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Report

Evolution of Mitochondrial Chaperones Utilized in Fe-S Cluster Biogenesis

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Summary

Biogenesis of Fe-S clusters is an essential process [1]. In both *Escherichia coli* and *Saccharomyces cerevisiae*, insertion of clusters into an apoprotein requires interaction between a scaffold protein on which clusters are assembled and a molecular chaperone system—an unusually specialized mitochondrial Hsp70 (mtHsp70) and its J protein cochaperone [2]. It is generally assumed that mitochondria inherited their Fe-S cluster assembly machinery from prokaryotes via the endosymbiosis of a bacterium that led to formation of mitochondria. Indeed, phylogenetic analyses demonstrated that the *S. cerevisiae* J protein, Jac1, and the scaffold, Isu, are orthologous to their bacterial counterparts [3, 4]. However, our analyses indicate that the specialized mtHsp70, Ssq1, is only present in a subset of fungi; most eukaryotes have a single mtHsp70, Ssc1. We propose that an Hsp70 having a role limited to Fe-S cluster biogenesis arose twice during evolution. In the fungal lineage, the gene encoding multifunctional mtHsp70, Ssc1, was duplicated, giving rise to specialized Ssq1. Therefore, Ssq1 is not orthologous to the specialized Hsp70 from *E. coli* (HscA), but shares a striking level of convergence at the biochemical level. Thus, in the vast majority of eukaryotes, Jac1 and Isu function with the single, multifunctional mtHsp70 in Fe-S cluster biogenesis.

Results and Discussion

SSQ1 Arose by Gene Duplication in the Fungi

To elucidate the origin of Ssq1, we examined ten sequenced fungal genomes, analyzing the relationship between Ssq1, the *S. cerevisiae* mitochondrial Hsp70 (mtHsp70) that specializes in Fe-S cluster biogenesis, and Ssc1, the multifunctional mtHsp70 involved in multiple processes including protein folding and protein translocation. Properties of Hsp70s facilitate identification of orthologous, as opposed to paralogous, genes among species. First, Hsp70s are highly conserved, making their complete annotation and identification in sequenced genomes relatively facile. Second, paralogs tend to retain conserved characteristic sequence features such that functional groups of genes are more likely to be correctly identified by reciprocal-best-blast scores and have been shown to form monophyletic clades in phylogenetic analyses [5–7]. Our blast analyses identified all previously annotated HSP70s in each genome, and orthology relationships among mtHsp70s were clear and consistent in all cases (Table S1 in the Supplemental Data online). In addition, yeast species closely related to *S. cerevisiae* have conserved gene order along their chromosomes (synteny), further facilitating the identification of true orthologs [8–10]. Hence, although we primarily identified orthology among genes through reciprocal best blast among taxa with a known evolutionary history [11, 12], we were also able to verify orthology among closely related yeast species through syntenic relationships among genes.

By these methods we found that three species, *Yarrowia lipolytica*, *Neurospora crassa*, and *Schizosaccharomyces pombe*, each had a single mtHsp70 gene, which was most similar to the multifunctional *SSC1* (Figure 1A). However, all species descended from the common ancestor of *Candida albicans* and *S. cerevisiae* had orthologs of both *SSC1* and *SSQ1*. In addition, we found three homologous mtHsp70s in the genomes of *S. cerevisiae*, *S. bayanus*, *S. castellii*, and *C. glabrata* (*ECM10*, in addition to *SSC1* and *SSQ1*). Analysis of gene order along the chromosomes of closely related species indicated that, consistent with previous findings [8–10], *ECM10* arose as a duplicate of the multifunctional *SSC1* during a whole-genome duplication, on the order of 100 million years ago.

Our findings indicate that in the common ancestor of *C. albicans* and *S. cerevisiae*, duplication of a multifunctional mtHsp70 gene gave rise to what eventually became the highly specialized Ssq1, which is therefore not orthologous to the bacterial HscA. However, it is also possible that the presence of only a single mtHsp70 homolog in yeast species distantly related to *S. cerevisiae* is due to multiple losses of the *SSQ1* ortholog in those lineages. Therefore, we searched fully sequenced, annotated genome databases in a wide range of eukaryotes to identify mtHSP70 homologs among more distantly related species. A single mt*HSP70* homolog was found in the genomes of a putative basal fungus (*Encephalitozoon cuniculi*), protozoans (*Plasmodium falciparum*, *Trypanosoma cruzii*, and *Dictyostelium discoideum*), metazoans (*Ceanorhabditis elegans*, *Drosophila melanogaster*, *Ciona intestinalis*, *Danio rerio*, *Gallus gallus*, *Canis familiaris*, *Mus musculus*, and *Homo sapiens*), or plants (*Oryza sativa*) (Figure 1A, Table S1).

