Stable Association of 70-kDa Heat Shock Protein Induces Latent Multisite Specificity of a Unisite-specific Endonuclease in Yeast Mitochondria*

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The multisite-specific endonuclease Endo.SceI of yeast mitochondria is unique among endonucleases because its 50-kDa subunit forms a stable dimer with the mitochondrial 70-kDa heat shock protein (mtHSP70), which otherwise fulfills a chaperone function by binding transiently to unfolded proteins. Here we show that the mtHSP70 subunit confers broader sequence specificity, greater stability, and higher activity on the 50-kDa subunit. The 50-kDa subunit alone displayed weaker activity and highly sequence-specific endonuclease activity. The 50-kDa protein exists as a heterodimer with mtHSP70 in vivo, allowing Endo.SceI to cleave specifically at multiple sites on mitochondrial DNA. Endo.SceI may have evolved from a highly specific endonuclease that gained broader sequence specificity after becoming a stable partner of mtHSP70.

Endo.SceI is a yeast mitochondrial endonuclease having multisequence specificity (1–3). Endo.SceI was purified as a heterodimer comprised of 50- and 75-kDa subunits (4), the latter of which was later identified as the mitochondrial 70-kDa heat shock protein (mtHSP70), also referred as Ssc1p (3). The 50-kDa subunit shares the consensus amino acid sequence LAGLIDADG (5, 6) for yeast sequence-specific endonucleases involved in genetic recombination (e.g. HO endonuclease and mitochondrial ω endonuclease), suggesting that this subunit is responsible for the core activity of the endonuclease. The recognition sequences of recombinational endonucleases are as long as 17–26 bp (7–10) and are unisequence-specific, which means that they cleave at only one unique site on the whole genome. The Endo.SceI endonuclease is unique among these endonucleases in that it tolerates degeneracy at several positions within the cleavage sequence (2). Because of its broad sequence specificity, Endo.SceI cleaves randomly at over 30 sites on yeast mitochondrial DNA both in vitro and in vivo (Ref. 11 and data shown below). The function of Endo.SceI is important during the process of mitochondrial fusion when haploid cells are mated to form zygotic cells. During this process, Endo.SceI cleaves mitochondrial DNA to induce genetic recombination among the heterogeneous mitochondrial DNAs inherited from the parents (12).

The 50-kDa protein is not the sole binding partner of mtHSP70. mtHSP70 is a member of the HSP70 family and has broad affinity for proteins in the unfolded state. Molecular genetics and biochemical analysis have revealed that mtHSP70 transiently binds to nuclear-encoded mitochondrial proteins which become unfolded during their transfer from the cytosol across the mitochondrial membrane. mtHSP70 is essential for the import of these proteins into mitochondria and their subsequent folding (13, 14). More than 10 homologs of HSP70 have been detected or cloned from the genome sequence of the yeast Saccharomyces cerevisiae (15–19). The HSP70 family functions in a diverse set of processes, including regulation of the heat shock response, quality control of proteins and protein folding, as well as protein translocation across organelar membranes (20, 21).

The 50-kDa protein is unique among the binding partners of HSP70 in that mtHSP70 does not chaperone the 50-kDa protein as a transient partner but becomes stably incorporated as part of the heterodimer complex. The diverse functions of HSP70 described above are accomplished through transient binding of HSP70 to proteins in an unfolded state. HSP70 prevents the unfolded proteins from aggregating and misfolding, until the proteins are either properly folded, transferred into organelles, or degraded by proteases (21). The chaperone function of mtHSP70 and the sequence motifs found in the 50-kDa subunit led us to speculate that mtHSP70 might somehow modulate the structure and activity of the 50-kDa subunit. It has been difficult, however, to examine if mtHSP70 has a regulatory role for the endonuclease activity because the heterodimer is so stable under nondenaturing conditions that the 50-kDa protein cannot be functionally analyzed in the absence of mtHSP70 (4).

