A Pool of Extramitochondrial Frataxin That Promotes Cell Survival*

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Frataxin is a mitochondrial protein involved in iron metabolism. Defective expression of frataxin causes Friedreich ataxia (FA), an inherited degenerative syndrome characterized by ataxia, cardiomyopathy, and high incidence of diabetes. Here we report that frataxin-deficient cells are more prone to undergo stress-induced mitochondrial damage and apoptosis, while the overexpression of frataxin confers protection to a variety of cell types. Moreover, we reveal the existence of an extramitochondrial pool of frataxin, which can efficiently prevent mitochondrial damage and apoptosis in different cellular systems. Remarkably, extramitochondrial frataxin can fully replace mitochondrial frataxin in promoting survival of FA cells.

Friedreich ataxia (FA)‡ is the most common inherited ataxia, with an estimated prevalence within the caucasian population of ~1:30,000 (1). Age at onset may range from 5 to 25 years, and loss of deambulation ensues 10–20 years from the onset. Progressive hypertrophic cardiomyopathy is a frequent cause of premature death. FA is caused by the defective expression of the mitochondrial protein frataxin, due to, in most cases, homozygous GAA triplet repeat expansion within the first intron of the FRDA gene (2).

Frataxin is required for animal development, since its complete loss results in embryonic lethality in the mouse (3) and in developmental arrest in the nematode Caenorhabditis elegans (4). Partial expression of frataxin allows organismal development and survival, yet results in progressive degeneration of specific tissues. In fact, frataxin deficiency in humans critically affects survival of large primary neurons of the dorsal root ganglia, cardiomyocytes, and pancreatic beta cells, accounting for FA syndromic features. Frataxin cooperates to the biogenesis of iron-sulfur clusters (ISC) (5, 6), important cofactors for a number of proteins. The extramitochondrial frataxin56–210 construct was synthesized into HindIII and BamHI sites of the expression vector pcDNA3 (Invitrogen). The extramitochondrial frataxin56–210 construct was synthesized by PCR using the primers 5′-CTAGGAAATTCATGTCGAACCA-3′ (EcoRI) and 5′-AGTTGGATCCGCATCA-3′ (BamHI), directly affecting energy metabolism. Frataxin itself might be involved in iron binding and storage (10), in a homopolymeric form capable of ferroxidase activity (11). Frataxin can also donate iron to ferrochelatase, which catalyzes the insertion of iron into protoporphyrin IX, thus helping in heme synthesis (12). Cells derived from FA patients have a partial defect in ISC-containing proteins, with consequent inefficient mitochondrial respiration (13), lower ATP production, and impaired iron utilization, leading to mitochondrial iron accumulation (14). This may cause higher sensitivity to oxidative stress induced by hydrogen peroxide (15, 16) or by serum withdrawal (17). Accordingly, FA patients experience oxidative stress (18, 19), and animal models of frataxin deficiency show accelerated cell death caused by enhanced oxidative stress (20, 21). In other models, however, iron accumulation and oxidative stress may have little pathogenic role (22).

Indeed, although current therapy for patients affected by Friedreich ataxia relies mostly upon the use of antioxidants, considerable controversy exists about the role of oxidative stress in FA pathogenesis (23). Here we show that an extramitochondrial form of mature frataxin autonomously contributes to oxidative stress resistance and overall cell survival.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The following reagents were used: C2-ceramide (Biomol), GD3 ganglioside, staurosporine, and carbonyl cyanide m-chlorophenylhydrazide (Sigma), Hoechst 33342, MitoTracker Red CMXRos, tetramethylrhodamine ethyl ester (TMRE), and dihydroethidium (HE) (Molecular Probes). The antibodies used were as follows: anti-frataxin clone I2G2 (Immunological Sciences), anti-voltage-dependent anion channel (VDAC) (Calbiochem), anti-Hsp60 clone LK-1 and anti-manganese-superoxide dismutase (StressGen), anti-cytchrome c clones 6H2.B4 and 7H8.2C12 (PharMingen), anti-α-tubulin (Sigma), and anti-p10 mitochondrial protein clone 2G2 (Kamiya Biomedical).

