxin in yeast resulted in heme formation defects which, however, were shown to be an indirect consequence of an impaired ISC biogenesis, thereby causing a reversible inhibition of ferrochelatase, the enzyme catalyzing the last step of heme biosynthesis (22,23).

FRDA is an autosomal recessive inherited mitochondrial disorder that is caused by frataxin deficiency and is characterized by progressive polyneuropathy and myopathy (24). Mammalian frataxin is expressed in all cells with highest levels in
nervous and muscle tissues, explaining why both tissues are preferentially affected in FRDA (25). A threshold level of frataxin seems to be essential (8,25), since in mice a homozygous gene disruption is lethal during embryogenesis (26). Humans develop clinical signs of FRDA, when frataxin levels drop by \( \sim 60\% - 70\% \) of normal.

On the cellular level, hallmarks of frataxin deficiency in humans, mice and yeast are mitochondrial iron accumulation, increased sensitivity to oxidants, depletion of mitochondrial DNA, decreased activities of ISC enzymes and impaired respiration (24,27,28). These observations are consistent with the idea of a vicious cycle in which reactive oxygen species are formed by mitochondrial iron deposits via Fenton chemistry and then may destroy ISCs. In turn, disassembly of Fe/S proteins might increase mitochondrial iron accumulation by a still unknown mechanism (29). However, the initial reason for this vicious cycle is still under debate. On the one hand, human frataxin and yeast frataxin (Yfh1p) were found to form high molecular mass complexes with iron \textit{in vitro}, indicating that the protein might be an iron storage protein (20,21). (See, however, note added in proof.) Without frataxin, iron precipitation and mitochondrial iron accumulation may lead to the FRDA phenotype. On the other hand, yeast frataxin interacts with members of the ISC assembly machinery and enhances the formation of ISC \textit{in vivo} and \textit{in vitro}, indicating an important role of frataxin in the maturation of Fe/S proteins (17–19,30). Moreover, depletion of yeast frataxin causes similar phenotypes as the depletion of proteins known to be involved in ISC assembly (1,23,29,31). Another clue that frataxin may play a direct role in ISC formation comes from mouse models for FRDA (26,32). Frataxin knock-out mice die at the embryonic stage without any histological signs of tissue-specific depletion of frataxin develop neuromyopathies (8,25), since in mice a homozygous gene disruption is lethal during embryogenesis (26). Humans develop clinical signs of FRDA, when frataxin levels drop by \( \sim 60\% - 70\% \) of normal.

Frataxin deficiency results in growth defects

Depletion of frataxin by RNAi reduced HeLa cell growth in comparison with the pSUPER-transfected control cells (Fig. 3A). Frataxin-deficient cells did not become confluent as fast as the control cells and their protein content as a measure of cell growth was reduced. Growth retardation appeared after the second transfection, and after 4–5 transfections the growth rate of the frataxin-depleted cells was <40% of the control cells (Fig. 2B, lanes 1 and 2). After re-transfection, frataxin levels dropped to <20% of the control cells (Fig. 2B, lanes 3–10), and the protein was hardly detectable by immunofluorescence (Fig. 2A). When the transfection series was aborted, the frataxin content increased and was restored to almost normal levels (Fig. 2B, lanes 11–14). The strong but reversible depletion of frataxin allowed us to study the phenotypical effects associated with frataxin deficiency.

RESULTS

Frataxin is efficiently depleted by RNAi

HeLa cells were transfected either with the hFraRNAi plasmid encoding siRNAs interfering with the frataxin mRNA or with the pSUPER control vector. A scrambled siRNA did not cause any detectable specific effects (data not shown). Transfection efficiency was assessed by co-transfection with the EGFP-N3 plasmid encoding a green fluorescent protein and resulted in 80–90% EGFP\(^{+}\) cells as indicated by flow cytometry on every 4th day after electroporation (Fig. 1; for further details see Materials and Methods). EGFP-fluorescence was similar for both cells transfected with the hFraRNAi or pSUPER plasmid (Fig. 1, insert). When the series of transfections was aborted and cells were cultured for another 4 or 8 days, the EGFP-fluorescence decreased over time (Fig. 1, +4 and +8 bars), until hardly any EGFP\(^{+}\) cells were detected.

