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Mitochondrial Isa2p plays a crucial role in the maturation of cellular iron–sulfur proteins

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Abstract The assembly of iron–sulfur (Fe/S) clusters in a living cell is mediated by a complex machinery which, in eukaryotes, is localised within mitochondria. Here, we report on a new component of this machinery, the protein Isa2p of the yeast *Saccharomyces cerevisiae*. The protein shares sequence similarity with yeast Isa1p and the bacterial IscA proteins which recently have been shown to perform a function in Fe/S cluster biosynthesis. Like the Isa1p homologue, Isa2p is localised in the mitochondrial matrix as a soluble protein. Deletion of the *ISA2* gene results in the loss of mitochondrial DNA and a strong growth defect. Simultaneous deletion of the *ISA1* gene does not further exacerbate this growth phenotype suggesting that the Isa proteins perform a non-essential function. When Isa2p was depleted by regulated gene expression, mtDNA was maintained, but cells grew slowly on non-fermentable carbon sources. The maturation of both mitochondrial and cytosolic Fe/S proteins was strongly impaired in the absence of Isa2p. Thus, Isa2p is a new member of the Fe/S cluster biosynthesis machinery of the mitochondrial matrix and may be involved in the binding of an intermediate of Fe/S cluster assembly.

Key words: Mitochondrion; Isa/IscA protein; ABC transporter; Iron homeostasis; *Saccharomyces cerevisiae*

1. Introduction

Fe/S clusters are cofactors of numerous proteins that perform versatile functions in metabolism, electron transport and regulation of gene expression [1,2]. During the past 2 years considerable progress has been made in elucidating the molecular machinery and mechanisms of iron–sulfur (Fe/S) cluster formation within a living cell [3–5]. In prokaryotes, the components of Fe/S cluster assembly are encoded by genes of the *isc* and *nif* operons [6,7]. In eukaryotes, mitochondria are the site of synthesis of Fe/S clusters. They contain some tens of proteins that are similar in amino acid sequence to the bacterial Isc and Nif proteins and hence were proposed to function in assembling Fe/S clusters [8–12]. According to a current working model, the assembly process in mitochondria is started by binding of iron to the proteins Isu1p/Isu2p [5,13]. The cysteine desulfurase Nfs1p then generates elemental sulfur to produce an [2Fe–2S] cluster on a dimer of the Isu proteins. Upon reduction, presumably by the mitochondrial ferredoxin Yah1p [12], the cluster is released and may be either directly or indirectly inserted into apoproteins. The requirement of further proteins in the process has been demonstrated, but their molecular function is not yet clear [8,10,14,15]. For instance, the two heat shock proteins Ssq1p and Jac1p are members of the Hsp70/DnaK and Hsp40/DnaJ families [16,17], respectively, and may perform a chaperone function during Fe/S cluster assembly.

Mitochondria are involved in the maturation of both mitochondrial and cytosolic Fe/S proteins. Up to now, a function in the assembly of Fe/S clusters for cytosolic apoproteins has been demonstrated for the mitochondrial matrix proteins Nfs1p, Yah1p, and Isa1p and for the inner membrane ABC transporter Atm1p [9,12,14]. It was suggested that mitochondria pre-assemble an Fe/S cluster which then is exported by Atm1p from the organelles for incorporation into cytosolic apoproteins [5].

We recently characterised a novel member of the Fe/S cluster biosynthesis machinery termed Isa1p [14]. It contains two characteristic heptapeptide motifs (termed IsaI and IsaII; [14]) that are highly conserved in a number of eukaryotic proteins and in the bacterial IscA proteins [7,15,18]. Isa1p is localised in the mitochondrial matrix and was shown to be involved in the maturation of mitochondrial and cytosolic Fe/S proteins [14]. However, in contrast to the essential nature of the *NFS1*, *JAC1* and *YAH1* genes, deletion of *ISA1* is not lethal. This may be due to the existence of a gene termed *ISA2* that exhibits similarity to *ISA1* (corresponding to 19% identical amino acid residues) and contains the IsaI and IsaII motifs. This renders it likely that the two yeast proteins perform similar functions.

