Sue1p Is Required for Degradation of Labile Forms of Altered Cytochromes c in Yeast Mitochondria*

Jun Wei‡ and Fred Sherman§

From the Department of Biochemistry and Biophysics, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642

Received for publication, Received for publication, April 5, 2004, and in revised form, April 28, 2004
Published, JBC Papers in Press, April 30, 2004, DOI 10.1074/jbc.M403742200

Previous studies on certain altered holo-isocytochromes c revealed a ρ⁺-dependent degradation (RDD) phenotype, in which certain altered holo-iso-1-cytochromes c are at normal or nearly normal levels in ρ⁺ strains, but are at low levels or absent in ρ⁻ strains, although wild-type holo-iso-1-cytochrome c is present at normal levels in both ρ⁺ and related ρ⁻ strains. The diminished levels of altered holo-iso-1-cytochrome c are due to the rapid degradation that is carried out by a novel proteolytic pathway in the IMS of mitochondria. SUE1, a nuclear gene that encodes a mitochondrial protein, was identified with a genetic screen for mutants that diminish RDD. The levels of RDD and certain other types of altered holo-iso-1-cytochrome c were elevated in ρ⁺ sue1 strains. Also, ρ⁻ sue1 strains containing certain altered holo-iso-1-cytochromes c were better on non-fermentable carbon sources than the corresponding ρ⁺ SUE1 strains. These results indicate that Sue1p may play an important role in the degradation of abnormal holo-iso-1-cytochrome c in the mitochondria.

Intracellular proteolysis plays an important role in maintaining the integrity of the proper folded state of proteins. It ensures removal of damaged and misfolded polypeptides because they are prone to aggregation. A basic mechanism for control of protein degradation is compartmentalization (1). In eukaryotic cells, proteases have been detected in four compartments: the cytoplasm, nucleus, lysosome, and mitochondrion. The mitochondria have various subcompartments that possess ATP-dependent proteases as a quality control system for selectively removing unassembled or misfolded polypeptides. Several ATP-dependent proteases such as the Pim1 protease in the matrix or AAA (ATPases associated with a variety of cellular activities) proteases in the inner membrane of mitochondria have been identified (2–5).

Additional proteolytic pathways may exist in the other two subcompartments of mitochondria: the intermembrane space (IMS)³ and outer membrane. The existence of an ATP-dependent proteolytic activity in the mitochondrial IMS in mammals has been reported, although the ATP-dependent protease so far has not been identified (6, 7).

We report herein a proteolytic pathway in the IMS of mitochondria acting on certain altered holo-iso-1-cytochromes c (holo-1) of the yeast Saccharomyces cerevisiae. S. cerevisiae contains two forms of cytochrome c, iso-1-cytochrome c (iso-1) and iso-2-cytochrome c (iso-2), which are encoded by the nuclear genes CYC1 and CYC7, which normally compose 95 and 5% of total cytochrome c, respectively, in aerobically grown, derepressed cells (8) and which are 80% identical. The isocytochromes c are synthesized in the cytosol as apocytochromes c and subsequently imported into mitochondria. Heme is covalently attached to the apocytochromes c by cytochrome c heme lyase, which is encoded by the gene CYC3, resulting in the formation of the mature holocytochromes c (9). Import of the apocytochromes c is dependent on the action of cytochrome c heme lyase, and cyc3Δ mutants lacking cytochrome c heme lyase accumulate apocytochromes c in the cytosol (10). However, apo-iso-1-cytochrome c is not detected in cyc3Δ mutants, whereas apo-iso-2-cytochrome c is present at the corresponding level of holo-iso-2-cytochrome c (holo-2) in related normal CYC3 strains (11). Dumont et al. (12) demonstrated with pulse-chase experiments that unimported apo-iso-1-cytochrome c is rapidly degraded, and Pearce and Sherman (13) demonstrated that this apo-iso-1-cytochrome c degradation requires functional proteasomes and is mediated by the ubiquitin-dependent pathway. In contrast, holo-1 and holo-2 are highly stable. We have been identifying and characterizing mitochondrial protein degradation systems by investigating mutant forms of iso-1 that are rapidly degraded and by determining mutations of other genes that diminish the degradation.

Downie et al. (14) observed that certain mutated holo-2 are at normal or nearly normal levels in ρ⁺ strains, but are absent in ρ⁻ strains, although wild-type isocytochromes c are present at normal levels in both ρ⁺ and related ρ⁻ strains. Subsequently, Pearce and Sherman (15, 17) generated equivalent mutants of holo-1 and showed that amino acid replacements at several positions of holo-1 diminish the levels of holo-1, preferentially in ρ⁻ strains. This phenomenon is referred to as the ρ⁻-dependent degradation (RDD) phenotype. The iso-1 that shows RDD is referred to as RDD iso-1.