Standard overexpression systems have not been applied to the 50-kDa protein because it is encoded by a mitochondrial gene whose translation requires mitochondrial specific codon usage. To examine the role of mtHSP70 in the heterodimer, we established overexpression and purification systems for the 50-kDa subunit. To our surprise, the purified 50-kDa protein by itself showed endonuclease activity that was not multisequence-specific but unisequence-specific. The Endo.SceI heterodimer was easily reconstructed by incubating the 50-kDa protein with mtHSP70 in the presence of ADP. The 50-kDa protein did not appear to bind to the substrate binding domain of mtHSP70 but to the ATPase domain. By comparing the
enzyme activities of the 50-kDa protein with the reconstructed heterodimer, we found that mtHSP70 converts the uniqueness-specific 50-kDa endonuclease into a multisequence-specific enzyme. Thus, multisequence specificity, the characteristic feature of Endo.Sel, is dependent on the stable binding of mtHSP70.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—**Thirty three oligonucleotides were used to change the mitochondrial TGA codons in the 50-kDa subunit gene, ENS2 (5), into universal TGG codons, the 23 ATA codons into ATG codons, the 5 CTT codons into ACT codons, and the 4 CTA codons into ACC codons. Thirteen cycles of mutagenesis (PCR) were performed using the Mutan-K kit (Takara Biomedicals, Kyoto, Japan). Up to 16 mutagenic primers were used at a time to reduce the number of mutagenesis cycles. The DNA sequence of the entire gene was checked after each cycle to ensure that no secondary mutations had been introduced.

**50-kDa Protein—**The modified ENS2 gene containing universal codons (GenBankTM accession number AF159242) was cloned into the pRSET vector (Invitrogen, Carlsbad, CA). The recombinant 50-kDa subunit, which had a hexahistidine tag at the N terminus, was expressed in *Escherichia coli* BL21(DE3) pLysS cells with induction at 18 °C for 12 h with 0.4 mM isopropylthigalactospyranoside. The overexpressed protein was partially purified by the use of a ProBond Ni 2 + chromatography step on an SP-Sepharose FF ion exchange resin column (1 × 5 cm, Amersham Pharmacia Biotech), and bound proteins were eluted with a gradient of 200–500 mM NaCl in 50 mM sodium phosphate buffer (pH 8.0). The 50-kDa protein was eluted from the column at 300 mM of NaCl. The purified protein was stored in 50 mM Tris-HCl (pH 8.0) containing 500 mM NaCl at 0 °C or in the same buffer containing 50% glycerol at −20 °C.

**Endonuclease Assay—**Two types of pUC-based plasmid DNA were used for the endonuclease assay. pmITO is a pUC119-based plasmid that has the mitochondrial EcORI/EcoRI fragment containing the ol2 region (5). A pUC118-based plasmid, pBR1, contains the 200-bp HindIII/NheI region of pBR22. Samples were assayed for the Endo.Sel activity under the following conditions: 15 ng of the substrate DNA previously linearized by ScaI were incubated with 1–2 μl of the protein solution to be tested in 20 μl of 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, 1 mM dithiothreitol, and 10 mM MgCl2. Reactions were performed at 37 °C for 30 min unless otherwise stated and then stopped by the addition of EDTA and proteinase K (1). The samples were phenol-extracted and then analyzed by electrophoresis on 0.8% agarose gels in TAE buffer (23). The DNA in the gels was stained with the SYBR green I nucleic acid stain (Molecular Probes, Inc., Eugene, OR) at 10,000 dilution with gentle agitation at room temperature for 30 min. DNA cleavage was quantitatively analyzed with an FM-Bio imaging scanner (Takara Biomedicals).

**Measurement of Circular Dichroism—**The circular dichroism spectrum of the 50-kDa protein (103.5 μg/ml) was obtained on a model-J-720 spectrophotometer (Jasco, Tokyo, Japan) at 4 °C with a light path of 2 mm. The protein concentration was determined by amino acid analysis of the 50-kDa protein on a Hitachi i-8500 amino acid analyzer (Hitachi Koki Co., Tokyo, Japan) after acid hydrolysis (24).

**Reconstruction of the Heterodimer—**The purified mtHSP70 was overexpressed in *S. cerevisiae* and purified as described previously (25). The purified mtHSP70 was incubated at 4 °C overnight in a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM ADP. The ADP-bound mtHSP70 (23 μg/ml) and the 50-kDa subunit (13 μg/ml) were incubated at 25 °C for 90 min in 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl, 1 mM dithiothreitol, and 30 mM MgCl2 to allow complex formation before gel filtration. Gel filtration analysis of the complex was performed on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) at 4 °C with 30 mM Tris-HCl (pH 8.0) containing 500 mM NaCl as elution buffer. Eluted proteins were fractionated and detected by Western blot analysis and the Endo.Sel nuclease assay. The 50-kDa protein in the eluates was detected by both the endonuclease assay with the ol2 substrate and Western blot analysis with anti-50-kDa protein antibody.