Thus, our analysis indicates that utilization of a specialized mitochondrial Hsp70 in Fe-S biogenesis in eukaryotes is limited to a subset of yeast species, those descended from the common ancestor of *C. albicans*

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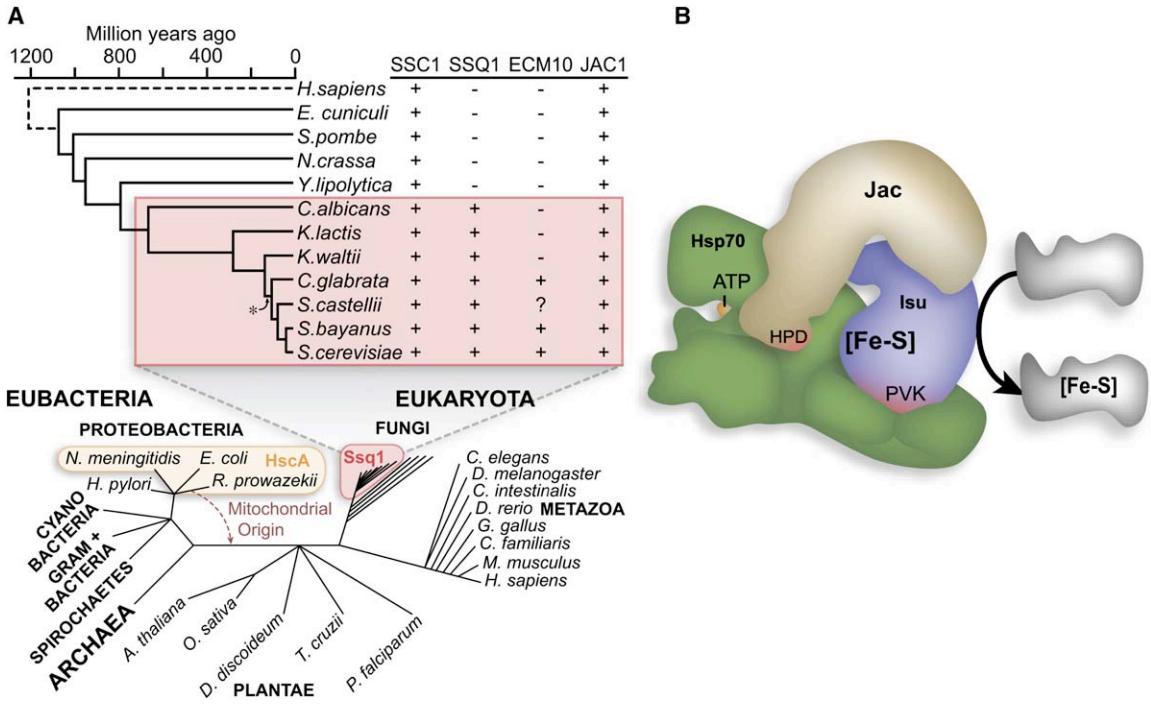

Figure 1. Orthology among Mitochondrial Hsp70s

(A) Evolutionary origins of mitochondrial Hsp70s and Jac1. (Top) Phylogenetic relationships, scaled to time, among ten fungal species, a putative fungus (microsporidia) (*Encephalitozoon cuniculi*), and humans are shown on the left (topology taken from [11, 12]; time scale taken from [29–32]). The presence (+) or absence (−) of orthologs for the indicated chaperones are indicated. The sequenced genome of *S. castelii* did not contain a *ECM10* ortholog, as indicated by (?); see Table S1. Species possessing a specialized Hsp70 are highlighted; the whole-genome duplication is indicated (*). (Bottom) Phylogenetic relationships among major groups from each of the three domains of life are shown. Only groups of eubacteria that are relevant to Fe-S metabolism are shown for clarity. All species of eukaryotes analyzed in this work are included in the tree topology. Proteobacteria having the specialized HscA are shaded in beige [4], and those fungi that independently evolved the specialized Ssq1 are in pink. All indicated higher eukaryotes had a single Ssc1 ortholog, with the exception of *Arabidopsis thalania*, which had four. Given the single ortholog found in *O. sativa*, these four are lineage specific.