cDNA Expression Constructs—Human frataxin cDNA containing EcoRI site at 5′-end/BamHI at 3′-end was obtained by PCR from the plasmid pTLX1 (ATCC) and inserted in pIRE2-EGFP (Clontech) bicistronic expression vector. Frataxin cDNA was furthermore cloned into HindIII and BamHI sites of the expression vector pcDNA3 (Invitrogen). The extramitochondrial frataxin56–210 construct was synthesized by PCR using the primers 5′-CTAGGAAATTCATGTCGAACCA-3′ (EcoRI) and 5′-AGTTGGATCCGCATCA-3′ (BamHI), to remove the first 55 amino acids from frataxin precursor, and inserted in pcDNA3 or pIRE2-EGFP vectors. pIRE2/Bcl-2 was generously provided from P. Bonini (University of Florence, Florence, Italy). Human 3-hydroxyisobutyrate dehydrogenase cDNA MGC-40361 was obtained from ATCC and subcloned in pIRE2-EGFP.

Cell Culture and Transfections—GM15850B lymphocytes, from a clinically affected Friedreich ataxia patient, homozygous for the GAA expansion in the FRDA gene with alleles containing ~700 and 1050 repeats, and GM15851B lymphocytes from a clinically unaffected brother of GM15850, with two FRDA alleles in the normal range of GAA trinucleotide repeats, were obtained from NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research. Cells were maintained in RPMI 1640 medium supplemented with 15% FBS.
and transfected by electroporation. Briefly, 10^7 cells were incubated in 0.4 ml of RPMI 1640 for 10 min on ice with 20 μg of pcDNA3 empty vector or pcDNA3/frataxin constructs. After electroporation at 260 V, 950 microfarads, cells were left 30 min on ice and resuspended in 5 ml of RPMI 1640, 15% FBS. After 4 h, live cells were recovered by Lympholyte-H (Cedarlane Laboratories) density gradient centrifugation and replated. Stable transfecteds were obtained from cultures in selection medium containing 400–600 μg/ml G418 (Invitrogen) for 15–20 days. Human embryonic kidney HEK-293 and cervical carcinoma HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Human T-cell lymphoma HuT78, T-cell leukemia Jurkat, and bone marrow neuroblastoma SH-SY5Y cells were maintained in RPMI 1640 medium containing 10% FBS. HEK-293 cells were transfected with the calcium phosphate precipitation method: cells were plated on 10-cm dishes and transfected with 15 μg of total DNA of indicated plasmids. After 16 h the medium was replaced with a fresh one and cells harvested for protein extracts after 24 h from transfection. HuT78 cells were transfected by electroporation as described above, while for Jurkat and SH-SY5Y cells Lipofectamine 2000 (Invitrogen) was used, following the manufacturer’s instructions.

**Apoptosis, Mitochondrial Depolarization, and Reactive Oxygen Species (ROS) Production**—Apoptotic FA and healthy lymphoblasts were detected after staining with annexin V-PE (PharMingen) following the supplier’s instructions; mitochondrial inner membrane depolarization was detected after incubation of treated cells with 50 nM TMRE for 20 min at 37 °C, whereas ROS production was quantitated by incubation with 4 μM HE for 20 min at 37 °C. Apoptosis of transiently transfected cells was determined by counting Hoechst 33342-stained fragmented nuclei among GFP-positive cells. Blind counting by two investigators, of at least 300 cells, was performed for each group. ROS production in transfected SH-SY5Y cells was determined as described above, by dual color flow cytometry, analyzing red fluorescence among electronically gated green fluorescent cells.

**Analysis of Cytochrome c Release**—Lymphoblasts from FA-affected and healthy individuals were treated with 0.4 μM staurosporine, harvested after 4–6 h, and resuspended in 100 μl digitonin (50 μg/ml in phosphate-buffered saline (PBS) with 100 mM KCl) for 5 min on ice. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed three times in PBS, and incubated in blocking buffer (3% BSA, 0.05% saponin in PBS) for 1 h. After fixation overnight at 4 °C with anti-cytochrome c or anti-Hsp60 1:200 in blocking buffer, cells were washed three times and incubated for 1 h at room temperature with PE-labeled secondary anti-mouse antibody (PharMingen), diluted 1:200 in blocking buffer. Quantitative analysis was performed by flow cytometry.

