The Yeast Frataxin Homologue Mediates Mitochondrial Iron Efflux

EVIDENCE FOR A MITOCHONDRIAL IRON CYCLE*

(Received for publication, November 6, 1998, and in revised form, December 9, 1998)

Derek C. Radisky†‡, Michael C. Babcock‡§ and Jerry Kaplan

From the Department of Pathology, University of Utah, Salt Lake City, Utah 84112

Mutations in the nuclear gene encoding the mitochondrial protein frataxin are responsible for the neurological disorder Friedreich ataxia (FA). Yeast strains with a deletion in the frataxin homologue YFH1 accumulate excess iron in mitochondria and demonstrate mitochondrial damage. We show that in the absence of YFH1, mitochondrial damage is proportional to the concentration and duration of exposure to extracellular iron, establishing mitochondrial iron accumulation as causal to mitochondrial damage. Reintroduction of YFH1 results in the rapid export of accumulated mitochondrial iron into the cytosol as free, non-heme bound iron, demonstrating that mitochondrial iron in the yeast FA model can be made bioavailable. These results demonstrate a mitochondrial iron cycle in which Yfh1p regulates mitochondrial iron efflux.

Friedreich ataxia (FA) is a neurodegenerative disease transmitted as an autosomal recessive trait with a prevalence of 1 in 50,000 individuals (1). The FA gene was identified by positional cloning and found to encode a 210-amino acid mitochondrial protein designated frataxin (2). Most cases of FA are because of the expansion of a polymorphic GAA trinucleotide repeat located in the first intron of the frataxin gene, resulting in lowered frataxin mRNA levels (3, 4). The defect responsible for FA also affects non-neuronal organs, and patients usually succumb to a cardiomyopathy in the fourth decade. Gait ataxia is the most common presenting symptom, and most patients eventually manifest dysarthria, areflexia, pyramidal weakness of the legs, extensor planar responses, and distal loss of joint position and vibration sense (1). The frataxin protein is localized to the mitochondria (5), but its function has not been determined. The frataxin protein is highly expressed in neuronal and heart tissue (2), both of which are postmitotic and highly dependent upon mitochondrial respiration (1). Iron deposits have been found in the myocardium of FA patients, and myocardial mitochondrial respiration has been found to be defective (6).

Yeast disrupted for YFH1 (yeast frataxin homologue) accumulate iron in mitochondria (7). Mitochondrial DNA (mtDNA) is damaged and mitochondrial respiratory activity is impaired (8, 9). Mitochondrial iron accumulation in yeast with YFH1 deletions is associated with subnormal cytosolic iron concentrations (7). Lowered cytosolic iron concentrations induce transcription of FET3, a component of the plasma membrane high affinity iron uptake system (10). The increased rate of iron uptake results in a doubling of cellular iron content relative to wild-type cells, but the excess iron is abnormally localized to mitochondria.

Mitochondrial defects in patients with FA and in the yeast model could be a direct result of mitochondrial iron accumulation. Alternatively, a deficiency of frataxin protein could result in mitochondrial damage, and iron overload may be one manifestation of the mitochondrial damage. This phenomenon has been observed in two patients with acquired idiopathic sideroblastic anemia (11). To determine whether iron accumulation is the cause or consequence of the mitochondrial defect, we constructed a yeast strain that contains respiration-competent mitochondria and regulatable YFH1. Using this approach, we found that YFH1 maintains mitochondrial iron homeostasis at the level of iron efflux. This result indicates that under normal conditions there is a dynamic flux of iron through mitochondria, which is disrupted by the loss of YFH1.