(B) Model of chaperone function in Fe-S cluster biogenesis. The J protein Jac1 interacts with the scaffold, Isu, targeting it to Hsp70, where its conserved PVK tripeptide binds in Hsp70s peptide binding cleft. Simultaneously, the J domain of Jac1 (conserved HPD motif highlighted) interacts with Hsp70's ATPase domain, stimulating conversion to ADP and stabilizing the Isu:Hsp70 interaction. Subsequently, the Fe-S cluster is transferred to the recipient apoprotein (gray).

and *S. cerevisiae*. However, our analyses for *JAC1* and *ISU* revealed their presence as single-copy genes in all eukaryotes, supporting the idea that the mechanism of Fe-S cluster biogenesis has been maintained throughout eukaryotic evolution. The results of our evolutionary analysis are consistent with the following scenario: When mitochondria arose via endosymbiosis by an ancestral eukaryote of a bacterium ancestral to *R. prowazekii* [13], the machinery of Fe-S cluster biogenesis, including the orthologs of the bacterial cochaperone HscB (Jac1) and scaffold client IscU (Isu), was inherited and maintained [4]. But a gene encoding an ortholog of HscA, the highly specialized member of the Hsp70 family from bacteria, was either not transferred or not maintained in ancestral eukaryotes.

Although consistent with available data, this scenario also raises a conundrum. The J protein Jac1 functions in Fe-S cluster biogenesis in *S. cerevisiae* and has been conserved throughout eukaryotic evolution. However, J proteins have never been found to function alone [14]; rather, they always partner with Hsp70s, serving to stimulate Hsp70s ATPase activity and thus stabilize interaction with substrate proteins (Figure 1B). Sometimes, as is the case with Jac1, they interact with and help "deliver" the substrate protein (e.g., Isu, in the case of Jac1) to Hsp70. We reasoned that Jac1 in the absence of a specialized mtHsp70, Ssq1, functions with the multifunctional mtHsp70 to facilitate Fe-S cluster biogenesis. Below we describe a series of biochemical and genetic experiments designed to test this idea.

Peptide Binding Selectivity Differentiates Multifunctional from Specialized mtHsp70s

Both Ssq1's and HscA's function in Fe-S cluster biogenesis requires interaction with a PVK tripeptide of the Isu/Iscu scaffold [15, 16], a sequence motif conserved in all mitochondrial Isu orthologs. We reasoned that if Ssc1 is involved in Fe-S cluster biogenesis in organisms lacking the specialized Ssq1, it would also interact with this sequence. The ability of an Hsp70's peptide binding domain (PBD) to interact with a given peptide can be monitored by its interaction with a fluorescein-labeled peptide, as has been done with the Ssq1 PBD and a peptide containing the PVK motif of Isu [17]. Therefore, we purified PBDs of Ssc1 and Ssq1 from yeast species whose genomes encode both orthologs (*S. cerevisiae* and *C. albicans*), as well as species that encode only Ssc1 (*Y. lipolytica* and human). All PBDs tested bound

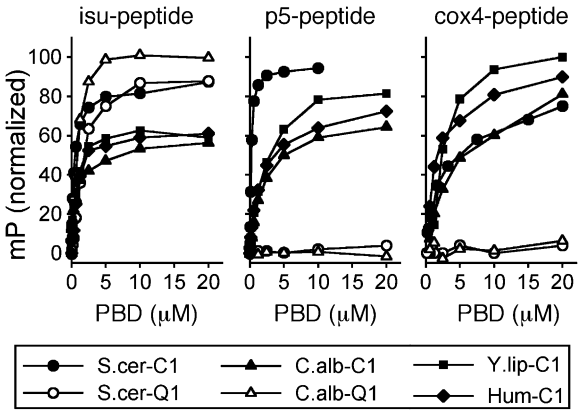

Figure 2. Peptide Binding Specificity of PBD of Orthologs of Ssc1 and Ssq1

Fluorescein-labeled peptides (Isu [LSLPPVKLHC], P5 [CALLLSAPRR], and Cox4 [MLSLRQSIRFFKPTRRLC]) at 10 nM were incubated in the presence of the indicated concentrations of purified PBDs of Ssc1 (C1) or Ssq1 (Q1) from *S. cerevisiae* (S.cer) and *C. albicans* (C.alb) and of Ssc1 from *Y. lipolytica* (Y.lip) and human (Hum). Fluorescence anisotropy measurements were taken. After subtraction of background polarization, minipolarization (mP), normalized to 100% of the maximum reading with each peptide, was plotted versus PBD concentration.