In addition, pulse-chase experiments with a variety of altered forms of cytochrome c revealed two other degradation pathways acting on other altered forms of cytochrome c, labile dependent degradation (LDD) and amphipathic dependent degradation (ADD). LDD, exemplified by Gly⁶ replacements,
Degradation of Labile Yeast Cytochromes c

Table I

| Strain | Genotype | Parental strain | Source/ref. |
|--------|---------|----------------|-------------|
| B-7528 | p+ MATa cyc1-31 cyc7-67 ura3-52 lys5-10 | B-8354 | 16 |
| B-7889 | p+ MATa cyc1-31 cyc7-67 ura3-52 leu2-3,112 can1-100 | B-7528 | 17 |
| B-8353 | p+ MATa CYC1 cyc7-67 ura3-52 leu2-3,112 | B-7528 | 17 |
| B-8514 | p+ MATa CYC1 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-8312 | p+ MATa cyc1-1120 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-8301 | p+ MATa cyc1-1113 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-8344 | p+ MATa cyc1-1299 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-8513 | p+ MATa cyc1-1195 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-8530 | p+ MATa cyc1-1205 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-8631 | p+ MATa cyc1-1206 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-7734 | p+ MATa cyc1-868 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-7735 | p+ MATa cyc1-865 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-7686 | p+ MATa cyc1-860 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-12711 | p+ MATa cyc1-1388 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-12712 | p+ MATa cyc1-1388 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-12713 | p+ MATa CYC1-1389 cyc7-67 ura3-52 lys5-10 | B-7528 | 17 |
| B-12714 | p+ MATa CYC1-1389 cyc7-67 ura3-52 lys5-10 sue1-1 | B-7528 | 17 |
| B-12719 | p+ MATa cyc1-1388 cyc7-67 ura3-52 lys5-10 sue1-1 | BY4741 | Research Genetics |
| B-14650 | p+ MATa cyc1-1388 cyc7-67 ura3-52 sue1-1 | B-7528 | 17 |
| B-14689 | p+ MATa hiz3-1 leu2-2 mut15-1 ura3-5 sue1-1:kanR | B-7528 | 17 |
| B-14705 | p+ MATa CYC1 cyc7-67 ura3-52 lys5-10 sue1-1:kanR | B-7528 | 17 |
| B-14746 | p+ MATa cyc1-868 cyc7-67 ura3-52 leu2-3,112 can1-100 | BY4741 | Research Genetics |

**X. Chen, R. P. Moerschell, D. A. Pearce, D. D. Ramanan, and F. Sherman, unpublished data.**

**EXPERIMENTAL PROCEDURES**

**Genetic Nomenclature—**CYC1 and CYC7 encode iso-1 and iso-2, respectively, the two isozymes of cytochrome c in the yeast S. cerevisiae. Dominant alleles are denoted with uppercase letters, and recessive alleles with lowercase letters. CYC1 refers to the wild-type allele, whereas the mutant allele of the CYC1 gene is designated cyc-x, such as the cyc1-1388 mutant allele. The cyc7-67 allele corresponds to a partial deletion that results in the loss of iso-2. SUE1, identified in this study, refers to the wild-type gene that encodes a mitochondrial protein. The sue1-1 mutant allele completely lacks Sue1p, and the sue1-2 mutant allele contains a combined nonsense and frameshift mutation at position 9, resulting in a nonfunctional Sue1p. The yeast strains used in this study are listed in Table I, and the cyc1 alleles used in this study are listed in Table II. Iso-1 encoded by, for example, cyc1-1388 is designated Cyc1-1388p.

**Media—**Escherichia coli cells containing plasmids were grown in LB medium (10 g/liter Bacto-tryptone, 10 g/liter NaCl, and 5 g/liter yeast extract plus 100 μg/ml ampicillin or 25–50 μg/ml kanamycin) (20). Standard YPD, YPS, YPG, and YPE media; synthetic minimal medium; synthetic complete medium, synthetic complete medium/Ura-, and synthetic complete medium/Leu-; and other omission media have been described by Sherman (21). The media contained 1% Bacto-yeast extract, 2% Bacto-peptone, and either 2% ethanol (YPE), or 2% sucrose (YP5). Medium A was composed of synthetic minimal medium supplemented with 0.2% (w/v) casamino acids, 20 μg/ml uracil, and 2% Bacto-agar. Synthetic lactate contained 0.67% Bacto-yeast nitrogen base without amino acids, 2% Bacto-agar, and 3.3% of a 30% lactate solution. Medium B was composed of synthetic lactate supplemented with 0.2% (w/v) casamino acids and 20 μg/ml uracil. Synthetic complete medium/Leu- and supplemented with 0.1% 5-fluoroacetic acid (22) was also used.