**Detection of in Vivo Cleavage—**Mitochondrial DNAs were prepared from YKN1423 and YKN1424 (12). The DNAs were digested with restriction enzymes and then run on a 6% polyacrylamide gel for Southern blot analysis (12). DNA probes used were the 0.25-kb MboII/EcoRI fragment of ol2, the 5'-CTCTAAATTATAATATATATTATGATC-3' oligonucleotide which contains the sequence downstream of the *BanHI* site within the 15 S rDNA gene, and the 1.1-kb Xba/HincII fragment which contains the first exon of cob-box. Radiolabeling of the DNA probes was performed as described previously (5, 12). [α-32P]ATP (110 TBq/mmol) and γ-[32P]ATP (110 TBq/mmol) were purchased from Amersham Pharmacia Biotech.

**Antibodies—**Anti-50-kDa protein antibody was generated by immunization of the mice with purified 50-kDa protein. Anti-6xHis monoclonal antibody was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). Anti-DnaK monoclonal antibody was from StressGen (British Columbia, Canada). Anti-mtHSP70 monoclonal antibody was prepared as described previously (4). Peroxidase-labeled secondary antibody was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

**Binding Assay—**The products of a series of GST-mtHSP70 fusion genes cloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) were overexpressed in *E. coli* JM109, and the GST fusion proteins were purified according to the manufacturer's protocol. Either ATP or ADP was loaded onto the fusion protein by incubating them with 10 mM nucleotide at 4 °C overnight. The fusion protein was assayed by gel electrophoresis on 0.8% agarose gels in TAE buffer (23). The DNA in the gels was stained with the SYBR green I nucleic acid stain (Molecular Probes, Inc., Eugene, OR) at 10,000 dilution with gentle agitation at room temperature for 30 min. DNA cleavage was quantitatively analyzed with an FM-Bio imaging scanner (Takara Biomedicals).

**Modulation of Enzymatic Activity by a Molecular Chaperone**

**RESULTS**

**Overexpression of the 50-kDa Subunit in *E. coli***

The 50-kDa subunit is encoded by a mitochondrial gene whose coding sequence contains 37 mitochondrial specific codons (5). The 50-kDa subunit gene was modified so that the whole amino acid sequence could be directed by universal codons and expressed in widely used overexpression systems. Briefly, 13 cycles of site-directed mutagenesis (22) were performed by using mutagenic oligonucleotide primers. Up to 16 primers were used at a time to reduce the number of mutagenesis cycles, unless the primer sequences overlapped with one another. For easier purification of the 50-kDa protein, its N terminus was tagged with a hexahistidine sequence. We overexpressed the hexahistidine-tagged 50-kDa protein in *E. coli* and purified it by affinity chromatography and ion exchange chromatography (Fig. 1A). Since DnaK, a bacterial HSP70, is present in *E. coli* cells, the 50-kDa protein sample was examined for contamination with DnaK. Western blot analysis with
an anti-DnaK monoclonal antibody showed that bacterial HSP70 could not be detected in the 50-kDa protein sample (Fig. 1A). For reconstruction of Endo.SceI in vitro, mtHSP70 was purified from the isolated mitochondria of yeast cells which overexpress mtHSP70 (Fig. 1A) as reported previously (25). We used a yeast strain that lacked the functional gene for the 50-kDa protein to avoid possible contamination of the mtHSP70 sample with the 50-kDa protein (5, 12).

The quaternary structure of the purified 50-kDa protein was analyzed by gel filtration chromatography. The purified 50-kDa protein was eluted as a single symmetrical peak between the elution positions of bovine serum albumin (67 kDa) and ovalbumin (43 kDa), suggesting a globular and monomeric structure (Fig. 1B).

The Purified 50-kDa Protein Is a Unisequence-specific Endonuclease

In general, the function of HSP70 is accomplished through its transient binding to unfolded or denatured proteins (13, 14, 28, 29). According to this rationale, mtHSP70 binding to the 50-kDa protein was eluted as a single symmetrical peak between the elution positions of bovine serum albumin (67 kDa) and ovalbumin (43 kDa), suggesting a globular and monomeric structure (Fig. 1B).