**Confocal Microscopy Analysis**—HeLa cells were plated onto glass chamber slides (Lab-Tek) and grown in complete medium for an additional day after plating. Viable cultures were either first exposed for 20 min at 37 °C to 100 nM MitoTracker Red CMX diluted in control...
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FIGURE 3. Processing and localization of frataxin. A, schematic representation of the 1–210 frataxin precursor, its intermediate (42–210) processing product, and the mature (56–210) mitochondrial frataxin. MIS, mitochondrial import sequences. B, HEK-293 cells, HeLa cells, and SH-SY5Y cells were transiently transfected with empty vector (ev), the wild type frataxin (F), or the extramitochondrial frataxin56–210 (EF). The different products were visualized by SDS-PAGE and immunoblot analysis with anti-frataxin antibodies. The asterisk indicates a frataxin degradation product. C, HEK-293 cells were transiently transfected with wild type frataxin (F) or extramitochondrial frataxin56–210 (EF). Total cell lysates (T), cytosolic (C), and mitochondrial (M) fractions were analyzed by SDS-PAGE and immunoblotted with anti-tubulin, anti-VDAC, anti-cytochrome c, and anti-frataxin antibodies.

RESULTS

To investigate the role of frataxin in promoting cell survival, we took advantage of lymphoblastoid cells derived from a FA patient, which express very low levels of frataxin, and control lymphoblastoid cells derived from a healthy brother of the patient (Fig. 1A). Lymphoblasts were exposed to death-inducing doses of ceramide, as a general and conserved mediator of cellular stress responses (24, 25), to ganglioside GD3, which specifically targets mitochondria and promotes apoptosis (26, 27), to the kinase inhibitor staurosporine and to UVB. Fig. 1B shows that FA lymphoblasts were more sensitive to ceramide, to GD3, and to staurosporine, as measured by apoptosis induction, than their related controls. However, frataxin deficiency did not affect their sensitivity to UVB (over a wide range of doses; data not shown). A similar spectrum of sensitivity was observed when mitochondrial inner membrane depolarization (Fig. 1C) and production of ROS (Fig. 1D) were measured. Importantly, FA lymphoblasts released cytochrome c from mitochondria more readily than their healthy controls, when exposed to staurosporine (Fig. 2). Collectively, these results indicate that frataxin-deficient cells are more sensitive to several, yet not all, major inducers of cellular stress.

We then directly addressed the ability of frataxin to suppress apoptosis by overexpressing frataxin in different cell lines. Frataxin is normally synthesized as a 210-amino acid precursor. Subsequently, N-terminal mitochondrial localization sequences allow the import of the precursor into the mitochondria, where a mitochondrial processing peptidase (MPP) sequentially cleaves the precursor after Gly41 and Ala55 releasing an intermediate product, and eventually the 155-amino acid mature, functional mitochondrial frataxin (28, 29) (Fig. 3A). To verify whether the mitochondrial localization is obligatory for its function, we generated a frataxin deletion mutant, extramitochondrial frataxin56–210, lacking the first 55 residues, including the N-terminal mitochondrial import sequences. This mutant therefore corresponds to the mature frataxin and cannot be recruited into the mitochondria. Although this mutant was produced according to the reported MPP cleavage site (RA55S) yielding the mature product (28), when transiently expressed in several

medium or directly fixed for 15 min with 4% paraformaldehyde in PBS. Coverslips were rinsed once with PBS and permeabilized for 30 min at 37 °C with 60 μM digitonin, followed by a blocking step, with PBS containing 10% FBS, overnight at 4 °C. Samples were then incubated for 1 h at 37 °C with 1:100 dilution of anti-frataxin mAb, anti-p110 mitochondrial protein mAb or anti-Hsp60 mAb, followed by secondary BODIPY-conjugated goat anti-mouse antibody (Molecular Probes), washed, and mounted on glass slides with Prolong Antifade (Molecular Probes). Confocal images were acquired by using a PCM-2000 confocal microscope (Nikon) and were then processed by using EZ C1 software (Nikon).