**Construction of a Yeast Strain Containing the cyc1-1388 Allele—**The desired CYC1 mutations were obtained by transforming the defective cyc1 mutant strain B-7528 (MATa cyc1-31 cyc7-67 ura3-52 lys5-10) directly with PCR-generated fragments and selection of at least partially functional cyc1 transformants, followed by sequencing the CYC1 locus of these transformants to confirm that the selected transformants contained the desired cyc1 alleles.

First, the primer pair OL256 and OL257 (Table III) was used to amplify 1.7-kb fragments containing either cyc1-1209 (T78S/C102A) or CYC1-820 (C102A). Subsequently, a primer of 92 oligonucleotides was designed that contained five amino acids (RRASV) inserted between residues 4 and 5 of the CYC1 gene (primer RRA5-cyc1). Primer RRA5-cyc1 (Table III) was paired with primer OL257 to generate 653-bp fragments containing either the cyc1-1388 or CYC1-1389 allele (Table II). The B-7528 yeast strain was then transformed with either the cyc1-1388 or CYC1-1389 fragments following the procedures described by Yumamoto et al. (23).

---

**Endnotes:**

1. The introduction of the global suppressor N52I (18) into both RDD and LDD holo-1 in p strains (15, 17). ADD, containing amphipathic structures in the N-terminal region, also does not have diminished levels in p strains. The cytochromes c degraded by these pathways are designated LDD and ADD cytochromes c, respectively.

2. Elevated levels of altered holo-1 (T78S RDD, G6I LDD, and ADD holo-1) were observed in strains. The color intensity is proportional to the level of holo-1chrome. Mutants with diminished degradation. Strain RDD holo-1 in the IMS of mitochondria could be a novel ATP-dependent mitochondrial proteases (Yme1p, Afg3p, and LDD holo-1 may occur in the IMS of mitochondria. All known physiological partner of cytochrome c.
Construction of Three Sets of Isogenic Strains Containing Different \( \text{cyc1} \) Alleles Encoding LDD, ADD, and RDD—
The chromosomal DNAs were extracted from these strains containing different \( \text{cyc1} \) alleles (see Table IV). The OL256 and OL257 primer pair (Table III) was used to amplify the 1.7-kb fragments containing the \( \text{cyc1} \) allele of interest. Three host strains (B-7528, B-7889, and B-8354) were transformed with these 1.7-kb fragments carrying the \( \text{cyc1} \) allele of interest. The procedures were described by Yamamoto et al. (23). The corresponding \( /\text{H}9267 \)/\( /\text{H}11002 \) strains were obtained by growing the \( /\text{H}9267 \)/\( /\text{H}11001 \) mutant strains in YPD plates containing ethidium bromide.

Construction of the sue1-1::kanR Mutant Strain by the PCR-based One-step Disruption Method—
A sue1-1::kanR mutant strain was purchased from Research Genetics (24). The chromosomal DNA was prepared from this strain and served as a template, and the primer pair SUEF1 and SUER1 (Table III) was used to amplify the PCR-based disruption fragments carrying the sue1-1::kanR mutant gene flanked by 100 bp homologous to the region directly upstream of the start codon and downstream of the stop codon of the SUE1 gene. Three sets of isogenic \( /\text{H}9267 \)/\( /\text{H}11001 \) cyc1 mutant strains, which were constructed as described above (see Table IV), were transformed with the amplified fragments, and G418-resistant colonies were obtained on YPD plates containing 300 \( \mu \)g/ml G418.