In this report, we investigated the consequences of deleting the *ISA2* gene either alone or together with the *ISA1* gene, analysed the subcellular localisation of Isa2p, and examined its functional role in the maturation of cellular Fe/S proteins.

2. Materials and methods

2.1. Yeast strains and cell growth

The following strains of *Saccharomyces cerevisiae* were used: Strain W303 (*MATα*, *ura3-1*, *ade2-1*, *trp1-1*, *his3-11,15*, *leu2-3,112*) and its isogenic diploid form served as wild-type. The *ISA2* gene was deleted in Δisa1 diploid cells by a PCR-based method employing the histidine resistance marker [14,19]. The haploid Δisa2 and Δisa1Δisa2 strains were obtained after sporulation and tetrad dissection. Exchange of the endogenous promoter of the *ISA2* gene for a galactose-inducible promoter (strain Gal-ISA2) was performed as described previously [9,20]. PCR fragments corresponding to the coding region (nucleotides 1–558) and the 5′-upstream region (nucleotides −81–105) of *ISA2*

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were cloned into the *Bam*HI/*Hin*dIII and *Hin*dIII/*Hpa*I restriction sites, respectively, of the Yep51 vector carrying the *GAL10* promoter. Cells were grown as detailed previously using rich (YP) or minimal media [21–23] and lactate medium [24] containing the required carbon sources. For experiments involving cell labelling with radioactive ^{55}Fe, no iron salt was added to the synthetic minimal medium.

2.2. Generation of Isa2p fusion proteins

To attach a typical mitochondrial targeting sequence to the conserved domain of Isa2p, the coding region of the presequence part of the β-subunit of yeast F_1-ATPase (amino acid residues 1–40) was combined with the region encoding residues 51–185 of Isa2p by using PCR-generated DNA fragments. The fragments were inserted into plasmid pBluescript and the coding region of the entire fusion protein was cloned into the *Cla*I/*Hin*dIII sites of the yeast expression plasmid pRS416-MET [25]. To facilitate the analysis of in vitro import of Isa2p into mitochondria, a C-terminal tag of five amino acid residues (MMSMM) was added to Isa2p by introducing a PCR-generated DNA fragment encoding the tagged protein into the *Bam*HI and *Hin*dIII restriction sites of pGEM4.

2.3. Miscellaneous methods

The following published methods were used: Manipulation of DNA and PCR [26]; transformation of yeast cells [27]; isolation of yeast mitochondria [24,28] and post-mitochondrial supernatant (PMS) [9]; import of radiolabelled precursor proteins into isolated mitochondria [29–31]; raising antibodies using peptides coupled to maleimide-activated ovalbumin (Pierce Co., [32]); enzyme activities of malate dehydrogenase, aconitase [23], succinate dehydrogenase [33]). The standard error of the determination of enzyme activities varied between 5 and 15%. The labelling of yeast cells with radioactive iron (^{55}Fe) and the measurement of the incorporation of ^{55}Fe into the mitochondrial Bio2p or the cytosolic Leu1p by immunoprecipitation and liquid scintillation counting was performed as described earlier [9]. The standard error for detection of ^{55}Fe associated with Bio2p or Leu1p in a cell lysate was less than 15%, the cellular uptake of ^{55}Fe varied by 30%.

3. Results

3.1. Inactivation of the ISA2 gene impairs growth of yeast cells

The protein Isa2p of *S. cerevisiae* (encoded by open reading frame *YPR067w*) contains 185 amino acid residues corresponding to a molecular mass of 21 kDa. To initiate the investigation of Isa2p function, we deleted the *ISA2* gene either alone or together with the *ISA1* gene. The single mutants Δisa1 and Δisa2 and the double mutant Δisa1Δisa2 displayed identical growth characteristics. On glucose-containing rich medium (YPD) growth was retarded as compared to wild-type cells, whereas no colony formation was observed on glycerol-containing medium (YPG; Fig. 1). Apparently, deletion of *ISA2* severely affects growth under non-fermentative conditions indicating that Isa2p is important for maintaining functional mitochondria. Double deletion of both *ISA* genes did not exacerbate the growth defects observed for Δisa1 and Δisa2 strains on YPD medium. This suggests that the two proteins perform functions that are dispensable for viability of yeast cells.