Subcellular Fractionations—To isolate mitochondrial and cytosolic fractions, cells were harvested, washed twice with ice-cold PBS, and resuspended into an ice-cold isotonic buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin). Human peripheral blood mononuclear cells from different healthy donors were isolated by lymphoprep density gradient centrifugation at 800 × g and treated as above. Cells were then passed through a 30-gauge syringe needle several times. The cell suspension was centrifuged at 700 × g for 10 min at 4 °C to eliminate nuclei and unbroken cells. The supernatant was further centrifuged twice at 10,000 × g for 30 min at 4 °C and collected as a cytosolic fraction. The heavy membrane pellet, which contained mitochondria, was washed in the isotonic buffer and resuspended in a hypotonic buffer (PBS containing 1% Triton X-100) supplemented with protease inhibitors. To partition the cytosolic fractions into P100 and S100 fractions, further centrifugations were performed at 100,000 × g for 1 h at 4 °C. For total protein extracts, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA) supplemented with protease inhibitors. Total, mitochondrial, and cytosolic fractions were then analyzed by Western blotting and revealed by ECL (Amersham Biosciences).
cell lines it migrated slightly slower than mature frataxin proteolytically generated from the wild type construct (Fig. 3B). The latter corresponds to the genuine mature product, since it co-migrated with the endogenous frataxin present in each cell line. To verify the compartmentalization of the frataxin products, the wild type and the extramitochondrial frataxin56–210 were transiently expressed in HEK-293 cells, and cell fractionations were performed. SDS-PAGE and Western analysis of the fractionated cell lysates confirmed that the cytosolic protein tubulin partitioned within the cytosolic fractions and that the mitochondrial VDAC and cytochrome c associated with the mitochondrial fractions. As expected, the extramitochondrial frataxin56–210 generated a product that did not associate with the mitochondria (Fig. 3C). Unexpectedly, however, the mature product from wild type frataxin was not only found associated with the mitochondria but was also found in the cytosolic fraction (Fig. 3C).

The T-cell lymphoma HuT78 cells undergo massive apoptosis when exposed to ceramide. We found that the transient expression of frataxin protects HuT78 cells toward ceramide-induced apoptosis, to the extent achieved by the transient expression of the anti-apoptotic protein Bcl-2 (Fig. 4A). The overexpression of a different mitochondrial protein, 3-hydroxyisobutyrate dehydrogenase, was ineffective. HuT78 cells also undergo apoptosis in response to endogenous GD3 accumulation, as induced by the transient expression of GD3 synthase (ST8). The simultaneous co-expression of frataxin prevented ST8-induced apoptosis, similarly to the protection conferred by Bcl-2 co-expression (Fig. 4B). These findings were
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extended to additional cellular systems, such as the lymphoid cell line Jurkat and the neuroblastoma cell line SH-SY5Y. In both cases the transient expression of frataxin significantly suppressed ceramide-induced apoptosis (Fig. 4C). Both in Jurkat and in SH-SY5Y cells, however, frataxin could not block UVB-induced apoptosis.

Quite unexpectedly, in all instances the expression of the extramitochondrial frataxin\textsuperscript{56–210} was at least as effective in apoptosis protection as the expression of wild type frataxin (Fig. 4, A–C). Moreover, as detected by hydroethidine staining and FACS analysis, extramitochondrial frataxin\textsuperscript{56–210} transiently expressed in SH-SY5Y neuroblastoma cells suppressed ceramide-induced ROS production as efficiently as wild type frataxin (Fig. 4D).

Together these results suggest that mature frataxin may not be only confined to the mitochondria and that an extramitochondrial pool of frataxin can control ROS production and apoptosis. To exclude that the presence of mature frataxin outside the mitochondria observed in HEK-293 cells (Fig. 3C) was due to forced expression, we investigated the distribution of endogenous frataxin by immunostaining and confocal microscopic analysis in HeLa cells. Fig. 5 shows that a substantial amount of frataxin does not co-localize with the mitochondria, as revealed by simultaneous MitoTracker staining, while complete co-localization can be detected between mitochondria and the p110 mitochondrial protein or the mitochondrial matrix protein HSP60.