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ing (12). The sizes of the cleaved products (3.4 and 1.4 kb) suggest that the cutting site of the 50-kDa protein was identical to that of the Endo.SceI dimer. The 50-kDa protein did not digest the oli2 mutant sequence (12) containing an altered Endo.SceI cutting site (data not shown). These results, as well as the physical properties of the 50-kDa protein, indicate that the 50-kDa protein possesses a native structure and acts as a functional endonuclease. However, the 50-kDa protein did not exhibit multisequence specificity which is a characteristic feature of the Endo.SceI heterodimer. The 50-kDa protein could not cleave plasmid DNAs, phage DNAs, or yeast mitochondrial DNA except for the olig2 sequence, whereas all of these DNAs have been used to detect Endo.SceI endonuclease activity (see below). For instance, the purified 50-kDa protein did not cleave a cutting site in pBR322 DNA which has been used in the standard assay for Endo.SceI (2, 4). These data suggested that mtHSP70 possesses a novel function distinct from its normal role in protein folding.

Reconstruction of the Endo.SceI Heterodimer

To investigate the role of mtHSP70 in the Endo.SceI heterodimer, we tried to reconstruct the heterodimer from each subunit in vitro. HSP70 can be found in three states depending on the nature of the bound nucleotide as follows: ATP-bound HSP70, ADP-bound HSP70, and nucleotide-free HSP70. In general, ADP-bound HSP70 makes a more stable complex with unfolded proteins than ATP-bound HSP70. The dissociation constants of ADP-bound HSP70 with substrates are between 5- and 85-fold lower than those of ATP-bound HSP70, although the ADP-bound form has slower binding rates for substrates (for a review, see Ref. 31). As an initial trial, the active 50-kDa protein was incubated with ADP-bound mtHSP70 without prior denaturing treatment of the endonuclease. We chose 25 °C as the temperature for the complex formation reaction because the 50-kDa protein loses little activity in terms of its ability to cleave within the olig2 sequence when incubated at this temperature even after several hours (data not shown). After the 50-kDa subunit was incubated with a stoichiometric amount of ADP-mtHSP70, the protein mixture was loaded onto a gel filtration column to analyze complex formation. The 50-kDa subunit was detected in fractions from the column by Western blot analysis and the endonuclease assay. The elution profile suggested that the Endo.SceI heterodimer was efficiently formed in vitro from the 50-kDa protein and mtHSP70, as the position of the peak fraction (fraction 68) corresponded to a molecular size of around 125 kDa (Fig. 2). It should be noted that dimer formation did not require denaturing treatment of the 50-kDa protein. The endonuclease assay using the oli2 substrate revealed that the Endo.SceI activity could only be observed in the fraction containing the heterodimer. No complexes of the 50-kDa subunit appeared at the elution volume of the heterodimer when the 50-kDa subunit was incubated alone (data not shown). The nucleotide dependence of heterodimer formation, as well as the nature of the binding between the 50-kDa protein and mtHSP70, will be described below.

Effect of mtHSP70 Binding on the Endonuclease Activity of the 50-kDa Subunit

Substrates—After establishing the conditions for complex formation, we quantitatively analyzed the effect of mtHSP70 binding on the activity of the 50-kDa protein. Two DNAs were chosen as substrates in the nuclease assay. The first one contained the mitochondrial oli2 sequence, which is the natural substrate efficiently cleaved by Endo.SceI in vivo (12) and the 50-kDa protein in vitro (Fig. 1D). The other substrate contained the Endo.SceI-cleavable sequence of pBR322 (2). Since the cleavage sequence of Endo.SceI is not unique but multiple, we determined the consensus sequence for Endo.SceI cleavage by comparing the cleavable sequences found in phage and plasmid DNAs (2) (Fig. 3A). Unlike bacterial restriction enzymes, Endo.SceI recognizes and binds to at least 26-bp regions of DNA (10). The cleavable sequences of oli2 and pBR322 used in the present experiment contained four and six mismatches to the consensus sequence, respectively (Fig. 3A).

Thermostability and Specific Activity—We have observed that Endo.SceI is a heat-sensitive enzyme that loses its activity at 37 °C within 2 h (1).2 Thus, we expected that the 50-kDa protein would be even more heat-labile. Since one of the biological functions of HSP70 is to protect unfolded proteins from aggregation and irreversible inactivation (for a review, see Ref. 21), we examined the thermostability of the 50-kDa protein in the presence or absence of mtHSP70. After preincubation of the 50-kDa protein at 37 °C the endonuclease reaction was done for 1 min to compensate for the possible inactivation of the 50-kDa protein during the reaction (note that the standard assay for Endo.SceI uses 30–60 min for digestion). Fig. 3B shows that the endonuclease activity of both the 50-kDa monomer and the reconstructed Endo.SceI decreased during the preincubation at 37 °C. However, the 50-kDa protein displayed reduced thermostability in the absence of the other subunit. After 15 min of preincubation, the residual activity of the 50-kDa monomer was less than 40% of the original activity, and it further decreased below 20% after 30 min preincubation. Under the same conditions, the reconstructed dimer was more resistant to inactivation, as its endonuclease activity remained stable with over 80% of the original activity remaining after 30 min of preincubation. These results indicate that the 50-kDa protein is heat-labile and that the mtHSP70 subunit confers thermostability on the 50-kDa protein. In a separate experiment,