Transformation of Δisa2 cells with a plasmid carrying the wild-type *ISA2* gene did not restore wild-type growth suggesting that the Δisa2 cells had lost the mitochondrial DNA (mtDNA). We therefore tested whether deletion of *ISA2* results in rho° cells lacking mtDNA [34]. Staining of DNA with 4′,6-diamidino-2-phenylindole dihydrochloride; (DAPI) did not detect mtDNA in Δisa2 cells, i.e. deletion of the *ISA2* gene was accompanied by a concomitant loss of the mtDNA (Fig. 2, middle). This resembles the situation reported for Δisa1 cells [14] and shows the requirement of functional Isa proteins for mtDNA maintenance.

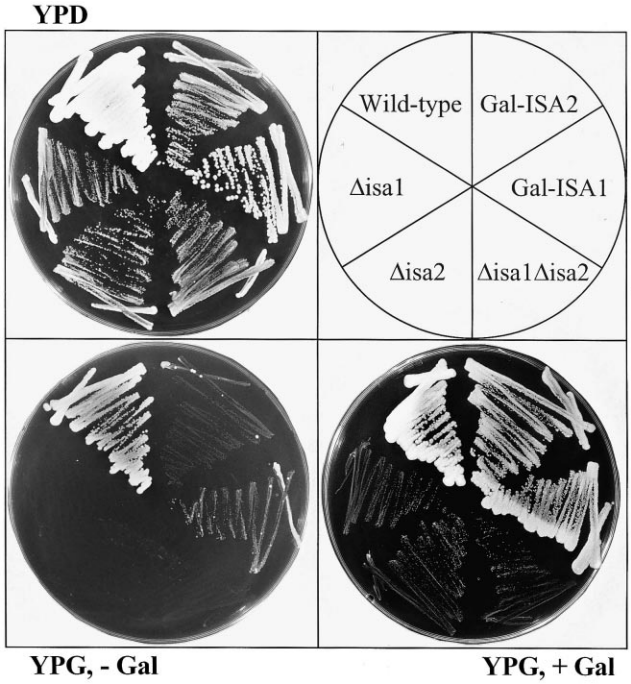

Fig. 1. Genetic inactivation of *ISA2* impairs growth of yeast cells. Wild-type, Δisa1, Δisa2, Δisa1Δisa2, Gal-ISA1, and Gal-ISA2 cells were grown on rich media containing glucose (YPD), glycerol (YPG) or glycerol plus 0.2% galactose (Gal) for 3 days at 30°C.

Rho° cells are deficient in respiration and exhibit numerous pleiotropic phenotypes including defects in mitochondrial Fe/S proteins [14,34]. This made it necessary to construct a strain in which the expression of the *ISA2* gene could be regulated. To this end, the *GAL10* promoter was inserted at the 5′-end of *ISA2* by homologous recombination. The resulting Gal-ISA2 cells did not lose mtDNA in the absence of galactose, even after growth for four days (Fig. 2, bottom). Nevertheless, we observed a slight growth defect on YPD medium and hardly any growth on YPG medium (Fig. 1). Growth was fully restored to wild-type rates by adding galactose to the media. The growth retardation of Gal-ISA2 cells was significantly stronger than that of Gal-ISA1 cells on both media. Together, these observations indicate an important cellular function of Isa2p.

3.2. Isa2p is targeted to the mitochondrial matrix in vivo and in vitro

To determine the sub-cellular localisation of Isa2p, an antibody was raised using a peptide of the middle part of Isa2p. The antibody recognised a protein of 21 kDa in isolated mitochondria, but not in the PMS of wild-type cells (Fig. 3A). No immunostaining was detected in mitochondria and the PMS of Δisa2 cells demonstrating that the antibody is specific for Isa2p. These findings suggest a mitochondrial localisation of Isa2p.