Depletion of frataxin was investigated by immunofluorescence microscopy and immunoblot analysis (Fig. 2). Already 4 days after the first transfection, HeLa cells co-transfected with the hFraRNAi plasmid displayed considerably decreased levels of frataxin (Fig. 2A). Immunoblot analysis revealed a frataxin content of \(<40\%\) of the control cells (Fig. 2B, lanes 1 and 2). After re-transfection, frataxin levels dropped to \(<20\%\) of the control cells (Fig. 2B, lanes 3–10), and the protein was hardly detectable by immunofluorescence (Fig. 2A). When the transfection series was aborted, the frataxin content increased and was restored to almost normal levels (Fig. 2B, lanes 11–14). The strong but reversible depletion of frataxin allowed us to study the phenotypical effects associated with frataxin deficiency.
decreased mitochondrial membrane potential as an indicator for reduced viability (Fig. 3B). When the transfection series was aborted and the frataxin-depleted cells were further cultured, their growth rate increased, again indicating the reversibility of the RNAi effect (Fig. 3A, time points +4 and +8).

Frataxin deficiency reduces the activities of mitochondrial Fe/S proteins

The importance of frataxin for the function of mitochondrial Fe/S proteins was assessed by measuring the activities of the ISC-containing enzymes aconitase and succinate dehydrogenase (SDH). Aconitase and SDH activities of pSUPER-transfected control cells were ~25 and 60 mU/mg protein, respectively (Fig. 4). In frataxin-deficient cells, aconitase and SDH activities decreased with the time course of frataxin depletion. After four transfections, enzyme activities were up...
to 40 and 60% lower than in the control cells, respectively. In contrast, the activities of malate dehydrogenase and citrate synthase, two enzymes lacking ISCs, did not differ between frataxin-deficient and control-transfected cells, and amounted to 2250 and 225 mU/mg protein, respectively (Fig. 4). When the transfection series was aborted, and frataxin was replenished, aconitase and SDH activities were restored to almost normal levels. Thus, frataxin plays an important and specific role in the maintenance of mitochondrial Fe/S protein activities. Our results did not allow us to test whether frataxin plays an additional, iron delivery function in the maintenance of aconitase activity as suggested recently (34).

Frataxin deficiency increases the RNA-binding capabilities of cytosolic IRP1

Nothing is known so far about the maturation of cytosolic Fe/S proteins in human cells and about the fate of cytosolic Fe/S proteins in FRDA. The HeLa cell culture model for FRDA provided a useful system to study the consequences of frataxin deficiency for the function of cytosolic Fe/S proteins in humans. The IRP1 is a cytosolic Fe/S protein that can bind to specific mRNA stem-loop structures called IRE when it lacks its ISC (35,36). Binding can be analysed by a REMSA. IRP2 is a non-ISC-containing protein that cannot be distinguished in its IRE-binding capacity from IRP1 in human cells, but supershift assays with anti-IRP1 antibodies revealed that in our cell culture system IRP2 contributed only 22.5–32.5% of the total IRP-binding capacity (for details see Materials and Methods). Therefore, the amount of total IRP bound to [α-32P]CTP-labelled IRE of ferritin mRNA mainly reflected the amount of ISC associated with IRP1 (Fig. 5). Densitometric analysis of autoradiographs revealed an up to 2-fold increase of IRE-binding in frataxin-depleted HeLa cell lysates compared with control cells. The elevated amounts of IRP-bound IRE were not caused by higher amounts of the IRPs, since after disassembly of the IRP1-associated ISC with β-mercaptoethanol comparable IRE-binding was observed in frataxin-deficient and control cells (Fig. 5, top).

Similar to our observations for mitochondrial Fe/S proteins, ISC loss on cytosolic IRP1 was dependent on the time course of frataxin depletion. After the first and second transfections, IRE-binding to total IRP was only slightly elevated (Fig. 5, lanes 1–4). Only after the third transfection, IRE-binding was significantly increased, reaching levels twice as high as in the control cells after the fifth transfection (Fig. 5, lanes 5–10). When the transfection series was aborted, IRE-binding dropped to normal IRP levels (Fig. 5, lanes 11–14), thereby indicating the reversible character of the defect. Taken together, frataxin is required for maintenance of both mitochondrial and cytosolic Fe/S protein activities.