2 H. Mizumura, T. Shibata, and N. Morishima, unpublished data.
addition of mtHSP70 to the 50-kDa protein inactivated at 37 °C did not restore the endonuclease activity, suggesting that the loss of activity under these conditions is due to irreversible inactivation.

We next examined the specific activity of the 50-kDa protein with or without mtHSP70, as we observed that the endonuclease activity of the reconstituted dimer was always higher than that of the 50-kDa monomer. The assay was again done for 1 min because this short assay time contributed little to the time inactivation. In agreement with previous observations of Endo.SceI endonuclease activity, Fig. 3C shows an approximately 3-4-fold enhancement of endonuclease activity for the reconstituted dimer up to a concentration of 1 μg/ml. The specific activity of the reconstituted dimer is comparable to that of the endogenous Endo.SceI heterodimer within the similar range of protein concentrations (Fig. 3C). These results indicate that the binding of mtHSP70 to the 50-kDa protein confers higher specific activity as well as greater thermostability.

Sequence Specificity—With the oli2 DNA, the endonuclease activity of the monomeric 50-kDa subunit was approximately one-third that of the heterodimer (Fig. 3C). This suggests that we might be able to detect cleavages of other substrate DNAs by the 50-kDa subunit at similar efficiency unless the multiple sequence specificity is different from that of the Endo.SceI dimer. Preliminary experiments showed that the 50-kDa protein could not cleave pBR322, although Endo.SceI can specifically cleave it. A simple explanation for the result is that the apparent change in sequence specificity might be the result of mtHSP70 enhancement of the overall activity of the 50-kDa protein. Another possibility is that the 50-kDa protein has very narrow sequence specificity compared with the reconstituted mtHSP70/50-kDa heterodimer. To examine these possibilities, we quantitatively compared the endonuclease activity of the reconstituted dimer with that of the 50-kDa protein using the pBR322 substrate.

In agreement with previous observations of Endo.SceI endonuclease specificity, Fig. 3D shows that the reconstituted dimer cleaved the pBR322 substrate as well as the oli2 sequence (2, 12). However, the pBR322 substrate was not cleaved
by the 50-kDa subunit at all. Cleavage of the pBR322 substrate was not observed even at concentrations of up to 16 μg/ml of the 50-kDa protein, whereas the cleavage of the same substrate was evident with only 0.125 μg/ml of the 50-kDa protein included in the reconstituted dimer (Fig. 3D). Neither the 50-kDa protein alone nor mtHSP70 showed endonuclease activity on the pBR322 substrate (Fig. 3E), indicating that the 50-kDa protein gained multiple sequence specificity on association with mtHSP70. The interaction between the 50-kDa protein and mtHSP70 appears to be specific because the 50-kDa protein preincubated with ADP-loaded DnaK at a 1:1 molar ratio did not show cleavage activity on the pBR322 substrate (Fig. 3E).

To compare the sequence specificity of the reconstructed dimer with that of the 50-kDa monomer in a more comprehensive manner, we used yeast mitochondrial DNA as a substrate for the endonuclease assay. We prepared BamHI/PstI double restriction digests of mitochondrial DNA, which was then treated with either the 50-kDa protein or Endo.SceI. The mitochondrial DNA used has a unique PstI site within the 50-kDa protein gene, which is located at about 1.2 kb downstream of the Endo.SceI cleavage site in the oli2 gene (5, 12). Fig. 3F shows that the 50-kDa protein monomer (5.3 μg/ml) generated fragments of 1.2 and 8.4 kb from the mitochondrial DNA, indicating specific cleavage at the oli2 sequence. No additional fragment appeared even at concentrations up to 10.6 μg/ml of the 50-kDa protein (data not shown). This result favors the idea that the oli2 gene is the unique cleavage site for the 50-kDa protein within mitochondrial DNA. In contrast to the apparent unsequence specificity of the 50-kDa protein, the Endo.SceI heterodimer at a concentration equivalent to 1.0 μg/ml of the 50-kDa protein generated many fragments from the restriction digests. Under the conditions used, the 9.6-kb band was partially cleaved by both the 50-kDa protein and the heterodimer with similar efficiency, whereas only the latter generated fragments other than those of 8.4 and 1.2 kb (Fig. 3F). We thus conclude that the oli2 sequence is the only sequence within the mitochondrial DNA (~75 kb) that can be efficiently cleaved by the 50-kDa protein and that the 50-kDa protein exhibits different sequence specificity depending on whether it is complexed with mtHSP70.