To verify that the non-mitochondrial endogenous frataxin corresponded to the endogenous \textapprox 17-kDa mature product, different cell lines were subjected to cell fractionation followed by SDS-PAGE and immunoblot analysis. Fig. 6A shows that in HeLa cells, tubulin was recovered in the cytosolic fraction, while VDAC, the mitochondrial matrix chaperone HSP60, the mitochondrial matrix manganese-dependent superoxide dismutase, or cytochrome c were recovered in the mitochondrial fraction. Frataxin, however, was recovered both in the mitochondrial and in the cytosolic fractions. Further fractionation of the cytosolic fractions at 100,000 \times g revealed that extramitochondrial frataxin is mostly distributed into the S100-soluble cytosol (data not shown). Frataxin was similarly found in the cytosolic fraction in two additional cell lines, SH-SY5Y and Jurkat, as well as in resting peripheral blood mononuclear cells from normal donors (Fig. 6B). In all instances, the cytosolic fractions contained frataxin, and its molecular size corresponded to the mature mitochondrial frataxin.

To further analyze the functional capability of extramitochondrial frataxin, both wild type and extramitochondrial frataxin\textsuperscript{56–210} were stably expressed into the frataxin-deficient FA lymphoblasts (Fig. 7A). Stable transfectants were then exposed to ceramide and staurosporine. As shown in Fig. 7, the expression of the extramitochondrial frataxin\textsuperscript{56–210} was at least as effective as the expression of the wild type frataxin in granting protection to ceramide, measured by triggering of apoptosis (Fig. 7B), induction of mitochondrial inner membrane depolarization (Fig. 7C), and production of ROS (Fig. 7D). Similarly, protection was conferred by extramitochondrial frataxin\textsuperscript{56–210} to FA cells in response to staurosporine (Fig. 7, B–D). Therefore, extramitochondrial frataxin can functionally substitute for mitochondrial frataxin in promoting cell survival.

DISCUSSION

Here we show that a significant pool of mature frataxin not associated with the mitochondria exists in different cell types. Extramitochondrial frataxin is functional, as its expression can prevent stress-induced ROS accumulation and apoptosis in transformed cells. Moreover, it can fully replace mitochondrial frataxin in restraining stress-induced mitochondrial damage and apoptosis induction in FA cells.

We found that frataxin deficiency sensitizes cells to stress-induced apoptosis and that, perhaps more importantly, the enhanced expression of frataxin suppresses stress-induced apoptosis in different cellular systems. In particular, frataxin appears to efficiently suppress mitochondria-mediated apoptosis induced by staurosporine and by ceramide or GD3 ganglioside. These two structurally related lipid mediators of cellular stress, upon intracellular accumulation, cause mitochondrial membrane damage associated with ROS production and release of...
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apoptotic factors such as cytochrome c and apoptosis-inducing factor and activated caspase 9 (26, 27). Surprisingly, mitochondrial damage and apoptosis appear efficiently protected also by an extramitochondrial pool of mature frataxin.

Proteolytic processing by MPP is required for the production of a mature and functional frataxin (28). Although not directly addressed by the present work, it seems likely that extramitochondrial frataxin is exported from the mitochondria in its mature form, after processing by the MPP. Frataxin may therefore behave similarly to other nuclear-encoded proteins, which enter the mitochondria for maturation and are later exported (30), although the mechanisms of export and targeting to extramitochondrial location are still largely obscure.

An extramitochondrial re-localization of frataxin has been very recently described during the enterocyte-like differentiation of the human colon adenocarcinoma cell line Caco-2 (31). In this system, extramitochondrial frataxin was found to associate with IscU1, a component of the ISC assembly machinery. Frataxin is in fact involved in the biosynthesis of ISC, inorganic prosthetic groups contained in a variety of proteins (7). In mammalian cells, frataxin deficiency affects both mitochondrial and cytosolic ISC-containing proteins (6, 8, 32). ISC bio-

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