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# YGE1p, A EUKARYOTIC GRP-E HOMOLOG, IS LOCALIZED IN THE MITOCHONDRIAL MATRIX AND INTERACTS WITH MITOCHONDRIAL HSP70

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**SUMMARY :** Yeast Yge1p, the gene product of *YGE1*, is a eukaryotic GrpE homolog found recently (Ikeda *et al.*, 1994). We have revealed here that Yge1p is a soluble protein in the mitochondrial matrix. Depletion of Yge1p in yeast cells resulted in accumulation of the precursor of $F_1$-ATPase ß subunit *in vivo*, suggesting that Yge1p is involved in protein import into mitochondria. Overexpression of Yge1p in the temperature-sensitive mutant strains of mitochondrial hsp70, Ssc1p, caused hypersensitivity to temperature for cell growth, suggesting a genetic interaction between the *YGE1* and *SSC1* genes. A physical interaction between Yge1p and Ssc1p was directly demonstrated by co-immunoprecipitation of Ssc1p by the anti-Yge1p antibodies. We propose that Yge1p functions in cooperation with Ssc1p in a similar manner as bacterial GrpE with DnaK. 

The 70-kDa heat shock proteins (hsp70) have been highly conserved during evolution and are important for cell growth even under physiological conditions. Hsp70 functions as a molecular chaperone by binding to unfolded polypeptide chains and releasing them at the expense of ATP hydrolysis (1). Constitutively expressed hsp70 has been shown to interact transiently with newly synthesized polypeptide chains (2) and to facilitate protein translocation across organellar membranes (3-8). In *Escherichia coli*, the function of DnaK (bacterial hsp70) is regulated by two other heat shock proteins, DnaJ and GrpE, which synergistically enhance the ATPase activity of DnaK; DnaJ stimulates ATP hydrolysis and GrpE stimulates ADP/ATP exchange by DnaK (9). Biochemical studies suggested that DnaJ may also function as a molecular chaperone (10, 11).

In eukaryotic cells, hsp70s are localized in various subcellular compartments. For example, the yeast *Saccharomyces cerevisiae* has several different species of hsp70s: Ssa1-4p (3) and Ssb1-2p (12) in the cytosol, Kar2p in the endoplasmic reticulum (ER) (13, 14), Ssc1p in mitochondria (15, 16) and less related hsp70 homologs with undefined locations, Msi3p or Sse1p (17, 18), and Sse2p (18). Yeast cells also have several DnaJ homologs: Ydj1p or Mas5p in the

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cytosol (19, 20), Sis1p in the cytosol and nucleus (21), Zuotin in the nucleus (22), Sec63p in the ER membrane (23), and Scj1p (24) and Mdj1p (25) in mitochondria.

Yeast Yge1p is the first eukaryotic homolog of GrpE identified recently (26). The *YGE1* gene was originally isolated as a gene that caused a staurosporin-resistant phenotype when placed on a high copy number plasmid, and could suppress the temperature-sensitive phenotype of the *grpE* mutation in *E. coli*. *YGE1* is essential for the yeast cell growth and its gene product appears to be involved in the maintenance of mitochondrial functions, since depletion of Yge1p from the yeast cells resulted in aberrant mitochondrial distribution (26). However, precise roles of Yge1p in maintaining the mitochondrial functions is not clear at the moment.

In the present study, we have found that Yge1p is localized in the mitochondrial matrix and is likely involved in protein import into the mitochondria. Genetic and biochemical analyses have suggested that Yge1p functions in cooperation with mitochondrial hsp70, Ssc1p.