Interaction of the 50-kDa Protein with mtHSP70 in Vivo

Since the 50-kDa protein alone exhibited the endonuclease activity, we re-examined the quaternary structure of the 50-kDa endonuclease in vivo. The conventional purification method for Endo.SceI depends on assaying for endonuclease using the pBR322 substrate, which cannot be cleaved by the 50-kDa protein alone. Thus, the monomeric form of the 50-kDa endonuclease may have been overlooked during such purification procedures. We analyzed mitochondrial extracts by gel filtration chromatography, and we found that the 50-kDa protein could be recovered only in the dimer fraction of 125 kDa (Fig. 4A). Later fractions from the column containing proteins of lower molecular weight neither contained immunoreactive materials for the anti-50-kDa protein antibody nor endonuclease activity on the oli2 substrate. Control experiments showed that the anti-50-kDa protein antibody specifically detected the 50-kDa protein in mitochondrial extracts prepared from an Endo.SceI-producing strain but not in those from an Endo.SceI-deficient strain (Fig. 4A).

In vivo, cleavage at the oli2 region has been detected on mitochondrial DNAs of vegetative growing cells (12). To confirm that Endo.SceI exhibits multisite cleavage activity in mitochondria, we probed several regions within mitochondrial DNA, including oli2, 15S rRNA, and the cob-box (for a review, see Ref. 32). The mitochondrial DNAs of two yeast strains, YKN1423 and YKN1424 which lack and possess a functional 50-kDa protein, respectively (12), were subjected to Southern blot analysis. Fig. 4B shows that mitochondrial DNAs prepared from YKN1424 vegetative growing cells were partially cleaved at several sites (YKN1424, lane 2), whereas the mitochondrial DNA of YKN1423, which is isogenic to YKN1424 except for the 50-kDa protein gene, did not show any specific cleavages. Our previous results showed that mitochondrial DNA can be prevented from nuclease digestion during its preparation (12), suggesting that the DNA breaks observed were made in vivo. The mitochondrial DNA prepared from YKN1423 was digested in vitro by Endo.SceI at identical sites to those seen in vivo (compare lanes 2 and 4). These results indicate that the mitochondrial DNAs of Endo.SceI-positive yeast strains are partially cleaved at multiple sites by the endonuclease in the organelles, strongly suggesting that the 50-kDa protein functions as part of a complex with mtHSP70 in vivo. From the results described above, we conclude that the 50-kDa protein occurs primarily as a heterodimer with mtHSP70 in mitochondria.

The Nature of Binding between the 50-kDa Protein and mtHSP70

To examine further the nature of the binding between the 50-kDa protein and mtHSP70, we performed a GST pull-down experiment using mtHSP70 fused to glutathione S-transferase (GST-mtHSP70) and endonuclease assays using the pBR322 substrate. For the interaction between HSP70 and unfolded substrates, the following observations have been reported. ADP-bound HSP70 was found to make more stable complexes with unfolded substrates compared with ATP-bound HSP70 (31). The substrate binding domain within HSP70 has been located immediately after the 44-kDa ATPase domain (Fig. 5A) through analysis of truncated versions of HSP70 (33) and x-ray crystallographic analysis of an HSP70-substrate complex (34).