The N-terminus of Isa2p resembles a mitochondrial presequence (predicted probability of import into mitochondria 99%; [35]). We tested whether the protein is imported into isolated mitochondria [30,31]. Since Isa2p does not contain any internal methionine residues, a tag containing four methionine residues was added to the C-terminus and the fusion protein was synthesised in reticulocyte lysate in the presence

of [^{35}S]methionine. Upon incubation of the radiolabelled protein with mitochondria, the Isa2p precursor of 26 kDa was cleaved to a 21 kDa protein (Fig. 3B, top panel). The mature form of Isa2p, but not the precursor was resistant to proteinase K (PK) in mitochondria and mitoplasts (i.e. mitochondria in which the outer membrane was ruptured by a swelling procedure; [36]). Only upon lysis of the organelles with detergent, the protein was digested by the protease. Import of Isa2p and processing to the mature form was dependent on the presence of a membrane potential, a typical behaviour of mitochondrial preproteins (Fig. 3B, top panel). Together, these results indicate the import of the Isa2p precursor into the mitochondrial matrix. The cleavage of a 5 kDa prepeptide suggests a processing site after residue 50.

The sub-mitochondrial localisation of wild-type Isa2p was examined by immunostaining analysis of isolated mitochondria. Isa2p behaved similarly to the matrix protein Tim44p in that it was not protease-sensitive in mitochondria and mitoplasts, but became degraded after lysis of the organelles in detergent (Fig. 3B, bottom panel). The protein appeared as soluble protein of the mitochondrial matrix. It was released from mitochondria upon sonication, but remained associated with mitoplasts and thus behaved characteristically different from the soluble cytochrome *b*2 of the intermembrane space (not shown). Further, localisation of Isa2p in the mitochondrial matrix was supported by the restoration of wild-type growth of Gal-ISA2 cells upon expression of a fusion protein consisting of the presequence of the β-subunit of F_1-ATPase (a matrix protein) and residues 51 through 185 of Isa2p (not shown). In summary, all these experimental approaches identify Isa2p as a constituent of the mitochondrial matrix.

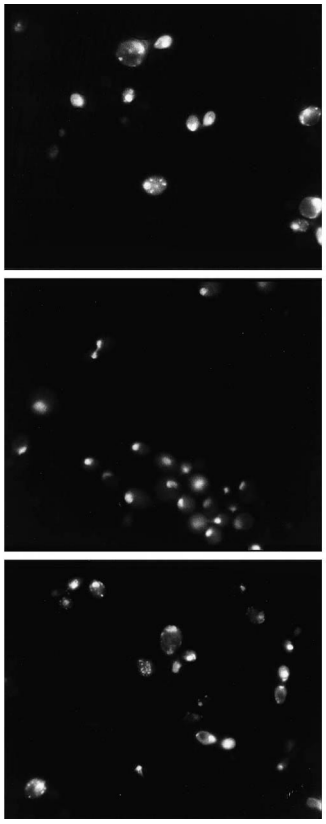

Fig. 2. Deletion of the *ISA2* gene causes the loss of mtDNA. Wild-type, Δisa2 and Gal-ISA2 cells were grown in rich media containing glucose and subjected to staining with DAPI [39]). This fluorescent dye stains both nuclear and mtDNA.