## MATERIALS AND METHODS

**Yeast strains, cell growth, and plasmids.** The following yeast strains were used in this study; D273-10B (*MATα* (ATCC 24657)), YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) (27), YPH501-1a (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 yge1::LEU2 URA3::pGAL1-YGE1(20-228)*) (26), MNSC-2A/CW (*MATα ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 can1-100 ssc1::LEU2 [pGAP-SSC1 (URA3)]*) (Kato, Y. *et al.*, unpublished), and MNSC-2A/C12 (*MATα ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 can1-100 ssc1::LEU2 [pGAP-ssc1$^{ts}$ (URA3)]*) (Kato, Y. *et al.*, unpublished). Temperature-sensitive alleles of the *SSC1* gene were generated by the low fidelity PCR technique (28; Kato *et al.*, unpublished). Yeast cells were grown on standard media as described by Daum *et al.* (29) and by Sherman *et al.* (30). pMRU1-YGE1 is a high copy number plasmid that contains a 2.5 kbp yeast genomic DNA fragment including the entire *YGE1* gene and its 5'- upstream and 3'-downstream region as well as the 2μ origin of replication and the *URA3* gene as a selectable marker. pYE-Ura3 (Clonetech) was used as a *URA3*-containing control plasmid.

**Subcellular and submitochondrial fractionation.** Subcellular fractionation, isolation of mitochondria, and submitochondrial fractionation were carried out as described previously (29, 31, 32). Determination of the intramitochondrial localization of proteins was performed by osmotic shock and protease treatment of mitochondria (32).

**Immunoprecipitation.** Mitochondria (20 mg/ml protein) were prepared from the wild-type cells (strain D273-10B). Mitochondria were lysed in a buffer containing 1% Triton X-100, 0.6 M mannitol and 20 mM HEPES-KOH, pH7.4, followed by centrifugation for 60 min at 100,000 x *g* for 60 min. The obtained mitochondrial extracts were diluted 50-fold with TENT buffer containing 1 % Triton X-100, 20 mM Tris-HCl, pH7.5, 150 mM NaCl and 5 mM EDTA, incubated with the anti-Yge1p antibodies or preimmune serum for 2 h at 4 °C, and subjected to incubation with the protein A-Sepharose FF beads (Pharmacia) under gentle mixing for 2 h at 4 °C. The immunoprecipitates were washed several times in the same buffer, dissolved in SDS sample buffer and analyzed by SDS-PAGE. SDS treatment of the mitochondrial extracts prior to immunoprecipitation was done by adding SDS to a final concentration of 2 %. For analyzing the nucleotide dependence of immunoprecipitation, the mitochondrial extracts were first incubated with 1 mM or 10 mM of AMP, ADP, ATP or GTP in the presence of 20 mM $MgCl_2$ for 30 min at 4°C, and then immunoprecipitated as described above except for the presence of 20 mM $MgCl_2$.

**Miscellaneous.** Published procedures were used for transforming yeast cells (30) and recombinant DNA technique (33). Crude yeast cell extracts were prepared as described by Nakai *et al.* (31). SDS-PAGE and immunoblotting with the enhanced chemiluminescence method with ECL detection kit (Amersham) for protein detection were performed as described previously (34). Immunoblots were quantified with a PD110 laser densitometer (Molecular Dynamics). The electrochemical potential across the inner membrane of Yge1p-deficient mitochondria was measured with a potential-sensitive fluorescent dye diS-$C_3$-(5) (35).

## RESULTS

**Ygelp is localized in the mitochondrial matrix.** To determine the subcellular localization of the *YGE1* gene product, we prepared an anti-serum against Ygelp using Ygelp overexpressed in *E. coli* as an antigen. When total cell extracts of the wild-type yeast strain was analyzed by immunoblotting, a protein of 26 kDa was specifically recognized by the anti-Ygelp antibodies, while the 26 kDa polypeptide was not detected in the extracts prepared from the *yge1::LEU2 [pGAL-YGE1]* cells, which had been depleted of Ygelp (data not shown). We therefore assigned the 26 kDa polypeptide to the *YGE1* gene product in the wild-type yeast cells.