Nucleotide State of mtHSP70—To examine whether the nature of the nucleotide bound to mtHSP70 affects the formation of Endo.SceI complexes, we incubated together the 50-kDa protein and GST-mtHSP70 bound to different nucleotides. Prior to the analysis, GST-mtHSP70 was loaded with either ATP or ADP or kept in the nucleotide-unbound state (see “Experimental Procedures”). Fig. 5B shows that the 50-kDa protein could stably bind to the ADP-bound form of GST-mtHSP70 (lane ABC), whereas the ATP-bound form of mtHSP70 could associate only weakly with the 50-kDa protein. It is possible that a complex formed by the ATP-bound form of mtHSP70 and the 50-kDa protein is unstable due to fast exchange rates that a complex formed by the ATP-bound form of mtHSP70 and the 50-kDa protein could associate only weakly with the 50-kDa protein. It is possible that a complex formed by the ATP-bound form of mtHSP70 and the 50-kDa protein is unstable due to fast exchange rates.
region AB seemed to be comparable with that of the whole molecule (region ABC). Further dissection of the truncated protein showed that region A alone stably interacted with the 50-kDa protein, whereas region B did not show any interaction (Fig. 5B). This is surprising as region B has been identified as the substrate binding domain of HSP70.

To confirm that the substrate binding domain (region B) is dispensable for 50-kDa protein binding to mtHSP70, we did a competition assay using a chemically denatured protein, RC-MLA (35), in a GST pull-down assay. It has been established that RCMLA binds to the substrate binding domain of various HSP70s (36, 37). Fig. 5C shows that the interaction between GST-mtHSP70 (region ABC) and the 50-kDa protein was not inhibited by the presence of RCMLA (at a concentration 100-fold higher than that of GST-mtHSP70) at all. These pull-down assays together with the results described above indicate that the binding mode within the Endo.SceI heterodimer is distinct from that used in other HSP70-substrate interactions.

Since the ATPase domain alone was found to make a complex with the 50-kDa protein, we examined if such a complex had multisite-specific endonuclease activity. Fig. 5D shows that the 50-kDa protein-GST-mtHSP70 complex exhibited the same level of endonuclease activity on the pBR322 substrate as the 50-kDa protein-mtHSP70 complex (lanes 3 and 4). Although the truncated versions “A” and “AB” could make complexes with the 50-kDa protein, they did not confer multisite specificity on the 50-kDa protein (lanes 1 and 2, respectively). This result suggests that the modulation of the endonuclease activity requires not only the association of the 50-kDa protein with the ATPase domain but also the function of other domains such as the substrate binding domain and/or the C-terminal domain.

**DISCUSSION**

In summary, we have shown the following: 1) the 50-kDa protein has very specific endonuclease activity; 2) the 50-kDa protein most likely interacts with the ATPase domain within mtHSP70; 3) the binding of mtHSP70 to the 50-kDa protein results in broadening of the DNA sequence specificity as well as enhancement and stabilization of the endonuclease activity; and 4) the 50-kDa protein is present as a heterodimer in vivo. These results have revealed a novel function for mtHSP70, that is modulation of an enzyme activity and stabilization of an active conformation through stable association. The association of mtHSP70 with the 50-kDa protein is essential for multisite specificity, which is a characteristic feature of Endo.SceI and probably relevant to its biological function. During zygoate formation, Endo.SceI cleaves mitochondrial DNA in fused mitochondria to induce genetic recombination between the heterogeneous mitochondrial DNAs inherited from the parents (12). We have also detected Endo.SceI-dependent cleavage
activity in vegetative growing cells (Ref. 12 and this study), suggesting that Endo.SceI initiates genetic recombination between homologous DNAs in the vegetative cell to maintain the homoplasmy of mtDNA (32).

The 50-kDa subunit of Endo.SceI shares sequence similarity with members of the homing endonuclease family (5, 6). Members of the homing endonuclease family are good examples of selfish genes because they are involved in site-specific gene conversion events of their own coding sequences into alleles lacking endonuclease genes. However, unlike the Endo.SceI endonuclease, these endonucleases display unique sequence specificity which limits gene conversion events to only a limited number of homing sites. Interestingly, however, the oli2 sequence is adjacent to the coding region of the 50-kDa protein (5). Endo.SceI may have evolved from an ancestral homing endonuclease that had its homing site right next to the oli2 gene. It is possible that the 50-kDa protein gained broader sequence specificity after it became a stable partner of mtHSP70. An interesting point here is that HSP70 can generate a diversity of an enzymatic activity without either mutation or post-transcriptional modification of the enzyme.