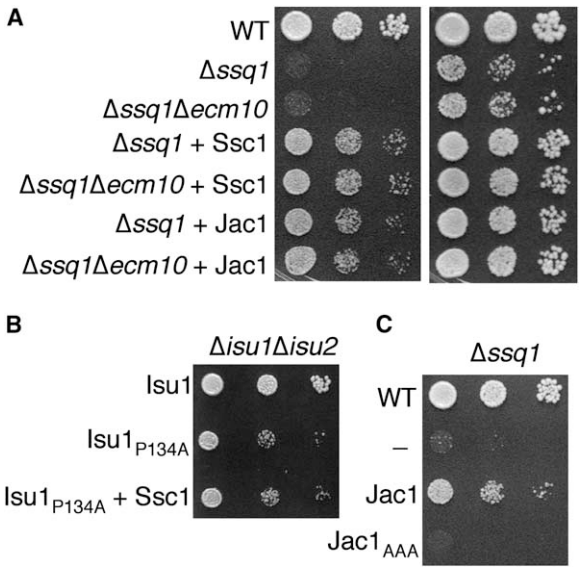

Figure 3. Overexpression of Ssc1 and Jac1 Suppress Growth Defects of *Δssq1*

Serial dilutions of cells were plated and incubated on indicated medium.

(A) Indicated strains expressing either Ssc1 or Jac1 from a cen or 2μ plasmid, respectively, were grown on rich glucose media at 23°C (left panel) and 30°C (right panel) for 3 days.

(B) *Δisu1Δ isu2* strain expressing wild-type Isu1, $Isu1_{P134A}$ alone, or $Isu1_{P134A}$ and Ssc1 from a plasmid were grown on glucose synthetic minimal (SM) media without tryptophan for 3 days at 30°C.

(C) *Δssq1* cells expressing wild-type Jac1 or $Jac1_{AAA}$ from a 2μ plasmid were grown on rich glucose for 3 days at 23°C.

the Isu peptide efficiently (Figure 2), regardless of whether they were from Ssq1- or Ssc1-type mtHsp70s, or whether they were from organisms that had one, or more than one, mtHsp70. These results are consistent with the hypothesis that Ssc1 is involved in Fe-S cluster biogenesis in species lacking Ssq1.

We also measured the ability of purified PBDs to interact with two other peptides, P5 and Cox4, known to bind *S. cerevisiae* Ssc1 [18]. As expected, all PBDs derived from orthologs of Ssc1 were able to bind both P5 and Cox4 peptides (Figure 2). In contrast neither the PBD of Ssq1 from *S. cerevisiae* nor that from *C. albicans* interacted with these peptides, indicating that Ssq1-type mtHsp70s specifically interact with Isu, but have lost the ability to interact with other protein substrates. These results are consistent with the involvement of Ssc1 in a broad range of processes, from protein folding to protein translocation across membranes, which require interaction with a very diverse array of hydrophobic amino acid sequences. On the other hand, the inability of Ssq1 to bind to the P5 and Cox4 peptides is consistent with Isu being its only identified substrate protein [19].

Interestingly, both specialized Hsp70s, HscA and Ssq1, are thought to interact specifically with a single substrate, IscU/Isu, recognizing the conserved PVK motif [15, 16] (Figure 1B). The striking functional similarities of Ssq1 and HscA, despite their independent origins, could be considered one of only a handful of examples of functional convergent evolution at the biochemical level [20]. Consistent with this idea, Ssq1 has a mixture of biochemical properties, some similar to those of the specialized HscA, whereas others resemble those of the multifunctional Ssc1 and its bacterial ortholog DnaK. Similar to HscA [15], Ssq1 recognizes the PVK motif of Isu1 as its unique binding site (Figure 2) [16]. On the other hand, Ssq1, like Ssc1 and DnaK, binds adenine nucleotide very stably, whereas HscA does not [19]. Ssq1, Ssc1, and DnaK require a nucleotide release factor, Mge1/GrpE, to complete a cycle of substrate binding and release, whereas HscA binds nucleotide transiently and is thought not to require a release factor. These differences also suggest that twice in evolution, specialized Hsp70s evolved to function solely in Fe-S cluster biogenesis.