EXPERIMENTAL PROCEDURES

Generation of MET-YFH Strain—pMET-YFH was generated by polymerase chain reaction using YFH1 open reading frame-flanking primers (5′ cga gat tag aat gta gca aag att aa 3′ and 5′ ccc gag ctc tta gcg 3′) and Escherichia coli (12). Constructs were checked by DNA sequencing. Yfh1Δ cells (7) were mated either to wild type or to the fet3Δ strain DY1297–6a (12). The resulting diploids were transformed with the pMET-YFH plasmid and sporulated onto complete media lacking uracil to select for yeast transformants, and the ampicillin resistance gene for selection in E. coli (12). Constructs were checked by DNA sequencing. Yfh1Δ cells (7) were mated either to wild type or to the fet3Δ strain DY1297–6a (12). The resulting diploids were transformed with the pMET-YFH plasmid and sporulated onto complete media lacking uracil and containing 2% glucose (CM-URA) and lacking methionine to maintain expression of YFH1. Spores were analyzed for the presence of the disruptions by polymerase chain reaction and Southern analysis. Plasmid was maintained by growth on media lacking uracil throughout all experiments.

Gradient Fractionation—MET-YFH cells and wild-type cells containing pTf63 were grown to log phase in CM-URAs (+met), then washed and resuspended in CM-URA (+met) supplemented with 35FeCl3 to a final iron concentration of 3.3 μM, and incubated for 20 min at 30 °C. Cells were then washed, resuspended in nonradioactive CM-URA (+met), and incubated with shaking at 30 °C for 3 h to allow the 35Fe to label the cellular iron pool. Cell samples were then grown for indicated times either in CM-URA (+met) or in CM-URA (~met) and placed on ice for the remainder of the experiment. Growth curves indicated that the cells were in log phase growth at all times, and time courses of wild-type and yfh1Δ cells indicated that incubation on ice did not affect cellular distribution of radioactive iron (data not shown). Washed cell samples were spheroplasted and Dounce-homogenized. Postnuclear supernatants were centrifuged (12,000 × g, 30 min) to generate organellar pellets, which were resuspended and layered onto preformed 0–25% iodixanol gradients. These gradients were spun at 10,000 × g for 2 h and then fractionated. Radioactivity in each fraction was measured, and the position of mitochondria in the gradients was determined by density gradient fractionation.
Western blotting and probing with monoclonal anti-porin antibody (Molecular Probes).

RESULTS

To determine whether iron accumulation is the cause or consequence of the mitochondrial defect in yfh1Δ cells, we constructed the MET-YFH strain, which contains respiration-competent mitochondria, a deleted chromosomal copy of YFH1, and a plasmid-based, methionine-regulated YFH1. Cells grown in the absence of methionine expressed abundant YFH1 message, whereas addition of methionine produced complete repression of YFH1 transcription within 2 h (Fig. 1A). Cells grown in the presence of methionine completely repressed YFH1 message, whereas removal of methionine resulted in full transcription of YFH1 (Fig. 1B). Correlated with YFH1 induction was a suppression of FET3 transcription (Fig. 1B), whereas repression of YFH1 resulted in increased expression of FET3 mRNA within 4 h (Fig. 1A). Increased iron uptake correlated with the expression of FET3 transcripts (Fig. 2). When cells were maintained in the absence of methionine, iron uptake remained low, whereas cells moved to media containing methionine rapidly induced the high affinity iron uptake system. This demonstrates that the cytosol becomes iron-depleted when YFH1 is repressed, because FET3 transcription and the high affinity iron transport system are positively regulated by iron deficiency (12).

To determine the relationship between cellular iron accumulation and mitochondrial damage, MET-YFH cells were grown in media lacking methionine and then incubated in media containing or lacking methionine that was supplemented with increasing concentrations of iron. Cell samples were then washed and plated on media that lacked methionine and contained glucose as the carbon source. The resultant colonies were then replica-plated onto media containing 1% glycerol and 1% ethanol as carbon sources. Mitochondrial damage was assessed as the percentage of colonies that were petite, i.e., unable to grow when glycerol and ethanol were the sole carbon sources. Mitochondrial damage was proportional both to the amount of iron in the growth media and to the time cells were exposed to iron (Fig. 3A). MET-YFH cells lacking FET3, which are defective for high affinity iron acquisition, demonstrated no iron-dependent mitochondrial damage even in the presence of 500 μM iron (Fig. 3B). These results indicate that the mitochondrial damage that follows YFH1 depletion is dependent upon cellular iron uptake.

