RNA Silencing of Mitochondrial m-Nfs1 Reduces Fe-S Enzyme Activity Both in Mitochondria and Cytosol of Mammalian Cells

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In prokaryotes and yeast, the general mechanism of biogenesis of iron-sulfur (Fe-S) clusters involves activities of several proteins among which IscS and Nfs1p provide, through cysteine desulfurization, elemental sulfide for Fe-S core formation. Although these proteins have been well characterized, the role of their mammalian homolog in Fe-S cluster biogenesis has never been evaluated. We report here the first functional study that implicates the putative cysteine desulfurase m-Nfs1 in the biogenesis of both mitochondrial and cytosolic mammalian Fe-S proteins. Depletion of m-Nfs1 in cultured fibroblasts through small interfering RNA-based gene silencing significantly inhibited the activities of mitochondrial NADH-ubiquinone oxidoreductase (complex I) and succinate-ubiquinone oxidoreductase (complex II) of the respiratory chain, with no alteration in their protein levels. Activity of cytosolic xanthine oxidase, which holds a [2Fe-2S] cluster, was also specifically reduced, and iron-regulatory protein-1 (IRP-1)3 acts as a cytosolic aconitase, whereas the apoprotein regulates translation of ferritin and transferrin receptor mRNAs by binding to iron-responsive elements in their 5'- and 3'-untranslated regions, respectively (4). Consequently, the IRP-1 [4Fe-4S] cluster, which has the ability to sense cellular iron status and the level of NO or reactive oxygen species, is the key regulator allowing the protein to commute between its two (aconitase/IRE binding) activities (7, 8). These numerous examples clearly show that Fe-S clusters are critical regulatory factors in controlling the functions of many proteins involved in various cellular metabolisms, and therefore it is essential to understand their de novo biogenesis.

The first insights into Fe-S cluster biogenesis have emerged from elegant studies on the metallocluster formation of A. vinelandii nitrogenase (9). In prokaryotes, Fe-S clusters are assembled in vivo through three multiprotein systems, referred to as ISC, NIF, and SUF (10). Within the ISC system, the proteins IscS and IscU are essential for the construction of the Fe-S core. The cysteine desulfurase IscS provides inorganic sulfide from L-cysteine, which is subsequently transferred to a nascent Fe-S cluster on the scaffold protein IscU (11, 12). This ISC machinery also exists in yeast and is exclusively localized in the mitochondria (13, 14). Once synthesized in this compartment, Fe-S clusters are incorporated into mitochondrial apoproteins. A mitochondrial ABC transporter, named Atm1p, probably exports assembled Fe-S clusters or at least components required for Fe-S assembly in the cytosol (15, 16). Based on knowledge gained in prokaryotes and lower eukaryotes, the first human and mouse genes homologous to the ISC machinery have recently been cloned (17–20). Among these, mouse m-Nfs1, which presents 57% identity with the E. coli IscS and yeast Nfs1p, was the first IscS-like protein identified in a mam-

Iron-sulfur (Fe-S) clusters are metalloprosthetic groups incorporated into proteins that serve a wide range of vital cellular functions (1, 2). These include electron transfer in oxidative phosphorylation of the respiratory chain, enzymatic catalysis of dehydratases, and stabilization of protein structure. Another important function ascribed to Fe-S clusters is the regulation of gene expression (3, 4). This role is well illustrated by the [4Fe-4S] cluster in fumarate nitrate reduction, which is an oxygen sensor controlling the synthesis of proteins required for anaerobic respiration in Escherichia coli (5). The [2Fe-2S] cluster of the bacterial transcription factor SoxR is also a good example of a gene regulator through its redox status or dinutryl iron conversion in response to oxygen and nitric oxide (NO), respectively (6). In mammals, Fe-S clusters can also be used to regulate expression of genes involved in iron homeostasis. When holding a [4Fe-4S] cluster, IRP-1 acts as a cytosolic aconitase, whereas the apoprotein regulates translation of ferritin and transferrin receptor mRNAs by binding to iron-responsive elements located in their 5'- and 3'-untranslated regions, respectively (4). Consequently, the IRP-1 [4Fe-4S] cluster, which has the ability to sense cellular iron status and the level of NO or reactive oxygen species, is the key regulator allowing the protein to commute between its two (aconitase/IRE binding) activities (7, 8). These numerous examples clearly show that Fe-S clusters are critical regulatory factors in controlling the functions of many proteins involved in various cellular metabolisms, and therefore it is essential to understand their de novo biogenesis.

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3 The abbreviations used are: IRP-1, iron-regulatory protein-1; siRNA, small interfering RNA; IRE, iron-responsive element; ISC, iron-sulfur cluster; complex I, NADH-ubiquinone oxidoreductase; complex II, succinate-ubiquinone oxidoreductase; mt-aconitase, mitochondrial aconitase.
malian system. m-Nfs1, which has a typical mitochondrial targeting presequence in its N-terminal end, has been found in the matrix of mouse liver mitochondria (17). In addition, the pyridoxal phosphate binding site and the cysteine residue essential for cysteine desulfuration have been conserved, which makes m-Nfs1 a plausible candidate for assembling Fe-S clusters in mouse proteins.