Ssc1 of *S. cerevisiae* Can Function in Fe-S Biogenesis with Jac1

Our biochemical analysis indicates that Ssc1 can interact with the PVK motif of Isu. However, can the multifunctional Hsp70 Ssc1, which plays essential roles in protein translocation into mitochondria and folding of import and mitochondrial synthesized proteins, actually function in Fe-S cluster biogenesis in *S. cerevisiae*? First, we constructed a strain, *Δecm10 Δssq1*, that had a single mtHsp70, Ssc1. Because Fe-S cluster-forming ability has been shown to correlate with rate of colony-forming ability on plates [21, 22], we compared the growth of *Δssq1* and *Δecm10 Δssq1* cells. The strains grew very similarly (Figure 3A, data not shown), suggesting that Ecm10 does not play a role in Fe-S cluster biogenesis.

Two-fold overexpression of Ssc1 partially suppressed the severe growth defect of *Δssq1 Δecm10* cells, as was the case with *Δssq1* cells [23] (Figure 3A). This suppression suggests that Ssc1 can function in Fe-S cluster biogenesis when it is the only Hsp70 in mitochondria. However, the fact that Ssc1, when overexpressed, can substitute to some extent for Ssq1 does not necessarily mean that the molecular mechanisms of Ssq1 and Ssc1

action are the same in promoting Fe-S protein biogenesis. A hallmark of Ssq1's function is its specific interaction with the Isu scaffold protein requiring the PVK motif [16]. To test whether the function of Ssc1 in Fe-S biogenesis is also dependent on this specific interaction with Isu, we asked whether the growth defect of a mutant having a scaffold with an alteration in the Ssq1 binding site could be suppressed by increasing Ssc1 levels. We utilized a mutant scaffold in which the proline of the PVK motif at position 134 is replaced by an alanine (P134A), which we had previously demonstrated to be defective in its interaction with Ssq1 [16]. *S. cerevisiae* has two *ISU* genes, *ISU1* and *ISU2*, paralogs that were generated during the whole-genome duplication [9, 10]. Cells expressing $Isu1_{P134A}$ as the only Isu protein grew more slowly than wild-type cells or *Δisu1 Δisu2* cells expressing Isu1 from a plasmid (Figure 3B). Overproduction of Ssc1 did not improve the growth of *Δisu1 Δisu2* cells expressing $Isu1_{P134A}$. Our approach was based on the rationale that, if the effect of Ssc1 on Fe-S cluster biogenesis was the result of interactions with Isu at a site different from the Ssq1 interaction site or of interactions with other proteins, suppression might well occur. The lack of suppression is consistent with the idea that similar to Ssq1, productive interaction of Ssc1 with Isu1 requires an intact PVK motif. Therefore, we propose that the mechanisms of action of Ssc1 and Ssq1 in Fe-S cluster biogenesis are the same.

The above results are consistent with the idea that Ssc1 can indeed function in Fe-S biogenesis. Next, we tested whether Jac1 functions as Ssc1's J protein partner in this process. First, approximately 8-fold overexpression of Jac1 partially suppressed the growth defect of *Δssq1* cells (Figure 3C). Similar levels of overexpression of Mdj1, an established J protein cochaperone of Ssc1 in the folding of proteins in the mitochondrial matrix, did not suppress the growth defect of *Δssq1* cells (data not shown). To assess whether the suppression by Jac1 was due to the classical mode of J protein function, we made use of a mutant Jac1, $Jac1_{AAA}$ [24], having three alanine substitutions in the highly conserved HPD tripeptide (Figure 1B), a motif found in virtually all functional J domains. This mutant Jac1 did not suppress the growth defect of *Δssq1*, indicating that Jac1 is functioning as a J protein partnering with Ssc1 to effect suppression.