When yeast cell extracts were subjected to subcellular fractionation, Ygelp was exclusively recovered in the mitochondrial fraction (Fig. 1A) with which mitochondrial hsp70, Sscl p, was associated (Fig. 1B). We thus analyzed the localization of Ygelp within the mitochondria. While selective removal of the mitochondrial outer membrane by hypotonic treatment exposed most cytochrome $b_2$ in the intermembrane space to externally added protease, Ygelp remained in a location protected from added protease (Fig. 1C). Ygelp as well as Sscl p only became accessible to added protease on disruption of the inner mitochondrial membrane of the mitoplasts with detergent (Fig. 1C). When mitoplasts were disrupted by sonication and the soluble and the membrane fractions separated by centrifugation, Ygelp was recovered in the soluble fraction

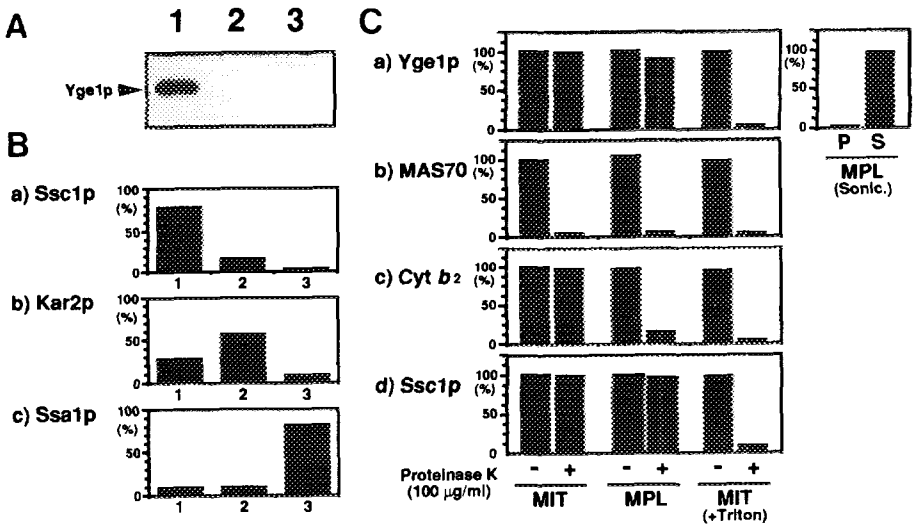


**Figure 1.** Ygelp is a soluble protein of the mitochondrial matrix. (**A**) Cell extracts of the wild-type yeast (strain D273-10B) were fractionated into mitochondria (lane 1), microsomes (lane 2), and cytosol (lane 3). Each fraction was analyzed by SDS-PAGE followed by immunoblotting with the anti-Ygelp antiserum. (**B**) The mitochondria (column 1), microsomes (column 2), and cytosol (column 3) were analyzed for distribution of the marker proteins of each fraction by immunoblotting: Sscl p (mitochondria), Kar2p (microsomes), and Ssa1p (cytosol). Immunoblots were quantified densitometrically. (**C**) Proteinase K treatment of mitochondria (MIT), mitoplasts (MPL) and solubilized mitochondria (MIT + Triton) according to the methods by Glick (32). Mitoplasts were prepared by osmotic shock and solubilized mitochondria by addition of 0.5% Triton X-100. After stopping the digestion by adding 1 mM phenylmethyl sulfonylfluoride, proteins were precipitated by addition of 5 % trichloroacetic acid, and analyzed by immunoblotting with various antisera as indicated. Mitoplasts disrupted by sonication (MPL Sonic.) were fractionated into membrane pellet (P) and soluble components (S) by centrifugation and analyzed for Ygelp by immunoblotting. The signal obtained with mitochondria without protease treatment was set to 100 %.