Functional studies of ISC machinery components in mammals have only recently begun (21–23). In the murine system, we have previously shown that Fe-S clusters of both cytosolic IRP-1 and mitochondrial aconitase (mt-aconitase), which are completely disassembled in cells exposed to NO, are rapidly repaired after shutting off NO flux (24). In addition, Fe-S cluster reassembly/insertion into these two proteins is dependent on mitochondria. The fact that bacterial IscS and yeast Nfs1p are among the most essential proteins in Fe-S cluster biogenesis is an exciting development for functional studies of the mammalian m-Nfs1. As yet, to our knowledge, no functional studies have linked the biosynthesis of Fe-S clusters to any mammalian cysteine desulfurase in living cells. To address this issue, we took advantage of siRNA technology to deplete m-Nfs1 in murine NIH3T3 fibroblasts (25). After achieving m-Nfs1 silencing, we observed an overall decrease in the activity of several mitochondrial Fe-S enzymes of the respiratory chain and the Krebs cycle without change in their protein levels. In addition, specific loss of Fe-S protein activities was also detected in the cytosol, and the siRNA-derived phenotype occurred even earlier in this compartment than in mitochondria. Moreover, depletion of m-Nfs1, which activated apo-IRP-1 for ferritin IRE binding, led to the down-regulation of the iron storage protein ferritin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal antibodies against complex I subunit NDUFS3 (NADH dehydrogenase ubiquinone Fe-S protein) and complex II subunit b (the Fe-S subunit of complex II) were from MitoSciences (Eugene, OR). Antibodies were kindly provided by Dr. R. B. Franklin (University of Maryland, Baltimore, MD) (mt-aconitase), Dr. J. Brock (Glasgow University, Glasgow, UK) (l-ferritin), and Dr. S. A. Anderson (University of Wisconsin, Madison, WI) (IRP-1). The rabbit anti-mouse m-Nfs1 affinity-purified polyclonal antibody was raised against the peptide sequence AQQEMEYDHKRISKL, corresponding to the amino acid residues 312–327 (Agro-Bio, La Ferté Saint-Aubin, France). All other chemicals were from Sigma unless otherwise stated.

**siRNA Preparation**—The targeting sequences of m-Nfs1 (accession number NM_010911), corresponding to the coding regions 589–610 AAGAGCGGGATCATTTGACTTA referred to as m-Nfs1-1, 727–748 AAGGTGACTTCCACACTGAT referred to as m-Nfs1-2, 768–789 AATCCCACTTGACGTA referred to as m-Nfs1-3, and 1008–1029 AAGCGGATCTCAAAGTTAGCA referred to as m-Nfs1-4, were designed using the Qiagen algorithm. Negative control siRNA, which has no homology with any mammalian sequence (AAT-TCTCCGAACTGTCAGGT), was from Qiagen-Xeragon. Annealed and deprotected double-stranded siRNAs were resuspended in 30 mM HEPES/KOH, pH 7.4, 100 mM potassium acetate, and 2 mM magnesium acetate (Qiagen-Xeragon) to obtain a 20 μM solution.

**Cell Culture and Transfection**—Murine NIH3T3 fibroblasts (ATCC clone CRL-1658) were kindly provided by Dr. D. Weil (Institut André Lwoff, Villejuif, France) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO2. The RAW 264.7 macrophage cell line (ATCC, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and gentamycin. NIH3T3 and RAW 264.7 cells were transfected with siRNAs by the calcium phosphate precipitation method (26) and the TransIT-TKO transfection reagent (Mirus), respectively. The absence of cellular damage after 48 h of transfection was monitored by flow cytometry using the nucleic acid dye propidium iodide. A titration experiment using m-Nfs1-specific siRNA (5, 15, 25, 50, 100 nM) was performed. Silencing was effective (~50% reduction) from 5 nM and was maximum at 100 nM. Negative control siRNA had no effect on m-Nfs1 expression under the same experimental conditions.

**Preparation of Mitochondria-enriched Fractions and Cytosolic Extracts**—RAW 264.7 macrophages and NIH3T3 fibroblasts (5 × 106/ml) were harvested at the indicated times after transfection and resuspended in 0.25% sucrose with 100 mM HEPES, pH 7.4, in the presence of an anti-protease mixture (1:100 final dilution; Calbiochem). Cells were then treated with 0.007% digitonin, and the lysate was centrifuged at 10,000 × g for 10 min at 4 °C. After several washes in sucrose/HEPES buffer, the resulting mitochondria-enriched pellet was lysed in 100 mM Tris, pH 7.5, 0.5% Triton X-100 plus protease inhibitors for 20 min at 4 °C. The lysate was then centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was either immediately tested for mitochondrial aconitase activity or aliquoted and kept at ~80 °C for further measurements.