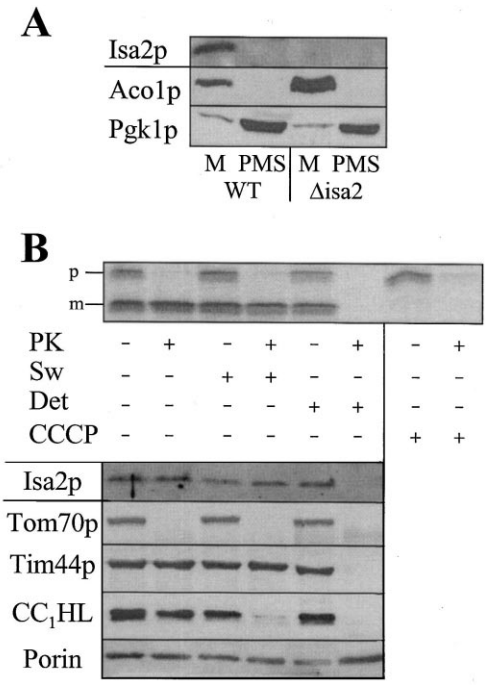

Fig. 3. Isa2p is localised in the mitochondrial matrix. A: Mitochondria (M) and PMS were isolated from wild-type (WT) and Δisa2 cells. Immunostaining was performed using antisera raised against Isa2p, mitochondrial aconitase (Aco1p) and cytosolic 3-phosphoglycerate kinase (Pgk1p; Molecular Probes). B: In vitro and in vivo targeting of Isa2p to the mitochondrial matrix. Isa2p carrying a C-terminal tag containing four methionine residues was synthesised in reticulocyte lysate in the presence of [^{35}S]methionine and used for in vitro import into isolated yeast mitochondria [30,31]. Import reactions were treated with 50 μg/ml PK. The sub-mitochondrial localisation of the imported (upper panel) and endogenous (bottom panel) Isa2p was tested by hypotonic swelling of the organelles (Sw) leading to the rupture of the outer membrane and by solubilisation of the organelles in buffer containing 1.25% octyl D-glucopyranoside (Det). To investigate the dependence of Isa2p import on the presence of a membrane potential (ΔΨ), the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 40 μM) was added before starting the import reaction. Import was analysed by separating the proteins by SDS–PAGE, blotting to nitrocellulose membranes, and autoradiography (top). In the bottom part, immunostaining was employed to visualise Isa2p and protease-sensitive proteins of the outer membrane (Tom70p), the intermembrane space (CC_1HL), and the matrix (Tim44p). The outer membrane protein porin is resistant to protease treatment. Abbreviations: p and m, precursor and mature forms of Isa2p.

3.3. A role of Isa2p in the maturation of cellular Fe/S proteins

Gal-ISA2 cells were used to examine the role of Isa2p in the maturation of mitochondrial Fe/S proteins. Cells were grown for up to three days in the absence of galactose to deplete Isa2p and mitochondria were isolated to examine the content of Isa2p. The protein was significantly decreased in concentration after 16 h of growth and was undetectable after 40 h (Fig. 4A). Other mitochondrial proteins such as Tim44p were hardly affected by the deprivation of Isa2p. To analyse the influence of the depletion of Isa2p on the maturation of mitochondrial Fe/S proteins, the enzyme activities of aconitase and succinate dehydrogenase and, as a control, of malate dehydrogenase were measured. A strong decrease in the activity

of the two mitochondrial Fe/S proteins was observed upon depletion of Isa2p, whereas the activity of malate dehydrogenase hardly changed (Fig. 4B). This demonstrates a crucial function of Isa2p in the assembly and/or repair of mitochondrial Fe/S proteins and may, at least in part, explain the growth defects observed for cells lacking functional Isa2p.

We next addressed the question of whether Isa2p function is required for the de novo synthesis of Fe/S clusters. In this context, we also investigated the potential involvement of Isa2p in the maturation of Fe/S proteins outside mitochondria. As an example of a cytosolic Fe/S protein, we used isopropyl malate isomerase (Leu1p; [37]), while overexpressed biotin synthase (Bio2p) was employed as a mitochondrial protein [38]. Wild-type and Gal-ISA2 cells were transformed with a yeast expression vector carrying the *BIO2* gene. These Gal-ISA2 cells were depleted in Isa2p as described in Fig. 4. Both cells were then labelled with radioactive [^{55}Fe]iron for 1 h and a cell extract was prepared [9]. To measure the incorporation of the Fe/S cluster into Leu1p or Bio2p, immunoprecipitation with antibodies against these proteins was performed and the amount of radioactive ^{55}Fe associated with the immuno-beads