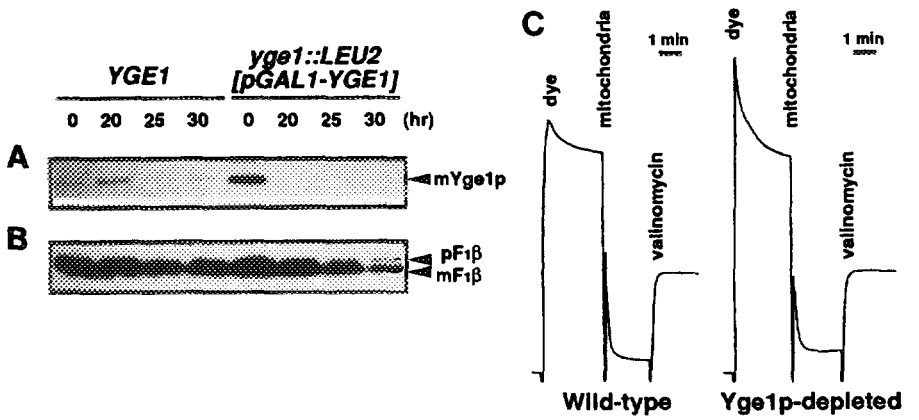


**Figure 2.** Depletion of Yge1p and accumulation of uncleaved mitochondrial precursor proteins. Strains YPH501-1a (*Yge1::LEU2, URA3::pGAL1-YGE1*) and YPH499(*YGE1*) were grown in rich medium containing 2 % galactose to late logarithmic phase and then diluted into fresh rich medium containing 2 % glucose instead of galactose. At the indicated times after dilution, cell extracts were prepared and aliquots were analyzed for Yge1p (A) and F1-ATPase ß subunit (B) by SDS-PAGE followed by immunoblotting: pFıß and mFıß, the precursor and mature forms. Mitochondria prepared from YPH499(*YGE1*) (wild-type) or YPH501-1a (Yge1p-depleted) yeast strain, which had been pre-grown in galactose-containing medium and then transferred to and cultivated for 30 hours in rich glucose-containing medium, were tested for maintaining the membrane potential across the inner membrane (C). Aliquots of mitochondria (200 µg/mL) were incubated in a buffer (20 mM HEPES-KOH, pH 7.4, 0.6 M mannitol, 0.25% (w/v) BSA, 2 mM ATP, 12.5 mM $MgCl_2$ and 20 mM K-Pi) containing the potential-sensitive fluorescent dye, diS-$C_3$-(5), (2.5 µM) and a time course of the fluorescence intensity at 670 nm (excited at 620 nm) was measured. Mitochondria and valinomycin (10 µg/mL) were added at the indicated times. A downward deflection signifies an increase in the membrane potential.

(Fig. 1C). These results indicate that Yge1p is a soluble protein of the mitochondrial matrix. Yge1p is probably processed to the mature form on its import into the mitochondrial matrix with the presequence proteolytically removed by processing protease in the matrix.

**Effect of Yge1p depletion on mitochondrial protein import *in vivo*.** A haploid yeast strain carrying the integrated plasmid pGAL1-YGE1 as well as a disrupted chromosomal *yge1::LEU2* allele could grow on galactose-containing medium but not on medium supplemented with glucose (26). These cells expressed the mature Yge1p at a level *ca.* 20 times higher than the wild-type cells (Fig. 2A). Yge1p and $F_1$-ATPase ß subunit ($F_1ß$ ) in the mutant cells were detected by immunoblotting at various times after transfer to glucose-containing medium (Fig. 2A). By 25 to 30 hours after transfer to glucose medium, Yge1p became immunologically undetectable and the cells started accumulating the uncleaved precursor form of $F_1ß$ (Fig. 2B). This defect in mitochondrial protein import was not simply due to cell inviability because the cells accumulating the precursor form of $F_1ß$ still continue to divide (data not shown). Mitochondria prepared from the Yge1p-depleted cells still maintain the membrane potential across the inner membrane, the level of which was comparable to that of the wild-type mitochondria (Fig. 2C). Import of the *in vitro*-synthesized $F_1ß$ precursor into the mitochondria from isogenic Yge1p-deficient cells was also much less efficient than that into mitochondria from wild-type cells (data not shown). These results suggest that Yge1p is involved in protein import into the mitochondria.