Cells with a deleted YFH1 gene demonstrated increased mitochondrial iron content. This accumulation could have resulted from either increased iron uptake or decreased iron efflux. To distinguish between these possibilities we monitored mitochondrial iron content following reintroduction of Yfh1p in yfh1Δ cells. MET-YFH cells grown in the presence of methionine were incubated with 59Fe for 20 min and then grown in the presence of methionine for 3 additional hours to label cellular iron stores. Following this, cells were either maintained in media containing methionine or transferred to media lacking methionine to induce expression of YFH1. Cells were then homogenized and organelles were fractionated on iodixanol gradients. Virtually all 59Fe was found in fractions that also contained the mitochondrial protein porin (Fig. 4A). When cells were incubated in medium lacking methionine, there was only a marginal, time-dependent decrease in mitochondria-associated iron (Fig. 4A). When cells were incubated in medium lacking methionine to induce expression of YFH1, there was a rapid decrease in mitochondria-associated iron (Fig. 4B). Iron mobilized from the mitochondria was immediately available as cytolic free iron as FET3 transcription was rapidly repressed (Fig. 1B). Although these data do not rule out an effect of YFH1 on mitochondrial iron uptake, they do establish that YFH1 mediates the efflux of iron from mitochondria.

DISCUSSION

In this study, we demonstrate a role for Yfh1p in maintaining mitochondrial iron homeostasis. Yfh1p is unlikely to be the
actual transporter, as sequence analysis indicates that it contains no transmembrane sequences. Yfh1p may mediate iron efflux by Atm1p, an ABC transporter located in the mitochondrial inner membrane (15). Although yeast strains deleted for ATM1 show a mitochondrial iron accumulation phenotype similar to YFH1 deletion strains (14), a direct biochemical interaction between Yfh1p and Atm1p has yet to be determined.

Our results suggest that the mitochondrial defect in tissues from patients with FA is the consequence of iron accumulation. These results are consistent with the hypothesis that mitochondrial damage results from iron-mediated oxygen radical production. Reintroduction of YFH1 resulted in the efflux of iron from mitochondria, suggesting that the excess mitochondrial iron in the similar FA syndrome may potentially also be mobilized. If this proves to be the case then perhaps chelation therapy should be considered for the treatment of FA, if a strategy for selectively chelating mitochondrial iron could be devised. Many drugs have been administered to patients with FA in an attempt to alleviate progression of the disease, including choline chloride, lecithin, thyrotropin-releasing hormone, γ-vinyl, γ-aminobutyric acid, 5-hydroxytryptophan, and benzerazide. None has had any beneficial effects, and current therapy of FA is limited to supportive care (1). Patients with hereditary aceruloplasminemia exhibiting basal ganglion dysfunction because of iron accumulation in neuronal tissues have been successfully treated by chelation with desferrioxamine (15), indicating that it can chelate iron in neuronal tissues. Excess iron in cardiac myocytes is also accessible to desferrioxamine as demonstrated in patients with cardiac iron overload because of thalassemia (16).

The observation that Yfh1p affects iron efflux indicates that, under normal conditions, iron can both enter and exit yeast mitochondria. This suggests the existence of a mitochondrial iron cycle. A mechanism must exist to regulate mitochondrial iron accumulation in response to iron need, either for mitochondrial proteins or for heme synthesis, the final step of which occurs within the mitochondrial matrix. Increased mitochondrial iron demand could be met by either increased influx or reduced efflux. A priori we expected regulation to occur at the level of uptake, but there is no theoretical reason why the site of regulation could not be at the level of efflux. Excessive mitochondrial iron accumulation is found in erythroid precursors of patients with sideroblastic anemia. Iron deposition results from deficiencies in heme synthesis, due either to mutations in heme biosynthetic enzymes or to pharmacologic inhibition of porphyrin synthesis (17). These observations have led to the suggestion that iron may only exit mitochondria as heme (18). As our results suggest the existence of a yeast mitochondrial iron efflux pathway independent of heme, we propose that the homologous frataxin-mediated pathway may support the high rate of heme biosynthesis required by this cell type.