Cytosolic extracts were prepared from digitonin-treated cells. The supernatant from lysate that was centrifuged at 10,000 × g was subsequently centrifuged at 100,000 × g for 1 h at 4 °C to remove any particulate material. The cytosolic extracts were aliquoted and kept at ~80 °C for further measurements.

**Immunoblot Analysis**—Equal amounts of proteins (30 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Specific proteins were detected by immunoblotting with the indicated primary antibodies and peroxidase-conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark). Blots were developed with an enhanced chemiluminescence detection system (Super Signal; Pierce). The protein content in the samples was estimated using the Bio-Rad protein assay with bovine serum albumin as a standard.

**Immunofluorescence**—NIH3T3 cells grown on microscopy slides were labeled with a mitochondrion-specific probe (MitoTracker Red® CMXRos; Molecular Probes). Cells were subsequently fixed and permeabilized in acetone for 10 min at 4 °C. After rehydration in phosphate-buffered saline, the cells were incubated in phosphate-buffered saline, 1% bovine serum albumin solution for 10 min at room temperature to block nonspecific binding. After several washes in phosphate-buffered saline, cells were incubated with a rabbit anti-mouse m-Nfs1
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antibody (1:100) and then with an fluorescein isothiocyanate mouse anti-rabbit antibody (1:20). Microscopy slides were mounted in Citifluor mounting medium (Citifluor, London, UK). Immunofluorescence was imaged on a Nikon TE 2000-E coupled with a Nikon digital camera DXM 1200 F.

Quantitative Reverse Transcription-PCR Analysis—Total RNA was extracted using the SV total RNA isolation kit (Promega). Quantitative real time PCR was performed using the Roche Light Cycler System and the FastStart DNA master DNA SYBR Green I kit (Roche Applied Science). cDNAs were prepared from 2 μg of total RNA using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Eurobio, Les Ulis, France) according to the manufacturer’s instructions. m-Nfs1 sequence-specific primers were designed to be upstream and close to the m-Nfs1 siRNA putative binding site (forward, CATTGCCCTGTTCCACTAGA; reverse, TGATGAGTGCGAGAATGAG). In addition, ferritin (forward, TCAGGCATTCGCCTGTTCACTCAGA; reverse, TGCATGAGTCGCTGCTGTTCACTGA) and IRP-1 primers (forward, CAGCCACCCAGTCCAAATGATG; reverse, GCCTGGAATACATCAAGGG) were designed to span intron-exon boundaries to generate around 100-bp amplicons. The generation of specific PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. Values were normalized to the relative amounts of glyceraldehyde-3-phosphate dehydrogenase cDNA (forward, GAGTATCTCTTTG; reverse, TGCCTAGTGGCTTGAGCGAGAATGAG).

Enzyme Activities—Enzyme activities were measured in cytosolic and mitochondria-enriched fractions. Complex I and complex II activities were measured as described by Rustin et al. (27). Briefly, complex I activity was determined spectrophotometrically at 340 nm by following the reduction of NADH at 37 °C in 50 mM Tris/HCl, pH 8, containing 5 mg/ml bovine serum albumin and 200 μM KCN. A sample containing 0.3 μM rotenone, a specific inhibitor of complex I, was used as the negative control. Complex II activity was measured spectrophotometrically at 600 nm by following the reduction of 2,6-dichlorophenolindophenol at 37 °C in the presence of inhibitors of complexes I, III, IV, and V. A sample containing 10 mM malonate (a specific inhibitor of complex II) was used as the negative control. Malate dehydrogenase activity was assessed according to Siegel and Englard (28). Mitochondrial and cytosolic aconitases were measured as described by Drapier and Hibbs (29). Xanthine oxidase and lactate dehydrogenase activities were determined using the Amplex Red xanthine/xanthine oxidase assay kit (Invitrogen) and the LD-L 10 kit (Sigma), respectively.

Electrophoretic Mobility Shift Assay—IRP-IRE interactions were analyzed as described (30, 31). Two μg of cytosolic proteins were incubated with an excess of [α-32P]UTP-labeled ferritin IRE probe in 10 mM HEPES, 40 mM KCl, 3 mM MgCl2, and 5% glycerol. In parallel experiments, samples were treated with 2% 2-mercaptoethanol to allow full expression of the IRE binding activity. The IRP-IRE complexes were resolved on 6% non-denaturing polyacrylamide gels, and radioactive bands were quantified with a PhosphorImager using ImageQuant software (Amersham Biosciences).

Detection of Carbonylated Proteins—Oxidized proteins were detected with 2,4-dinitrophenylhydrazine to derivatize protein carbonyl groups followed by immunoblot analysis using primary polyclonal antibodies to the 2,4-dinitrophenol moiety (Chemicon International).