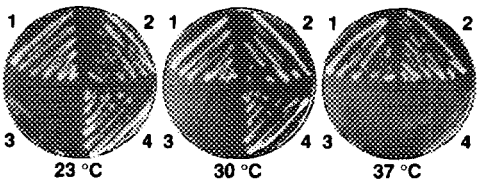


**Figure 3.** Hyper-temperature sensitivity of *ssc1*$^{ts}$ yeast mutant caused by overexpression of Ygelp. MNSC-2A/CW(*SSC1*)(sectors 1 and 2) or MNSC-2A/C12 (*ssc1*$^{ts}$)(sectors 3 and 4) yeast strains were transformed with either Ygelp-overexpression plasmid (pMRU1-YGE1)(sectors 1 and 3), or the control plasmid (pYE-Ura3)(sectors 2 and 4). Transformants were first selected onto synthetic glucose-containing medium lacking uracil at 23 °C then streaked onto the same medium and further incubated at the indicated temperatures for 5-7 days.

**Genetic interaction between *YGE1* and *SSC1*.** Since the *E. coli* GrpE protein functions in concert with DnaK, it is reasonable to assume that Ygelp functions in cooperation with a mitochondrial hsp70, Sscl p. To demonstrate that Ygelp functionally cooperates with Sscl p, we introduced the *YGE1* gene on a high copy number, 2μ-based plasmid (pMRU1-YGE1) into various yeast *ssc1*$^{ts}$ mutant cells (Kato *et al.*, unpublished), and analyzed the effects of overexpression of Ygelp on their phenotypes. One of the mutant strains, MNSC-2A/C12, with the *ssc1*$^{ts}$ allele as a control grew at 30 °C, but not at 37 °C (Fig. 3, sector 4). Surprisingly, the mutant strain could no longer grow even at 30 °C, and only grew slowly at 23 °C, when Ygelp was overexpressed 7-fold from a high copy number plasmid. Overexpression of Ygelp from a high copy number plasmid itself did not cause temperature hypersensitivity for growth of the cells bearing the wild-type *SSC1* allele (Fig. 3, sector 1). We tested three other mutant strains bearing *ssc1*$^{ts}$ alleles for the effects of overexpression of Ygelp; they also exhibited hyper-temperature sensitive phenotypes when Ygelp was overexpressed. These results indicate a genetic interaction between the *YGE1* and the *SSC1* genes.

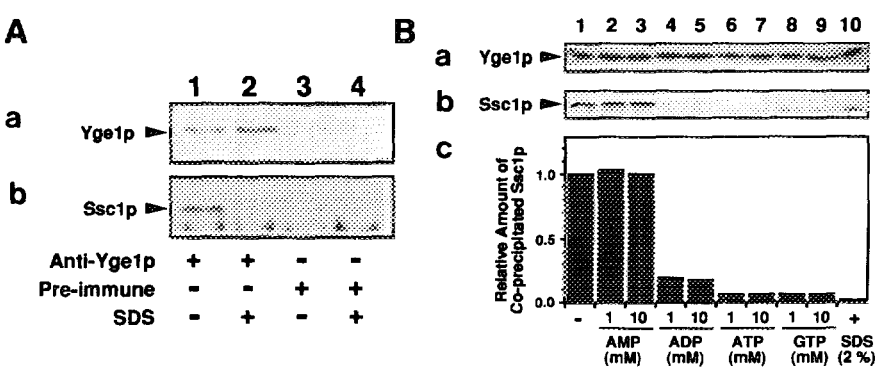


**Figure 4.** The mitochondrial hsp70, Sscl p, was co-immunoprecipitated with Ygelp. Mitochondria prepared from the wild-type cells (strain D273-10B) were solubilized as described in Materials and methods. (**A**) Soluble mitochondrial extracts were reacted with the antibody to Ygelp (lanes 1 and 2) or pre-immune serum (lanes 3 and 4), precipitated with protein A-Sepharose and analyzed by SDS-PAGE followed by immunoblotting using the anti-Ygelp serum (a) or the anti-Sscl p serum (b). Where indicated, mitochondrial soluble extracts were pretreated with 2 % SDS prior to immunoprecipitation (lanes 2 and 4). (**B**) Mitochondrial extracts were incubated with the indicated nucleotides (1 or 10 mM) before immunoprecipitation. Immunoprecipitates were analyzed for Ygelp (a) and Sscl p (b and c) as described above. The amounts of Sscl p co-immunoprecipitated with Ygelp were normalized by those of precipitated Ygelp (c).