Acknowledgments—We thank J. Leslie and D. Ward for advice on gradient fractionation and J. Kushner for numerous helpful discussions.

REFERENCES
1. Harding, A. E. (1993) in Advances in Neurology (Harding, A. E., and Deufel, T., eds) Vol. 61, pp. 1–14, Raven Press Ltd., NY
2. Campuzano, V., Montemini, L., Montermini, L., Molto, M. D., Pianese, L., Cova, L., Hindelang, C., Cavalcanti, F., Monros, E., Rodius, F., Duclus, F., Monicelli, A., Zara, F., Canizares, J., Koutnikova, H., Bidichandani, S. I., Gellera, C., Brice, A., Trouillas, P., De Michele, G., Filla, A., De Prutos, R., Palau, F., Patel, P. I., Di Donato, S., Mandel, J-L., Coozza, S., Koenig, M., and Pandolfo, M. (1996) Science 271, 1423–1427
3. Montemini, L., Andermann, E., Lahuda, M., Richter, A., Pandolfo, M., Cavalli, P., Pianese, L., Iodice, L., Farina, G., Monticelli, A., Turano, M., Filla, A., De Michele, G., and Coozza, S. (1997) Hum. Mol. Genet. 6, 1261–1266
4. Bidichandani, S. I., Ashizawa, T., and Patel, P. I. (1998) Am. J. Hum. Genet. 62, 111–121
5. Campuzano, V., Montermini, L., Lutz, Y., Cova, L., Hindelang, C., Jirarlerspong, S., Trevitt, Y., Kish, S. J., Gauchezux, B., Trouillas, P., Authier, F. J., Durr, A., Mandel, J-L., Vescovi, A., Pandolfo, M., and Koenig, M. (1997) Hum. Mol. Genet. 6, 1771–1780
6. Röting, A., de Lonlay, P., Chretien, D., Foury, F., Koenig, M., Sidi, D., Munnich, A., and Rustin, P. (1997) Nat. Genet. 17, 215–217
7. Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jirarlerspong, S., Montemini, L., Pandolfo, M., and Kaplan, J. (1997) Science 276, 1709–1712
8. Foury, F., and Cazzalini, O. (1997) FEBS Lett. 411, 375–377
9. Wilson, R. B., and Roof, D. M. (1997) Nat. Genet. 16, 352–357
10. Hassett, R. F., Romeo, A. M., and Kosman, D. J. (1998) J. Biol. Chem. 273, 7628–7636
11. Gattermann, N., Retzlaff, S., Wang, Y.-L., Hofhaus, G., Heinisch, J., Aul, C., and Schneider, W. (1998) J. Biol. Chem. 273, 7628–7636
12. Gattermann, N., Retzlaff, S., Wang, Y.-L., Hofhaus, G., Heinisch, J., Aul, C., and Schneider, W. (1998) J. Biol. Chem. 273, 7628–7636
13. Leighton, J., and Schatz, G. (1995) EMBO J. 14, 188–195
14. Kosman, D. J., and Deufel, T., eds) Vol. 61, pp. 1–14, Raven Press Ltd., NY
15. Miyajima, H., Takahashi, Y., Kamata, T., Shimizu, H., Sakai, N., and Gitlin, J. D. (1997) Ann. Neurol. 41, 404–407
16. Giardina, P. J., and Grady, R. W. (1995) Semin. Hematol. 32, 304–312
17. Kushner, J. P., Lee, G. R., Wintrobe, M. M., and Cartwright, G. E. (1971) Medicine 50, 139–159
18. Ponka, F. (1997) Blood 89, 1–25
19. Askwith, C. A., and Kaplan, J. (1997) J. Biol. Chem. 272, 401–405