Statistical Analysis—Student’s t test was performed using Excel (Microsoft). Results are expressed as mean ± S.E. A p value of <0.05 was defined as significant, and asterisks are used to denote significance as follows: * , p < 0.05; ** , p < 0.01; *** , p < 0.001.

RESULTS

Localization of m-Nfs1 in Murine NIH3T3 Fibroblasts—Like the yeast Nfs1p homolog, m-Nfs1 possesses a typical mitochondrial targeting sequence at its N terminus, which predicts a mitochondrial localization (17). First, to assess its localization in our cellular model, m-Nfs1 protein levels were examined in different cellular fractions of murine NIH3T3 fibroblasts by immunoblot analysis. We found that m-Nfs1 was present in the mitochondrial fraction, whereas no labeling was detected in the cytosol (Fig. 1A). The single band identified at...
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47 kDa in the immunoblot corresponded to the mature protein size described in the literature (17). Reliability of the subcellular fractionation was assessed by using mitochondrial aconitase and vinculin as mitochondrial and cytosolic markers, respectively (Fig. 1A, middle and bottom panels). In addition, NIH3T3 cells were costained with an anti-m-Nfs1 antibody and the mitochondrion-selective dye MitoTracker Red® and were subsequently evaluated by using fluorescence microscopy. We showed that m-Nfs1 staining presented a distinct punctate distribution similar to that of mitochondria and colocalized with the mitochondrial marker (Fig. 1B, left panels). Importantly, immunostaining of the same cell line with preimmune IgGs showed no mitochondrial labeling, thus confirming the specificity of staining for m-Nfs1 (Fig. 1B, right panels).

siRNA Silencing of m-Nfs1 Expression in NIH3T3 Cells—To investigate the participation of m-Nfs1 in assembling Fe-S clusters in mammalian cells, four siRNAs (m-Nfs1-1, m-Nfs1-2, m-Nfs1-3, and m-Nfs1-4) directed against distinct regions of the m-Nfs1 coding sequence were synthesized. To test the specificity of silencing, a negative control siRNA with no homology to any mammalian sequence was used. The double-stranded siRNAs at a concentration of 100 nM were transfected into cells that were subsequently cultured for 48 h. After this incubation time, both total RNAs and mitochondrial fractions were isolated and analyzed by quantitative PCR and immunoblot, respectively. As shown in Fig. 2A, all four m-Nfs1 siRNAs were effective in reducing m-Nfs1 mRNA levels by at least 50%, the third being the most potent with a 75% reduction. m-Nfs1 protein was also efficiently depleted by the four specific siRNAs (Fig. 2B, compare lanes 1 and 6 with lanes 2–5). The lower level of mitochondrial aconitase (taken here as loading control) seen in lane 6 by comparison with that of lane 1 illustrates the fact that loading was not equal but does not preclude the conclusion that cells treated with control siRNA (lane 6) and nontransfected cells (lane 1) exhibited a similar level of m-Nfs1 (see also supplemental Fig. S2). The m-Nfs1 level was reduced by at least 95% after 48 h of specific siRNA treatment, corresponding to the 95% transfection efficiency measured by flow cytometry using a rhodamine-labeled negative control siRNA (Fig. 2C). The lack of m-Nfs1 did not negatively influence the viability of the cells at least for 48 h, as determined by propidium iodide exclusion flow cytometry experiments (data not shown). Taking all of these data into consideration, all subsequent experiments were performed using m-Nfs1-3 siRNA to achieve the best m-Nfs1 silencing.

Role of m-Nfs1 in the Activity of Mitochondrial Fe-S Cluster-containing Enzymes—We then asked whether m-Nfs1 has a functional role in assembling mitochondrial Fe-S clusters. We thus performed an efficient silencing of m-Nfs1 over 7 days by transfecting NIH3T3 cells with m-Nfs1-3 siRNA every 48 h. Mitochondrial fractions of cells were obtained after 2, 4, and 5 or 7 days of siRNA treatment and analyzed by immunoblot for m-Nfs1. We first checked that cell transfection with control siRNA did not have any detectable effect on m-Nfs1 expression or on mitochondrial aconitase activity (used as an Fe-S protein reporter) when compared with untransfected cells (supplemental Fig. S3). After 7 days of m-Nfs1 silencing, cell density was lower, indicated by a consistent half-decrease in protein concentration in lysates from m-Nfs1-deficient cells as compared with control cells (data not shown). As shown in Fig. 3 (upper panel), m-Nfs1 silencing was strong and constant over the 7-day period, whereas no effect was observed using negative control siRNA. The activity of several mitochondrial Fe-S cluster-containing proteins was subsequently measured. We observed a significant decrease in the specific activities of three Fe-S cluster-containing enzymes: complex I by 32% (p < 0.05), complex II by 57% (p < 0.01), and mt-aconitase by 27% (p < 0.01) from 4 days of m-Nfs1 deficiency (Fig. 4). These activities were kept low or even declined (42%, p < 0.05; 39%, p < 0.001; and 40%, p < 0.01, respectively) after 5 or 7 days of silencing. Importantly, no change was detectable in the activity of malate dehydrogenase, which does not contain Fe-S clusters, upon m-Nfs1 depletion. The loss of activity of mitochondrial Fe-S cluster-containing proteins did not result from impaired synthesis or import of these proteins into mitochondria, since no changes in Fe-S protein levels were observed by immunoblot analysis (Fig. 3, lower panels).
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Figure 3. m-Nfs1 siRNA does not affect the steady-state level of mitochondrial Fe-S cluster-containing enzymes. NIH3T3 cells were transfected with m-Nfs1-3 or negative control siRNA for the indicated times. Then, mitochondria-enriched fractions were prepared, and proteins were separated on 10% SDS-PAGE. Blots were subsequently incubated with antibodies against m-Nfs1, subunit NDUFS3 of complex I, subunit SDHB of complex II, and mitochondrial Fe-S cluster-containing enzymes. The experiments were performed at least three times, and a representative result is shown.