No alterations in cellular and mitochondrial iron content upon frataxin depletion

Depletion of the yeast frataxin homologue Yfh1p leads to an increased cellular iron uptake and cellular iron content (9,10,37), and a hallmark of frataxin deficiency in yeast, mouse and man is the accumulation of iron within mitochondria resulting in the formation of iron precipitates (27). In the present study, cellular iron content of frataxin-deficient and of pSUPER-transfected control cells was analysed by a spectrophotometric assay and was found to be similar, ranging
between 1.5 and 2.0 nmol/mg protein (Fig. 6A). These findings fit to recent observations that IRP1 deletion in mice did not elicit severe effects on the cellular iron status (38). We further asked whether iron deposits were detectable in mitochondria of frataxin-depleted HeLa cells, but no iron precipitates were found by transmission electron microscopy combined with electron dispersion X-ray (EDX) spectroscopy (data not shown). The iron status of HeLa cells was not affected within the first 3 weeks of frataxin deficiency. In summary, the change in Fe/S protein activities was not due to alterations in the intracellular iron supply, but appeared to be a primary consequence of the depletion of frataxin.

Frataxin deficiency does not affect $^{55}$Fe incorporation into heme

As a final question we asked whether frataxin might play a role in heme formation. Since human ferrochelatase contains a C-terminal Fe/S cluster that is necessary for enzymatic function (39), an influence of the frataxin depletion on heme biosynthesis might be expected. The HeLa cell culture model offered the opportunity to examine the heme formation in human cells from the onset of frataxin depletion. Cells were incubated for 18 h with non-transferrin-bound $^{55}$Fe chelated by nitritotriacetic acid (NTA), leading to a comparable $^{55}$Fe uptake in hFraRNAi transfected and control cells (Fig. 6B).

This result supported the findings from earlier that the iron content of frataxin-deficient HeLa cells was not considerably altered. Similar to the $^{55}$Fe uptake, incorporation of $^{55}$Fe into heme was not changed during the first days and weeks of frataxin deficiency (Fig. 6C). Thus, in human cells frataxin depletion does not lead to an immediate defect in heme formation. As in yeast cells, human ferrochelatase might be in large excess so that its partial functional inactivation might not result in immediate consequences for the efficiency of heme synthesis (40).

DISCUSSION

A deficiency of the mitochondrial protein frataxin is the molecular basis of FRDA, one of the most prevalent inherited ataxias. The mechanisms leading to the phenotypical consequences, such as mitochondrial iron accumulation and Fe/S protein deficiency, are still objects of intense investigation (27). The present study introduces a human cell culture model for FRDA that provides insights into the molecular consequences of frataxin depletion. Additionally, our experimental set-up provides a general system to study Fe/S protein biogenesis in human cells. Applying the RNAi technology, a strong decrease of the cellular frataxin content was achieved in HeLa cells and was maintained over several weeks. Frataxin deficiency resulted in 2-fold reduced activities of the mitochondrial Fe/S proteins aconitase and SDH and in a 2-fold increased RNA-binding activity of the cytosolic protein IRP1, indicating the loss of its Fe/S clusters. Altered activities of Fe/S proteins were already observed 4 days after the initial transfection, indicating that these proteins were affected early after frataxin depletion. In contrast, disturbances in cellular iron content and iron incorporation into heme were not detected within the first 3 weeks of frataxin deficiency. This indicates that human frataxin plays a primary role in the maintenance of Fe/S protein activities suggesting that frataxin is a component of the human mitochondrial Fe/S protein biogenesis apparatus.