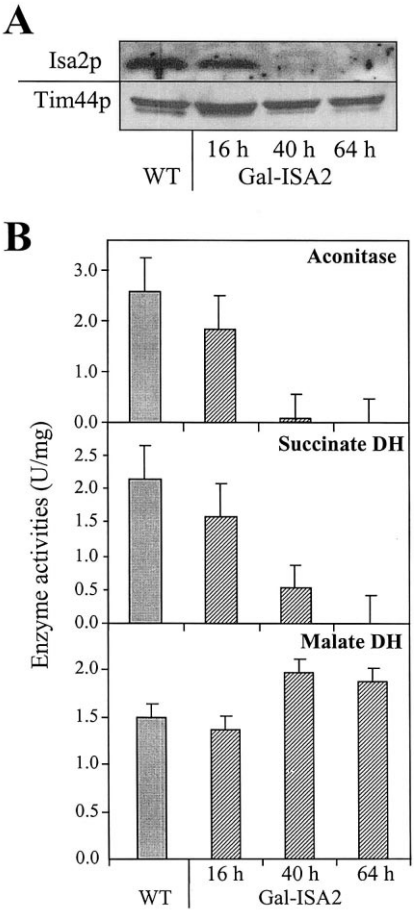

Fig. 4. Depletion of Isa2p causes a specific defect in the activity of mitochondrial Fe/S proteins. A: Gal-ISA2 cells were grown for the indicated times in lactate media containing 0.1% glucose. Mitochondria were isolated from these cells and from wild-type (WT) cells and analysed by immunostaining for the content in Isa2p or Tim44p. B: Isolated mitochondria from A were used to measure the enzyme activities of two Fe/S cluster-containing proteins (aconitase and succinate dehydrogenase) and of malate dehydrogenase. DH, dehydrogenase.

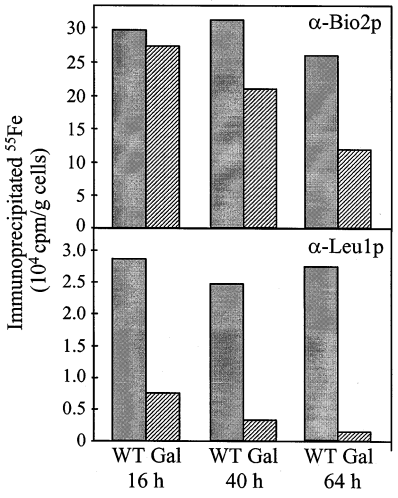

Fig. 5. Isa2p is required for de novo synthesis of Fe/S clusters of both mitochondrial and cytosolic Fe/S proteins. Wild-type (WT) and Gal-ISA2 (Gal) cells were transformed with the plasmid pRS426-GPD carrying the *BIO2* gene. Cells were grown in 'iron-free' minimal media containing glucose for the indicated times. Cells were radiolabelled with [^{55}Fe] iron chloride in the presence of 1 mM ascorbate for 1 h [9]. Immunoprecipitation with anti-Bio2p (α-Bio2p) or anti-Leu1p (α-Leu1p) antibodies was performed and co-immunoprecipitated ^{55}Fe was estimated by liquid scintillation counting. The background signal obtained for immunoprecipitation performed with preimmune serum ($2 \pm 0.5 \times 10^3$ cpm/g cells) was subtracted.

was quantitated by scintillation counting. A strong (more than tenfold) decrease in radioactive iron bound to Leu1p was observed upon depletion of Isa2p as compared to wild-type cells (Fig. 5) or Gal-ISA2 cells grown in the presence of galactose (not shown). Only a twofold, yet significant decrease was detectable for Fe/S cluster assembly into Bio2p. Gal-ISA2 cells displayed wild-type efficiency in iron uptake indicating that depletion of Isa2p did not impair the ability to import iron into the mutant cells (not shown). These findings show that (i) Isa2p, in addition to its participation in the assembly of mitochondrial Fe/S proteins is required for the maturation of cytosolic Fe/S proteins, (ii) the function of Isa2p is necessary for de novo synthesis of Fe/S clusters, and (iii) the assembly of a cytosolic Fe/S protein fails before defects in the maturation of mitochondrial Fe/S proteins become evident. In conclusion, the matrix protein Isa2p plays an important role in the biogenesis of Fe/S clusters for both mitochondrial and cytosolic apoproteins.