| Control siRNA | m-Nfs1-3 |
|---------------|----------|
| 50 kDa        | m-Nfs1   |
| 37 kDa        | NDUFS3   |
| 37 kDa        | SDHB     |
| 75 kDa        | mt-aconitase |

Transfection number (days after 1st transfection)

FIGURE 3 (24). Therefore, the potential role of m-Nfs1 in the biosynthesis of cytosolic Fe-S-containing proteins was carefully examined in our mammalian cell model. To address this question, NIH3T3 cells were transfected with negative control or m-Nfs1-siRNAs and maintained in culture for 2 and 4 days. Cytosolic fractions were prepared, and activities of [4Fe-4S]-containing aconitate (IRP-1) and [2Fe-2S]-containing xanthine oxidase were measured spectrophotometrically and fluorometrically, respectively. Our results show a rapid and dramatic decrease in aconitate activity of IRP-1 (by 71% after 2 days of m-Nfs1 depletion), which continued to decline by 79% after 4 days (Table 1). Likewise, xanthine oxidase activity was inhibited by 40 and 65% in m-Nfs1-deficient cells after 2 and 4 days, respectively. Protein levels were analyzed to determine whether loss of cytosolic Fe-S-containing protein activities was dependent on protein loss or defective Fe-S cluster assembly. Intriguingly, whereas the xanthine oxidase level was not significantly lower in specific m-Nfs1-siRNA treated cells as compared with its respective control, a 50% decrease in IRP-1 protein content was observed in m-Nfs1-silenced cells, as compared with the protein level in nonsilenced cells (Fig. 5, A and B). Nevertheless, despite the fact that IRP-1 protein level diminished, a significant increase in the RNA-binding capacity of IRP-1 was detected upon m-Nfs1 depletion, which positively signifies that IRP-1 was deprived of its Fe-S cluster (Fig. 6A). We also observed that IRE-binding activity of IRP-2, the second iron-regulatory protein that does not contain an Fe-S cluster, was low in NIH3T3 cells and did not change in cytosolic extracts from negative control and m-Nfs1-siRNA-treated cells. Finally, we observed no alteration in the activity of cytosolic lactate dehydrogenase, which does not contain an Fe-S cluster, upon m-Nfs1 deficiency (Table 1). We also performed efficient m-Nfs1 silencing in a macrophage cell line, another cell type that expresses m-Nfs1 in the mitochondrial compartment with no trace detectable in the cytosol, and examined cytosolic aconitate activity (Fig. 7, A and B). As shown in Fig. 7C, cytosolic aconitate activity declined in m-Nfs1-deficient macrophages after 48 h and was still low after 72 h of silencing, whereas no change in this activity was observed in nontransfected and control siRNA-transfected cells.

Regulation of Ferritin Expression in m-Nfs1-deficient Cells—One of the relevant features of m-Nfs1-deficient cells was the

| First transfection (2 days after first transfection) | Second transfection (4 days after first transfection) |
|-----------------------------------------------|-----------------------------------------------|
| Negative | m-Nfs1-3 | Negative | m-Nfs1-3 |
| IRP-1   | 24 ± 3  | 7 ± 4α  | 29 ± 1  | 6 ± 3α  |
| XO      | 15 ± 1  | 9 ± 0.4α | 17 ± 0.5 | 6 ± 1α  |
| LDH     | 1.9 ± 0.1 | 1.8 ± 0.1 | 1.7 ± 0.1 | 1.5 ± 0.2 |

α p < 0.01. β p < 0.001.
ability of IRP-1 to bind tightly to the ferritin IRE motif (Fig. 6A).