Construction of the sue1-2 Mutant Strain by Site-directed Mutagenesis—
The primer pair OL150WF and OL150WR (Table III) was used to produce the 1.5-kb fragments encompassing YPR150w and downstream of the termination codon of this gene. The 1.5-kb fragments were then inserted into the TA vector (Invitrogen). The construction was carried out with 10 ng of TA vector containing the 1.5-kb insert as a template and 125 ng of both 150FS1 and 150FS2 (Table III) as two complementary oligonucleotide primers for amplification in the presence of 2.5 units of \( \text{Pfu} \) Turbo DNA polymerase (Stratagene). The 40 nucleotides of primers 150FS1 and 150FS2 contained the desired sue1-2 mutation, a deletion of an A residue corresponding to nucleotide 9 of the YPR151c gene and to a position outside the open reading frame of YPR150w, 147 nucleotides downstream of the chain termination codon. The sue1-2 deletion of an A residue was introduced into the TA vector containing the 1.5-kb insert using a PCR-based site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. The TA vector with the 1.5-kb insert containing the sue1-2 allele was obtained. The 1.5-kb insert containing the sue1-2 gene was transferred to the pAB621 vector, a yeast shuttle vector containing the \( \text{URA3} \) gene. Subsequently, the pAB621 vector with the desired insert containing the sue1-2 gene was linearized with the NheI restriction enzyme within the

---

### Table II

**cyc1 alleles**

| Horse no. | -5 | -4 | -3 | -2 | -1 | 1 | 2 | 6 | 78 | 102 |
|---|---|---|---|---|---|---|---|---|---|---|
| Iso-1 no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 11 | 83 | 107 |

---

**Table III**

**Oligonucleotides**

| Primer | Sequence |
|---|---|
| OL256 | GTGACACATGTCGACAATCTTACATGG |
| OL257 | GCAGAGCTGCAAAATTAAAGGCTTCG |
| RRASV-cyc1 | CTATGAGCAACGCAAAAACAAACAAACACTAAATAATATATGACTGAATTCCGTCGTGCATCTGTTAAGGCCGGTTCTGCTAAGAAAGGTG |
| SUEF1 | CCCCCTGAGTGTGTGTCTGCAT |
| SUER1 | ACCTGGAACTCCTAATGGTTCA |
| 151F3 | TGAGAGATCTGCACTATAT |
| 151FOF | TGAGAGATCTGCACTATAT |
| 151WR | AAGGAAACAACAAAACGTT |
| 150FS1 | TATGAATAGGATGATTTTTAAAAAGAGGACAAAAATAG |
| 150FS2 | CTATTTTTCGTCTCTTAAATAACATCTCTTATCATA |
| 151AFH | TTTTCTTCTCTGCAATCTGCTTTATTTTTTCTTCTTCTTCTTCTT |
| 151AHFR | TTTTCTTCTCTGCAATCTGCTTTATTTTTTCTTCTTCTTCTTCTT |
| 151GFPR | TTTTCTTCTCTGCAATCTGCTTTATTTTTTCTTCTTCTTCTTCTT |
| 151GFP | TTTTCTTCTCTGCAATCTGCTTTATTTTTTCTTCTTCTTCTTCTT |
| CmycF | AAGCCGTTTGACAAGGCACTGCAAATGCGGTTGACACACAAAAGCAAAAAAAAGATGAAAAGGGAACAAAAGCTGGAG |
| CmycR | ATGTATGAGGAATCGTGCTATAAAATCATTTTTTCTCATGTATATGCCAAATATTTATTATTTACTATAGGGCGAATTGG |
| 151MYCR | TTTTCTTCTCTGCAATCTGCTTTATTTTTTCTTCTTCTTCTTCTT |

---

Construction of the sue1-2 Mutant Strain by Site-directed Mutagenesis—The primer pair OL150WF and OL150WR (Table III) was used to produce the 1.5-kb fragments encompassing YPR150w and ~1 kb of DNA sequence downstream of the termination codon of this gene. The 1.5-kb fragments were then inserted into the TA vector (Invitrogen). The construction was carried out with 10 ng of TA vector containing the 1.5-kb insert as a template and 125 ng of both 150FS1 and 150FS2 (Table III) as two complementary oligonucleotide primers for amplification in the presence of 2.5 units of \( \text{Pfu} \) Turbo DNA polymerase (Stratagene). The 40 nucleotides of primers 150FS1 and 150FS2 contained the desired sue1-2 mutation, a deletion of an A residue corresponding to nucleotide 9 of the YPR151c gene and to a position outside the open reading frame of YPR150w, 147 nucleotides downstream of the chain termination codon. The sue1-2 deletion of an A residue was introduced into the TA vector containing the 1.5-kb insert using a PCR-based site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. The TA vector with the 1.5-kb insert containing the sue1-2 allele was obtained. The 1.5-kb insert containing the sue1-2 gene was transferred to the pAB621 vector, a yeast shuttle vector containing the \( \text{URA3} \) gene. Subsequently, the pAB621 vector with the desired insert containing the sue1-2 gene was linearized with the NheI restriction enzyme within the
subclones. The levels of RDD holo-1 were examined in strain B-14650 harboring a subclone. The subclone decreasing the level of RDD holo-1 in transformed into strain B-14803. Ura
tagged SUE1 and spread onto 5-fluoroorotic acid plates. The 5-fluoroorotic acid-resis-
tion sites for SphI (E), NruI (E), flanked by EcoRI restriction sites were amplified, subsequently
was designed: CmycF, with the 5
(pAA1869) (25) was used as a template, and the following primer pair
vector (see Fig. 1).