To test whether our in vivo observations could be correlated with biochemical results, we measured Ssc1's ATPase activity in the presence of Isu and Jac1. We chose this assay because an increase in the ATPase activity of Hsp70 in the presence of both a J protein cochaperone and a substrate protein is a hallmark of productive chaperone interactions (Figure 1B). Three-fold stimulation of Ssc1 ATPase indicated that this multifunctional Hsp70 interacted with both Jac1 and Isu1, albeit not as effectively as did Ssq1 (Figure 4A). The lack of Ssq1 binding to general peptide motifs suggests that this function has been lost following retention as a gene duplicate. Similarly, the lower level of ATPase stimulation of Ssc1 by Jac1 could also be taken as evidence that Ssq1 and Jac1 may have coevolved, whereas Ssc1 has lost the ability to interact with Jac1 to some degree. These reciprocal, yet complimentary, losses of functions may well be a molecular example consistent

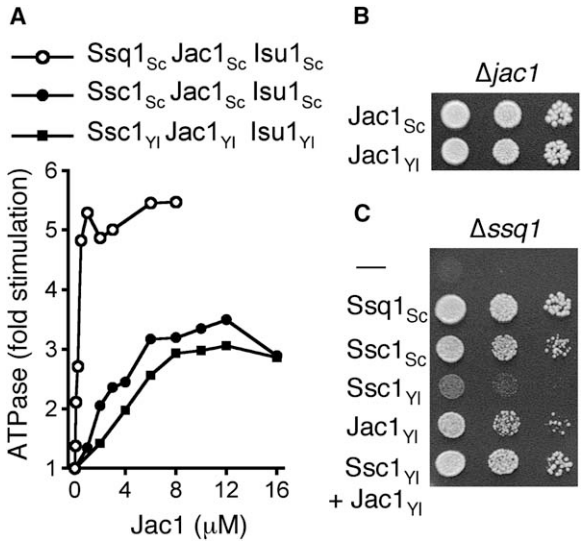

Figure 4. Orthologs from *Y. lipolytica* Are Functional in Fe-S Cluster Biogenesis

(A) Stimulation of Ssc1 ATPase activity by Jac1 and Isu1. Reactions contained indicated Hsp70 (0.8 μM), indicated Isu1 (16 μM), $Mge1_{SC}$ (0.8 μM), and different concentrations of indicated Jac1.

(B) *Δjac1* cells expressing Jac1 from *S. cerevisiae* ($Jac1_{Sc}$) or the Jac1 ortholog from *Y. lipolytica* ($Jac1_{Yl}$) were plated on rich glucose media and incubated for 3 days at 30°C.

(C) *Δssq1 cells* overproducing $Ssq1_{Sc}$, $Ssc1_{Sc}$, $Ssc1_{Yl}$, or $Jac1_{Yl}$ and $Ssc1_{Yl}$ as indicated. Cells were grown on rich glucose media at 23°C for 3 days.

with the subfunctionalization model of evolution following gene duplication. Although it appears that Ssq1 has completely lost the ability to act as a general chaperone, Ssc1 has retained some ability to function in Fe-S cluster biogenesis. Perhaps such redundancy increases robustness of this essential cellular machinery. These hypotheses are worthy of further testing.

Jac1 from *Y. lipolytica* is functional in *S. cerevisiae*

The biological function of Jac1 has yet to be rigorously established in eukaryotes other than *S. cerevisiae*. However, Jac1's high degree of conservation, and the fact that in *S. cerevisiae* it can function, albeit rather ineffectively, with Ssc1, suggests that it does indeed work in Fe-S cluster biogenesis in all eukaryotes. We chose *Y. lipolytica* to address this issue more directly, because it is the species that both shares the most recent common ancestor with *S. cerevisiae* (Figure 1) and has a single mtHsp70. The ATPase activity of the Ssc1 from *Y. lipolytica* ($Ssc1_{Yl}$) was stimulated in the presence of its cognate Jac1 ($Jac1_{Yl}$) and Isu1 ($Isu1_{Yl}$) to an extent similar to that observed for *S. cerevisiae* proteins (Figure 4A). $Jac1_{Yl}$ was also able to functionally replace $Jac1_{Sc}$ in *S. cerevisiae*, allowing robust growth of *Δjac1* cells (Figure 4B) and suppressing the slow-growth phenotype of *Δssq1* cells (Figures 3A and 4C, data not shown). In addition, even though overexpression of $Ssc1_{Yl}$ led to only a minor improvement in the growth of *Δssq1* cells, the suppression was more complete when both $Jac1_{Yl}$ and $Ssc1_{Yl}$ were expressed together than when $Jac1_{Yl}$ was expressed alone (Figure 4C). This enhancement of suppression when both *Yarrowia* proteins are expressed

is also consistent with the hypothesis that the mtHsp70 and J protein cochaperones are coevolving.