4. Discussion

The work presented here identifies the yeast protein Isa2p as a new component of the mitochondrial machinery for biogenesis of cellular Fe/S proteins. The properties of Isa2p characterised in our work closely resemble those previously reported for Isa1p [14,15]. Upon deletion of the *ISA1* or *ISA2* genes the mtDNA is lost. The reason for the failure to maintain the mtDNA is unknown. However, we noted that the mtDNA loss does not occur immediately after deletion of the *ISA* genes, but happens gradually (not shown). Further, both Gal-ISA1 and Gal-ISA2 cells do not loose mtDNA after cultivation for 4 days in the absence of galactose. Therefore, the loss of mtDNA may be an indirect phenotypical conse-

quence of the inactivation of the *ISA* genes. Cells lacking Isa1p or Isa2p display a growth defect which is most substantial upon growth under non-fermentative conditions. A direct comparison of the growth characteristics of Gal-ISA1 and Gal-ISA2 cells indicates a stronger retardation upon depletion of Isa2p. This finding fits nicely to the somewhat stronger effects on the maturation of cellular Fe/S proteins in the absence of Isa2p ([14], this work). Both proteins are required for the assembly of Fe/S clusters of both mitochondrial and cytosolic proteins. It appears from these findings that the proteins perform at least partially redundant functions in Fe/S cluster formation. The fact that simultaneous deletion of both *ISA* genes does not intensify the growth defect of yeast cells, may be explained by functional co-operation of the two Isa proteins. Thus, they differ from the Isu proteins which can replace each other [10,11]. Our observations suggest a function of the Isa proteins that is dispensable. Thus, the Isa proteins appear to improve the efficiency of Fe/S cluster assembly, but, unlike Nfs1p, Yah1p, Jac1p and the Isu proteins, they do not contribute to an essential step of Fe/S cluster formation. An auxiliary role of the Isa proteins fits well to the observations on IscA, the bacterial homologue of the Isa proteins [18].

In a parallel investigation, a different subcellular localisation of Isa2p was found [15]. An overexpressed, epitope-tagged version of Isa2p was localised to the mitochondrial intermembrane space. The explanation for the difference to our findings is not known at present. In our study, localisation of Isa2p in the matrix space was observed in four independent approaches, namely by in vitro protein import, by localisation of the native protein in wild-type cells using immunostaining, by association of the soluble Isa2p with mitoplasts, and by complementation of the growth defects of Gal-ISA2 cells with a fusion protein directing Isa2p into the mitochondrial matrix.

The identification of the Isa proteins as important members of the mitochondrial Fe/S cluster biosynthesis machinery opens the way to analyse the molecular function of these proteins. The Isa proteins contain three conserved cysteine residues which have been shown to be important for function of the proteins [14,15]. It is likely that these residues may interact with an Fe/S cluster either during or immediately after its assembly on the Isu proteins. In this speculative view, the Isa proteins might play a role in the transfer of the Fe/S cluster from the initial site of its synthesis on the Isu proteins to the apoproteins. An alternative view might be that the Isa proteins bind iron and serve as a donor of this metal to the Isu proteins. Future mechanistic analysis of Isa protein function will be facilitated by an in vitro approach to unravel the initial steps of Fe/S cluster biosynthesis by NifU/IscU und NifS/IscS proteins [13].

So far, all components known to participate in Fe/S cluster assembly in yeast are localised within mitochondria. The striking sequence similarity of these proteins with the bacterial Isc and Nif proteins suggests that during evolution these organelles have maintained the archaic Fe/S cluster biosynthesis machinery to satisfy their needs for Fe/S proteins. At some point, mitochondria must have acquired the capability to participate in the maturation of cytosolic Fe/S proteins. To date, a participation in the process of generating cytosolic Fe/S proteins has been documented for Nfs1p, Yah1p, the Isa and Isu proteins and the ABC transporter Atm1p ([9,12,14], this work, unpublished data). Evidently, the entire mitochondrial Fe/S cluster biosynthesis apparatus is engaged in maturation of cytosolic Fe/S proteins. It is therefore probable, even though not yet shown, that Fe/S clusters are pre-assembled in the matrix space and then exported via Atm1p. It will be a challenge to unravel the function of the components (including Isa2p) participating in this process and to identify extramitochondrial proteins mediating delivery of exported Fe/S clusters into cytosolic apoproteins.

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