As described earlier, the 50-kDa protein formed a stable dimer with ADP-bound mtHSP70 without any need for denaturation of the 50-kDa protein in vitro. It is possible in vivo, however, that de novo synthesized 50-kDa protein associates with mtHSP70 either during or just after its translation in mitochondria to prevent irreversible inactivation of its activity. This may explain, in part, why we could not detect the monomeric form of the 50-kDa protein in vivo. In accord with this idea, newly synthesized Var1 protein together with Mdj1 (yeast homolog of E. coli DnaJ) has been reported to associate with mtHSP70 (38, 39). The lifetime of the 50-kDa protein may be regulated through the binding of mtHSP70. Without mtHSP70, it is possible that the 50-kDa endonuclease may not be able to function in vivo due to low thermostability and/or even rapid degradation.

Heterodimerization between mtHSP70 and the 50-kDa protein showed a marked preference for ADP-bound mtHSP70 over ATP-bound mtHSP70. ATP-induced conformational changes have been detected in HSP70s upon binding of the nucleotide to the protein in vitro (40). The changes are thought to lead to substrate release (for a review, see Ref. 41). Considering that active mitochondria contain ATP at high concentrations, we are investigating whether the interaction between mtHSP70 and the 50-kDa protein is stable in the presence of ATP. Preliminary results show that addition of ATP after heterodimer formation does not affect the endonuclease activity of the reconstituted dimer in terms of cleavage of the pBR322 substrate (data not shown), suggesting that the Endo.SceI heterodimer, once formed, is stable enough to be resistant to high concentrations of ATP. Before, we observed resistance of endogenous Endo.SceI to ATP (1).

The following observations suggest that the binding mode of mtHSP70 to the 50-kDa protein is distinct from mtHSP70 binding of other substrates. The GST pull-down assay showed that the 50-kDa protein interacted with the ATPase domain but not with the substrate binding domain that has been implicated in the binding of other HSP70 substrates. Inclusion of RCMLA in the binding assay did not affect the efficiency of binding between the 50-kDa protein and mtHSP70. The Endo.SceI heterodimer is resistant to 1 mM ATP, whereas other HSP70-substrate complexes are unstable in the presence of ATP. Since the 50-kDa protein seems to interact with the ATPase domain, the above difference may be due to the incapacity of ADP-mtHSP70 to undergo conformational change when complexed with the 50-kDa protein.

The ATPase domain of HSP70 is also known to make specific complexes with other proteins. These include the co-chaperone, GrpE (42, 43), and proteins BAG-1 (44) and Hip (45). All of these proteins are believed to be modulators of HSP70. For instance, binding of GrpE to ADP-bound DnaK triggers the release of ADP and thereby accelerates substrate dissociation from DnaK. In contrast to GrpE, Hip appears to stabilize the ADP-bound form of HSP70, thereby prolonging substrate in-
interaction with HSP70. In this respect, the nature of the interaction between the 50-kDa protein and mtHSP70 would appear to be unique, as mtHSP70 appears to be involved in modulating the enzymatic activity of the endonuclease. The effect of 50-kDa protein binding on the chaperone activity of mtHSP70 remains to be examined.

One of the probable roles for mtHSP70 in the Endo.SceI heterodimer would be to keep the 50-kDa protein in an active form. The 50-kDa protein shows increased stability in the presence of mtHSP70 during incubation at 37 °C. Furthermore the specific activity of the 50-kDa protein increases on association of mtHSP70. These results imply that the tertiary structure of the 50-kDa protein in the heterodimer is more rigid or at least different from that of the 50-kDa protein monomer. On the other hand, however, the sequence specificity of the reconstituted dimer is less strict than that of the 50-kDa protein monomer. Since the role of mtHSP70 within the Endo.SceI complex seems to be distinct from those of HSP70s in other substrate complexes, it is possible that a novel enzyme mechanism is involved.

The endonuclease assay showed that the ATPase domain alone was unable to confer multisite specificity on the 50-kDa protein (Fig. 5D), suggesting that the C-terminal region including the substrate binding domain is involved in modulation of the endonuclease activity of the 50-kDa protein. Crucial questions that still need to be answered are as follows. 1) What is the nature of the 50-kDa protein structure recognized by mtHSP70? 2) Where is the binding site of the 50-kDa protein within the ATPase domain? 3) What mechanism governs the modulation of the endonuclease activity of the 50-kDa protein by mtHSP70? To address these questions, we are currently trying to locate the binding interface between the 50-kDa protein and mtHSP70 and also to crystallize both the 50-kDa protein and the Endo.SceI dimer for x-ray analysis. Information about the three-dimensional structure of the 50-kDa protein should give us further insights into how a molecular chaperone like mtHSP70 can convert a unisequence-specific endonuclease into a multisequence-specific one.

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