**Ygelp exists in a complex with Sscl p.** Next we examined if Ygelp and Sscl p physically interact with each other in the mitochondrial matrix. Using antibodies against Ygelp, we could co-immunoprecipitate Sscl p after lysis of mitochondria with detergent (Fig. 4A). Treatment of the mitochondrial extracts with 2% SDS inhibited co-immunoprecipitation of Sscl p with Ygelp. This co-immunoprecipitation provides strong evidence for a physical interaction between the two proteins. Incubation of mitochondrial extracts with ADP, ATP or GTP prior to immunoprecipitation appeared to abolish the formation of a stable complex between Sscl p and Ygelp (Fig. 4B). This means that, in the mitochondrial matrix, Ygelp interacts with Sscl p only transiently. Ygelp likely stabilizes the nucleotide-free form of Sscl p by binding to it and enhances the exchange between the nuceotide-free and nucleotide-bound forms; this may promote efficient ADP/ATP exchange by Sscl p. Since Ygelp and Sscl p represent roughly 0.03% and 0.8% of total mitochondrial proteins, respectively (data not shown), the molar ratio of Ygelp:Sscl p after co-immunoprecipitation was estimated to be approximately 1 : 0.7. This figure suggests that equimolar amounts of Ygelp and Sscl p form a complex.

## DISCUSSION

Yeast Ygelp is a eukaryotic GrpE homolog and is suggested to play a pivotal role in maintaining mitochondrial functions (26). In this study we have shown that Ygelp is localized in the mitochondrial matrix and likely facilitates protein import into mitochondria in cooperation with Sscl p.

The findings that depletion of Ygelp in the mitochondrial matrix results in accumulation of the precursor form of a mitochondrial protein suggest that Ygelp is involved in protein import into mitochondria. Besides we reason that Ygelp functions in cooperation with mitochondrial hsp70, Sscl p, on the basis of the following findings. First, Sscl p was co-immunoprecipitated by the anti-Ygelp antibodies, suggesting that Ygelp can directly interact with Sscl p. Since the amount of Sscl p is 10-fold excess over that of Ygelp and Sscl p and Ygelp form a complex with a 1:1 mole ratio, most Ygelp may be complexed with Sscl p. Second, overexpression of the wild-type Ygelp resulted in hypersensitivity to temperature for cell growth of mutant yeast strains carrying the *ssc1*$^{ts}$ allele. This observation may indicate that Sscl p$^{ts}$ is defective in the ATPase function and that enhanced ADP/ATP exchange by the overexpressed Ygelp shifts the equilibrium of Sscl p$^{ts}$ from the ADP-bound form to the ATP-bound form in the mitochondrial matrix and hence interferes with the normal turnover of Sscl$^{ts}$p.

In *E. coli*, GrpE was shown to interact transiently with DnaK (36) and to stimulate ADP/ATP exchange; the accelerated ADP/ATP exchange may enhance the rate of turnover of polypeptide binding and release by DnaK (9, 10). In yeast mitochondria, Sscl p was suggested to have dual roles in protein translocation across the membranes; Sscl p facilitates unfolding of the translocating polypeptide chain and driving the completion of the transport of a matrix-targeted preproteins across the inner membrane (6, 37, 38). Since translocation of precursor proteins across the mitochondrial membranes may well require multiple cycles of polypeptide binding to and release by Sscl p (39), which are coupled with ATP hydrolysis, the ADP/ATP exchange rate may limit the rate of protein transport into the mitochondrial matrix. We thus propose that, in

analogy with bacterial GrpE and DnaK, Yge1p stimulates the rate of ADP/ATP exchange, thereby stimulating ATP hydrolysis by Ssc1p. Depletion of Yge1p would suppress the ATPase activity of Ssc1p, resulting in the retarded protein import into the mitochondria. The Yge1p-Ssc1p complex found in the present study may represent an intermediate form in the cycle of reactions including formation and dissociation of the Ssc1p-polypeptide complex.

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