Alterations in cellular iron homeostasis have long been known to be associated with FRDA and frataxin deficiency (9,41), implying a possible role for frataxin in iron metabolism. In vitro findings that human and yeast frataxin can self-assemble to regular spherical multimers containing several thousands of iron atoms prompted the idea that frataxin might be a mitochondrial iron storage protein (20,21).
However, evidence for a functional role of frataxin multimers in vivo has not been provided yet (see note added in proof). Also, the present HeLa cell culture model for FRDA does not support the hypothesis of an iron storage function of frataxin. First, the cellular iron content of frataxin-deficient HeLa cells was normal. Mitochondria isolated from cell culture were not used, since they are known to be highly contaminated by other organelles. Such results therefore may be incorrect or misleading. Second, the normal uptake of at least non-transferrin-bound $^{55}$Fe, and the lack of mitochondrial iron deposits upon frataxin depletion render it unlikely that alterations in the cellular iron status as well as mitochondrial iron precipitation and accumulation are early consequences of frataxin deficiency.

Several observations have pointed to a possible function of frataxin in heme formation. Yfh1p-deficient yeast cells display a heme synthesis defect and a reduced cytochrome content (18,22,23). In vitro studies have shown that heme formation by human ferrochelatase occurs at slightly increased efficiencies in the presence of frataxin (42). This had led to the idea of an iron delivery function of frataxin to ferrochelatase. However, recently the heme synthesis defect in yeast cells was shown to be a general consequence of an impaired Fe/S protein biogenesis in mitochondria and not specific for a defect in Yfh1p. All mutants defective in Fe/S protein biogenesis displayed a substantial reversible inhibition of ferrochelatase (23). Human ferrochelatase is an ISC-containing enzyme (39) and, in light of frataxin’s function in Fe/S protein biogenesis, one might expect that it is defective upon impairment of Fe/S protein biogenesis. However, FRDA patients exhibit a normal erythroid development (43), and the present HeLa cell culture system did not reveal any impairment in heme synthesis upon frataxin depletion. The lack of an in vivo defect in heme formation upon frataxin depletion may be due to the presence of high amounts of human ferrochelatase exceeding the needs for heme synthesis under normal conditions (40).

Depletion of frataxin not only decreased the activities of the mitochondrial Fe/S proteins but also affected the IRE-binding capabilities of cytosolic IRP1. For the first time, the present study demonstrated that frataxin deficiency in human cells increased the IRP-binding to ferritin IRE, suggesting the disassembly of the IRP1-associated ISC. These results point to a role of frataxin in the maturation of both mitochondrial and cytosolic Fe/S proteins. The involvement of a mitochondrial protein in the function of cytosolic Fe/S proteins resembles the situation described in yeast, where virtually all components of the mitochondrial ISC assembly machinery are required for Fe/S protein maturation in the cytosol (1). The cell culture system developed here will allow us to study the role of other members of the mammalian mitochondrial ISC assembly machinery and of two recently identified cytosolic components in the maturation of cytosolic Fe/S proteins (1).

The influence of frataxin on the function of cytosolic Fe/S proteins in both yeast (18) and human cells (this study) underscores the important role of frataxin in the maturation of Fe/S proteins and raises the question about the link to the phenotype observed in FRDA. IRPs modulate translation of proteins that are involved in the transport, storage and use of iron, including the divalent metal transporter 1, the iron regulated transporter 1, the transferrin receptor (TfR) and ferritin (35). Despite the fact that in the present study the depletion of frataxin resulted in an increased IRE-binding of IRPs, there was no evidence for altered cellular levels of both ferritin and TfR (data not shown). Thus, other mechanisms might exist for the fine-tuning of the biogenesis of these proteins. On the one hand, HeLa cells are transformed and have been maintained in culture for many years. This might affect their response to altered cellular IRP1 levels. On the other hand, the development of clinical symptoms of FRDA is a long-lasting process (24,27,32), and alterations in iron homeostasis might not be detectable within the first 3 weeks of frataxin deficiency. This is supported by findings on frataxin knock-out mice. The homozygous deletion of the frataxin gene is lethal in early embryogenesis (26), but no iron accumulation was observed during embryonic resorption, suggesting a severe defect independently of an altered iron homeostasis. Mice specifically depleted for frataxin in muscle and neuronal tissues through a conditional gene-targeting approach are viable and accumulate iron only late after the inactivation of SDH (32).