Conclusions

Before this study, it was generally thought, on the basis of analysis of only two model systems, that specialized Hsp70s were utilized in all eukaryotic organisms for Fe-S cluster biogenesis. We now conclude that twice during evolution, specialized Hsp70s arose as the result of the duplication of a gene encoding a multifunctional Hsp70. Thus, bacterial HscA and mitochondrial Ssq1 serve as an example of convergence at the biochemical level, and, on the basis of our functional data, the retention of both Ssc1 and Ssq1 is a potential example of subfunctionalization. In other eukaryotes, including humans, Fe-S cluster biogenesis is likely carried out by utilizing the generic mtHsp70 Ssc1.

The results reported here raise a broader question: Do other examples of specialized Hsp70s exist? Yeasts do have a specialized ribosome-associated Hsp70, Ssb, that interacts with nascent polypeptides as they exit the ribosome tunnel, whereas other eukaryotes appear to use the multifunctional cytosolic Hsp70, orthologs of *S. cerevisiae* Ssa [25]. Further study is needed to determine whether examples of Hsp70s devoted to particular functions have evolved to function in physiological processes in specialized tissues of multicellular eukaryotes.

Experimental Procedures

Evolutionary Sequence Analyses

Homologs of mtHsp70s Jac1 and Isu1 were identified by TBLASTN of each *S. cerevisiae* peptide sequence as a query against the completed genome sequence for each of the species listed in the text. When available, the refseq protein database for each species was also searched, by using PBLAST. In each case, the translations from all significant hits were then used as query sequences in a PBLAST search of the *S. cerevisiae* protein database to identify reciprocal-best-blast sequences as orthologs. Orthology was further verified on the basis of syntenic relationships among genes from the following species: *S. cerevisiae*, *S. bayanus*, *C. glabrata*, *S castellii*, *K. waltii*, and *K. lactis*. Syntenic relationships were determined manually by using BLASTN of the *S. cerevisiae* gene of interest plus four neighboring genes, two each from centromere distal and proximal portions of the chromosome, respectively. These results were then compared to previously published synteny maps [9, 10] and visually inspected on the Yeast Gene Order Browser [26]. Orthology among fungal mtHsp70 homologs, to the exclusion of other family members, was further verified via phylogenetic analyses of both the peptide and nucleotide sequences, and the complete analysis will be published elsewhere.

Biochemical and Genetic Methods

S. cerevisiae strains used are haploid derivatives of PJ53 (W303) containing various gene deletions. *JAC1*, *ISU1*, *ISU2*, and *SSQ1* deletions have been described previously [24, 27]. A deletion of *ECM10* was constructed by transforming with DNA containing *TRP1* in place of sequences −289 to +1592. The open reading frames for $SSC1_{Yl}$ and $JAC1_{Yl}$ were amplified by PCR from chromosomal DNA (CLIB99) and cloned into p414TEF and p415TEF, respectively [28]. *C. albicans* genes were obtained from chromosomal DNA (SC5314), and *H. sapiens* genes from total RNA (HEK293 cells). Plasmids for purification of PBDs of $Ssq1_{Ca}$, $Ssc1_{Ca}$, $Ssc1_{Yl}$, and $Ssc1_{Hs}$ (Mot2) were made by amplifying sequences starting at aa 396, 411, 410, and 433, respectively, by PCR from cloned genes. A BspHI site was engineered at the 5′ end and a XhoI site at the 3′ end in order to clone into pET21d digested with NcoI and XhoI (Novagen, Madison, Wisconsin). PBDs and full-length Hsp70s were purified from *E. coli* and *S. cerevisiae*, respectively, as described previously [16, 18].

Supplemental Data

Supplemental Data include one table and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/16/1660/DC1/.

Acknowledgments

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