We therefore decided to investigate whether m-Nfs1, via IRP-1 activation, has an impact on iron metabolism by determining the ferritin protein content in cytosolic extracts from negative control and m-Nfs1-siRNA-treated cells. As shown in Fig. 6, A and B, the up-regulation of IRE-binding capacity of IRP-1 in m-Nfs1-deficient cells was accompanied by a marked reduction of ferritin levels. In addition, no regulation of the ferritin mRNA was observed after 2 days of m-Nfs1 silencing (data not shown), which therefore indicates that m-Nfs1 silencing reduced ferritin content, most likely by a post-transcriptional regulation involving the trans-regulatory activity of apo-IRP-1.

**DISCUSSION**

Our study provides the first direct evidence that m-Nfs1 is required for full activity of mitochondrial Fe-S cluster-containing enzymes. We have shown that strong reduction in the level of m-Nfs1 in murine NIH3T3 cells over a period of 7 days results in a significant decrease in the specific activities of complex I and complex II of the respiratory chain, as well as the mt-aconitase of the Krebs cycle, from 4 days of m-Nfs1 deficiency. No change was detectable in the activities of proteins that do not contain Fe-S clusters, pointing to the specificity of action of m-Nfs1 on Fe-S-containing enzymes. Importantly, the loss of Fe-S protein activities occurred without significant
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The majority of Fe-S cluster-containing proteins are located within the mitochondria of mammalian cells, but some are also present in other cellular compartments. In the cytosol, xanthine oxidase activity was strongly reduced upon m-Nfs1 silencing, and IRP-1 accumulated as an IRE-binding protein through its conversion from Fe-S aconitase to an apo-form. This conversion was not due to oxidative stress-induced Fe-S disruption upon m-Nfs1 deficiency, since no increase in oxidized proteins was observed as compared with nontransfected and control siRNA-transfected cells (supplemental Fig. S7). Interestingly, unlike all other Fe-S proteins studied here, the level of apo-IRP-1 was decreased upon m-Nfs1 deficiency. This was confirmed by a similar decrease in the total IRP-1 IRE binding capacity obtained in the presence of 2% 2-mercaptoethanol. These results suggest a role for m-Nfs1 in IRP-1 biosynthesis or that the apo-form of IRP-1 may be more susceptible to proteolysis than its holo counterpart. Eisenstein and co-workers (33) provided evidence that the absence of clusters means that apo-IRP-1 is more accessible to several proteases and thus more susceptible to degradation. In support of this argument, downregulation and increased sensitivity to degradation of apo-IRP-1 have been proposed in several other reports (34–36). IRP-1 protein loss would be a secondary consequence of Fe-S cluster defects resulting from m-Nfs1 depletion. Finally, we reveal that m-Nfs1 deficiency affects the two main types of Fe-S clusters in mammals. Thus, activity of both xanthine oxidase, which contains a [2Fe-2S] cluster, and aconitase, a [4Fe-4S] enzyme, was reduced upon m-Nfs1 depletion. Altogether, these data demonstrate that m-Nfs1 plays an important and specific role in the maintenance of both mitochondrial and cytosolic Fe-S protein activities.

Rouault and co-workers (18) have identified NifS proteins (homologous to m-Nfs1) in the cytosol and the nucleus of human cells, suggesting that Fe-S cluster assembly is compartmentalized in mammalian cells. These isoforms of human NifS are smaller than their mitochondrial counterparts, since they are translated from a single transcript by initiation at alternative in-frame AUG codons. Very recently, Li et al. (37) showed that human cytosolic NifS (referred to as IscS), when overexpressed in yeast, exhibits desulfurase activity and participates in Fe-S cluster formation of IRP-1 in vitro. The relative abundance of mitochondrial and cytosolic forms of NifS varies depending on cell type (18). For instance, K562 erythroleukemia cells exhibit both forms (at ratio 1:1), whereas renal oncocytoma cells predominantly express the mitochondrial form of NifS. Moreover, the ratio of long/short NifS proteins differs according to the pH of the culture medium. In our experiments, NIH3T3 and RAW 264.7 cells were cultivated at pH 7.5, which favors the synthesis of the cytosolic isoform of NifS (18). However, despite a favorable pH, we did not detect m-Nfs1 in cytosolic fractions, strongly suggesting that these two murine cell types express solely or at least primarily the mitochondrial form of m-Nfs1 (Figs. 1 and 7A). Since suppression of mitochondrial m-Nfs1 in both macrophages and fibroblasts resulted in specific modulation of cytosolic aconitase/IRP-1 activity, our findings highlight a cooperative connection between mitochondria and cytosol in the assembly of cytosolic mammalian Fe-S proteins. This is in agreement with what has been described in yeast, where Nfs1 of the mitochondrial ISC machinery contributes to the maturation of extramitochondrial Fe-S cluster-containing proteins (14, 38). It is worth noticing that a small frac-