Several model systems have been developed in order to elucidate the cellular role of frataxin and the mechanisms leading to the FRDA phenotype. The present study is the first to apply the RNAi technique for frataxin depletion and for examination of the consequences on the function of Fe/S proteins in mammalian cells. The HeLa cell culture system described here complements existing patient cell culture (44), yeast (1) and animal models (26,32), and facilitates the investigation of the initial cellular events during the pathogenesis of FRDA. Studies on biopsies or cultured cells from FRDA patients are only of limited significance to address the question of the primary frataxin function because the protein is deficient for years, precluding the discrimination between primary and secondary effects of frataxin deficiency. Human frataxin has been shown to be a functional homologue of yeast Yfh1p (11). However, depletion of Yfh1p does not allow the discrimination between the time of occurrence of decreased formation of ISCs and mitochondrial iron accumulation, since in yeast both effects occur simultaneously (18), indicating that the regulation of intracellular iron distribution in yeast and mammals is different.

Both the mouse and the cell culture models strongly indicate that the entry point into the vicious cycle of Fe/S protein deficiency and mitochondrial iron accumulation in FRDA is due to an impaired formation of ISCs and not due to alterations in cellular iron metabolism. Future efforts for the treatment of FRDA therefore may have to be focussed on the ISC assembly process. Approaches on the basis of primary cultured cells similar to our RNAi depletion model for FRDA might help to develop new therapies for this mitochondrial disease. The HeLa cell culture model introduced here will be invaluable for investigating other members of the ISC biogenesis machinery in mitochondria and the cytosol and for testing the similarities and differences to the yeast system.

MATERIALS AND METHODS

Vector design

Frataxin was depleted in human HeLa cells by RNAi using a pSUPER vector-based (hFraRNAi) approach (33). The 19 nt
long frataxin gene-specific targeting sequence (position 167–185 of the coding sequence; GTTCGAAACCGTGGCCT) was separated by a short spacer (TTCAAGAGA) from the reverse complement of the same sequence. The 64 nt long oligonucleotides containing the coding and complementary sequence of these components, together with BglII and HindIII restriction site sequences at the 5' and 3' ends, respectively, were synthesized (Metabion, Martinsried) and hybridized. This short double-stranded DNA was cloned into the pSUPER vector via the BglII and HindIII restriction sites. Expression was directed by a H1 promotor and terminated by five thymidine residues.

**Cell culture, transfection and flow cytometry**

Human cervix carcinoma cells (HeLa) were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 7.5% fetal calf serum (FCS), 1 mM glutamine and 50 μg/ml gentamycin (complete DMEM). Cells grown to near confluency on an area of 40 cm² (~15 x 10⁶ cells) were harvested by trypsinization, washed twice in PBS, resuspended in 500–600 μl transfection buffer (21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄ and 6 mM dextrose) (45) and supplemented with 15–22.5 μg of each of the required plasmids. All cells were co-transfected with the pEGFP-N3 vector (Clontech, Heidelberg) in order to determine the transfection efficiency in combination with either the hFraRNAi construct (depletion of frataxin) or the pSUPER vector (control). Transfections were carried out by electroporation (250 V, 1500 μF, 25–30 ms duration) using an EASYJect+ device. Cells were immediately cultured in complete DMEM and were re-transfected every 4th day in order to prolong the time period of frataxin depletion.

Transfection efficiency was analysed by flow cytometry (FACSscan, Becton Dickinson, equipped with a 488 nm argon laser and 530, 585 and 650 nm band pass filters), each time the cells were harvested and resulted in 80–90% EGFP⁺ cells (Fig. 1). EGFP-fluorescence was up to 500 times stronger than the autofluorescence, indicating a simultaneous strong expression of the frataxin mRNA-specific siRNAs (Fig. 1, inset). Re-transfection prolonged plasmid expression and slightly increased the overall transfection efficiency. After abortion of the transfection series, fade out of EGFP fluorescence strongly depended on the amount of DNA used for the previous transfections, explaining the large variability in the time course of fluorescence decline (Fig. 1, +4 and +8 bars).

Cell viability was determined by assessing the mitochondrial membrane potential (∆Ψ) (46) using the red fluorescent dye rhodamine 6G (R6G) at a final concentration of 275 nM. R6G is selectively accumulated by mitochondria in a ∆Ψ-dependent manner (47), allowing the evaluation of cell death-associated loss of ∆Ψ by flow cytometry.

**Immunostaining**

Human frataxin was labelled by a polyclonal rabbit antibody raised against recombinant human frataxin produced in *Escherichia coli*. Transfected HeLa cells were grown on cover slips and frataxin was detected by indirect immunofluorescence using the affinity-purified rabbit anti-frataxin serum and a TRITC-labelled goat anti-rabbit secondary antibody (Dionova). For SDS–PAGE and subsequent electroblotting, 30–50 μg HeLa cell proteins were applied. Frataxin was immunostained by the rabbit anti-frataxin serum and its amount was quantified relative to cellular α-tubulin detected by a mouse anti-alpha-tubulin monoclonal antibody (DM1α). Visualization of antigens was performed by peroxidase-conjugated secondary antibodies in combination with a chemiluminescence assay.

**Enzyme assays**

Enzyme assays were performed with whole HeLa cells that had been shock-frozen in liquid nitrogen and stored at −80°C. Aconitase activity was determined by a coupled aconitase–isocitrate dehydrogenase assay (48). SDH activity was assessed by the DCIP assay in combination with decyl ubiquinone (49). Malate dehydrogenase and citrate synthase activities were analysed as described (50,51).

**IRE-binding of IRP**

RNA-binding capabilities of IRPs (36) were determined by REMSA as described (52,53). In principle, [α-32P]CTP-labelled IREs of human ferritin mRNA were incubated with HeLa cell lysates and unbound RNA was removed by digestion with RNase T1. RNA-binding was determined after PAGE and autoradiography. RNA electrophoretic mobility supershift assays using polyclonal rabbit α-human IRP1 antisem (kindly provided by M. Hentze, EMBL, Heidelberg) demonstrated that the contribution of IRP2-bound IREs to the autoradiography-signal was ranging between 22.5 and 32.5% (data not shown). Addition of the iron chelator desferoxamine (DFO; 12.5–250 μM) to the cell culture medium increased the autoradiography-signal and reduced the contribution of IRP2-bound IRE to 10% in a DFO-concentration dependent manner (data not shown). Since the non-ISC-containing IRP2 is less abundant than the ISC-containing IRP1 (35), the amount of total IRP bound to [α-32P]CTP-labelled IRE of ferritin mRNA was an indicator for the amount of ISC associated with IRP1. As a loading control, HeLa samples were pre-treated with 2% β-mercaptoethanol in order to disassemble the ISC especially from IRP1 and to achieve maximal IRE-binding.

**Uptake, incorporation into heme and cellular concentration of iron**

HeLa cell cultures of 25 cm² were grown in complete DMEM with a reduced FCS content of 3.75%, supplemented with 150 μM ascorbate and with 1 μCi/ml ⁵⁵FeCl₃ premixed with NTA (54). After 18 h, cells were harvested and washed twice with PBS/2.5 mM EDTA. Uptake of ⁵⁵Fe into HeLa cells and incorporation into heme was determined under conditions described previously for yeast cells (55,56). Analysis of total ⁵⁵Fe content of cell lysates as a measure of iron uptake and incorporation into heme determined by butyl acetate extraction required data normalization, since both values reciprocally depended on HeLa cell culture density.
Results were normalized by plotting the values for $^{55}$Fe uptake and incorporation into heme against the corresponding cell density. For the control cells, a linear regression was calculated, and used for correction of the measurements for both uptake of $^{55}$Fe and incorporation into heme by frataxin-depleted HeLa cells. Cellular iron concentration was spectrophotometrically analysed essentially according to Loenberg et al. (57), using the dye Nitro-PAPS as an iron chelator (58) at a final concentration of 130 μM.

Electron microscopy

For electron microscopy, cells were fixed, scraped off with a rubber policeman, dehydrated and embedded in Epon according to standard procedures. Sections were investigated either by an EM 109 electron microscope (Zeiss) or by an analytical scanning and transmission electron microscope ARTEM H-600 (Hitachi) equipped with an EDX spectrometer (Kevex).

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NOTE ADDED IN PROOF

After submission of our manuscript, Craig and co-workers (59) showed using yeast YFH1 mutants that oligomerization of Yfh1 is dispensable in vivo adding to the notion that iron storage is not a critical cellular function of frataxin/Yfh1.

REFERENCES