FIGURE 7. Effect of mitochondrial m-Nfs1 knockdown in RAW 264.7 macrophages on cytosolic aconitase activity. A, equal amounts (30 μg) of cytosol, mitochondria-enriched fraction, and total cell lysate from RAW 264.7 macrophages were analyzed by immunoblot using anti-m-Nfs1 antibody. Anti-m-aconitase and -vinculin antibodies were used as mitochondrial and cytosolic markers, respectively. B, mitochondria-enriched fractions of RAW 264.7 macrophages, either nontransfected (NT) or transfected with m-Nfs1-3-specific siRNA or negative control siRNA for 48 and 72 h, were immunoblotted with anti-m-Nfs1 antibody and then with anti-SDHB to control for sample loading. C, cytosolic aconitase activity of IRP-1 was measured spectrophotometrically 48 and 72 h after siRNA transfection and in nontransfected cells. These experiments were repeated twice.

change in protein levels. These findings rule out a role of m-Nfs1 in the regulation of Fe-S protein expression and rather imply a defect in assembly of Fe-S clusters, since these are crucial for the electron transfer activity of both complex I and complex II as well as for the enzymatic catalysis performed by aconitase (32).

The majority of Fe-S cluster-containing proteins are located within the mitochondria of mammalian cells, but some are also present in other cellular compartments. In the cytosol, xanthine oxidase activity was strongly reduced upon m-Nfs1 silencing, and IRP-1 accumulated as an IRE-binding protein through its conversion from Fe-S aconitase to an apo-form. This conversion was not due to oxidative stress-induced Fe-S disruption upon m-Nfs1 deficiency, since no increase in oxidized proteins was observed as compared with nontransfected and control siRNA-transfected cells (supplemental Fig. S7). Interestingly, unlike all other Fe-S proteins studied here, the level of apo-IRP-1 was decreased upon m-Nfs1 deficiency. This was confirmed by a similar decrease in the total IRP-1 IRE binding capacity obtained in the presence of 2% 2-mercaptoethanol. These results suggest a role for m-Nfs1 in IRP-1 biosynthesis or that the apo-form of IRP-1 may be more susceptible to proteolysis than its holo counterpart. Eisenstein and co-workers (33) provided evidence that the absence of clusters means that apo-IRP-1 is more accessible to several proteases and thus more susceptible to degradation. In support of this argument, downregulation and increased sensitivity to degradation of apo-IRP-1 have been proposed in several other reports (34–36). IRP-1 protein loss would be a secondary consequence of Fe-S cluster defects resulting from m-Nfs1 depletion. Finally, we reveal that m-Nfs1 deficiency affects the two main types of Fe-S clusters in mammals. Thus, activity of both xanthine oxidase, which contains a [2Fe-2S] cluster, and aconitase, a [4Fe-4S] enzyme, was reduced upon m-Nfs1 depletion. Altogether, these data demonstrate that m-Nfs1 plays an important and specific role in the maintenance of both mitochondrial and cytosolic Fe-S protein activities.

Rouault and co-workers (18) have identified NifS proteins (homologous to m-Nfs1) in the cytosol and the nucleus of human cells, suggesting that Fe-S cluster assembly is compartmentalized in mammalian cells. These isoforms of human NifS are smaller than their mitochondrial counterparts, since they are translated from a single transcript by initiation at alternative in-frame AUG codons. Very recently, Li et al. (37) showed that human cytosolic NifS (referred to as IscS), when overexpressed in yeast, exhibits desulfurase activity and participates in Fe-S cluster formation of IRP-1 in vitro. The relative abundance of mitochondrial and cytosolic forms of NifS varies depending on cell type (18). For instance, K562 erythroleukemia cells exhibit both forms (at ratio 1:1), whereas renal oncocytoma cells predominantly express the mitochondrial form of NifS. Moreover, the ratio of long/short NifS proteins differs according to the pH of the culture medium. In our experiments, NIH3T3 and RAW 264.7 cells were cultivated at pH 7.5, which favors the synthesis of the cytosolic isoform of NifS (18). However, despite a favorable pH, we did not detect m-Nfs1 in cytosolic fractions, strongly suggesting that these two murine cell types express solely or at least primarily the mitochondrial form of m-Nfs1 (Figs. 1 and 7A). Since suppression of mitochondrial m-Nfs1 in both macrophages and fibroblasts resulted in specific modulation of cytosolic aconitase/IRP-1 activity, our findings highlight a cooperative connection between mitochondria and cytosol in the assembly of cytosolic mammalian Fe-S proteins. This is in agreement with what has been described in yeast, where Nfs1 of the mitochondrial ISC machinery contributes to the maturation of extramitochondrial Fe-S cluster-containing proteins (14, 38). It is worth noticing that a small frac-
tion of yeast Nfs1 found in the cytosol/nucleus is more prone to post-transcriptional modification of tRNAs (39). Cooperation between both compartments is also illustrated by the important part played in yeast by the recently discovered cytosolic Fe-S cluster assembly machinery in concert with the mitochondrial ISC machinery (14). Remarkably, the cluster assembly components have been conserved in the mammalian system (14). Furthermore, the fact that IRP-1 reacts to the mitochondrial m-Nfs1 deficit is also reminiscent of previous data showing that the yeast Aft1p transcription factor is responsive to Fe-S level in mitochondria (40) and that the zebrashiraf Shiraz mutant deficient in glutaredoxin 5, a member of the mitochondrial ISC machinery, expresses an apo-IRP-1 (41). As a result, translation of erythroid aminolevulinate synthase, the first and rate-limiting enzyme of heme synthesis, whose mRNA carries an IRE at its 5′-end, is repressed. Our results are also in accordance with recent data showing that frataxin and the ABCB7 transporter, two other mitochondrial proteins, participate in cytosolic Fe-S cluster assembly in *Drosophila* and mammals (21, 22, 42, 43).

Intriguingly, the m-Nfs1-siRNA-induced phenotype was displayed earlier and was more severe in the cytosol than in mitochondria. Cytosolic aconitase and xanthine oxidase activities were already reduced by 70 and 40%, respectively, from 2 days of m-Nfs1 depletion, whereas mitochondrial Fe-S protein activities were not yet affected. Differential protein turnover could be an explanation but does not apply to mitochondrial and cytosolic aconitases, since they have roughly the same half-life (44, 45). Rather, protection of mitochondrial Fe-S protein activities after 2 days of m-Nfs1 silencing may be explained by the presence of a low residual fraction of m-Nfs1. siRNA gene silencing is usually not complete and, in our experimental conditions, m-Nfs1 knocked down cells exhibited 25% residual m-Nfs1 mRNA. Therefore, m-Nfs1 left over in the vicinity of mitochondrial Fe-S cluster enzymes would favor maintenance of their activities rather than those of enzymes in distal cellular compartments. Interestingly, these data suggest that mitochondrial vital functions can be preserved for 2 days even at low m-Nfs1 level. Mitochondria are essential organelles that generate energy in the form of ATP via oxidative phosphorylation. Defective mitochondrial Fe-S cluster assembly automatically damages cells by depleting their energy production, which leads to multiple dysfunctions and eventually cell death (46). Moreover, activity of the mitochondrial ATP-binding cassette transporter ABCB7, which is thought to participate in the export of preassembled Fe-S clusters required for cytosolic Fe-S proteins, is probably affected by ATP depletion (15, 47). As a result, this would block Fe-S cluster delivery from mitochondria to other cellular compartments, giving another meaning to the upholding of the integrity of mitochondrial Fe-S proteins compared with their cytosolic counterparts, upon m-Nfs1 deficiency. The residual m-Nfs1 upon silencing would be primarily dedicated to the biogenesis of mitochondrial Fe-S clusters in order to maintain a threshold of energy availability and function as a protective device.

Misregulation of intracellular iron has been reported in yeast when the ISC protein machinery is defective (13, 14, 48). In addition, iron accumulation in mitochondria has been reported in a mouse model for Friedreich ataxia, a disease associated with abnormalities in Fe-S cluster synthesis (49). Since iron metabolism is different in yeasts and mammals (*e.g.* yeasts do not have IRP and ferritin), it was worth analyzing the fate of IRP-1 and the level of ferritin (which is under the post-transcriptional control of IRP-1) upon m-Nfs1 deficiency. In the cytosol of m-Nfs1-deficient cells, there was an increase in IRP-1 binding to ferritin IRE, which was concomitant with a strong reduction in ferritin levels. In addition, no decrease in ferritin transcripts was seen under the same experimental conditions. Therefore, loss of ferritin may be explained by the conversion of the cytosolic Fe-S aconitase to apo-IRP-1, which in turn specifically binds to ferritin mRNA, blocking translation. This cytosolic phenotype, which was found upon m-Nfs1 deficiency, was also recently reported in *Drosophila* after suppression of frataxin (43). Since ferritin is the main iron storage protein in mammalian cells, its down-regulation induced by m-Nfs1 deficiency may be the first step in an increase in cellular iron availability.

To conclude, we have developed the first mammalian cellular model in which endogenous mitochondrial m-Nfs1 was depleted, presenting evidence for a specific role of m-Nfs1 in the maturation of several mammalian Fe-S cluster-containing enzymes with a significant bearing on iron metabolism. Moreover, we recently showed that m-Nfs1 is down-regulated in murine macrophages exposed to immunological stimuli. Our results may therefore have important implications in physiology and pathophysiology. By limiting the activity of various Fe-S cluster proteins, down-regulation of m-Nfs1 may indirectly affect several metabolisms, particularly since the number of known Fe-S proteins continues to grow, notably with the identification of an Fe-S cluster in human glutaredoxin 2 involved in mitochondrial redox homeostasis (50). In mammals, further study on the functional role of both the ISC machinery members and the putative cytosolic Fe-S cluster assembly components upon cell stimulation is likely to provide important insights into communication between mitochondria and cytosol.

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