Construction of the C-terminal Green Fluorescent Protein (GFP)-
 fused SUE1 Gene—Approximately 640-bp fragments containing the SUE1 gene (YPR151c without a stop codon) flanked with EcoRI restriction sites were amplified using the primer pair 51ADHF and 151DFR (Table III) and subsequently digested with the EcoRI restriction en-
yme. The gel-purified EcoRI fragments were then ligated to the EcoRI site in the polylinker of plasmid pGFP-C-FUS (pAA1901), giving rise to the C-terminal GFP-fused SUE1 gene under the control of the P
promoter. Plasmid pAB1901 carrying C-terminal GFP-SUE1 (pAB2935) was obtained from the ampicillin-resistant colonies. Both plasmids pAB1931 and pAB2935 were transformed into strain B-14705,
an isogenic strain of B-7528 containing the CYC1 and sue1-Δ1:kan" genes. Colonies containing plasmid pAB1931 or pAB2935 were selected on synthetic complete medium/Ura-
plates.

Construction of the C-terminal Myc-tagged SUE1 Gene—Initially, the yeast strain bearing the C-terminal (Myc)-tagged SUE1 gene was constructed. A (Myc)-tagging cassette in plasmid pMPY-3·Myc (pAA1869) (25) was used as a template, and the following primer pair was designed. CmycP, with the 5‘-60 bases homologous to sequences immediately prior to the stop codon of SUE1 and the 3‘ 18 bases comple-
mentary to unique sequences in the (Myc)-tagging cassette; and
CmycR, with the 5‘-63 bases homologous to sequences immediately 3‘ of the stop codon of SUE1 and the 3‘ 16 bases complementary to unique sequences in the (Myc)-tagging cassette. PCR conditions were as de-
scribed by Schneider et al. (25). The gel-purified 1.5-kb fragments were transformed into strain B-14853. Ura-
colones were selected and then grown overnight in YPD medium, and cultures were washed with water and spread onto 5-fluoroorotic acid plates. The 5-fluoroorotic acid-resis-
tant colonies were selected and subsequently confirmed by sequencing to contain the integrated C-terminal (Myc)-tagged SUE1 gene without the UR33 gene.

We did not detect any signal of Myc-tagged SUE1 in whole cell extracts using monoclonal antiserum (purchased from NeoMarkers) against the Myc tag. Thus, we tried to construct C-terminal (Myc)-tagged SUE1 in the 2μ-based yeast expression vector pBEVY contain-
ing the UR33 gene (pAB2324) (26), and we also constructed SUE1 in the same vector (pAB2324) as a control. The total DNA prepared from the strain containing (Myc)-tagged SUE1 was used as a template, and the primer pair 51ADHF and 151MYCR (Table III) was designed. Approximately 820-bp fragments containing C-terminal (Myc)-tagged SUE1 flanked by EcoRI restriction sites were amplified, subsequently digested with the EcoRI restriction enzyme, and ligated to the EcoRI site in the polylinker of the pAB2324 vector. Meanwhile, wild-type SUE1 was also amplified using the primer pair 51ADHF and 151ADHR (Table III). Approximately 650-bp fragments containing SUE1 flanked by EcoRI restriction sites were amplified, digested, and ligated to the EcoRI site in the polylinker of pAB2324 as well. The plasmids containing either (Myc)-tagged SUE1 (pAB2936) or SUE1 (pAB2937) under the control of the ADH1 (glohol dehydrogenase-1) promoter were obtained from the ampicillin-resistant colonies and transformed into strain B-14705, an isogenic strain of B-7528 containing the CYC1 and sue1-Δ1:kan" genes. Strain B-14705 with pAB2936 or pAB2937 was selected on plates of synthetic dropout medium/Ura.

Determination of holo-1 Content—holo-1 levels in intact cells were screened by the benzidine staining method (17) and examined visually with a spectroscope as described by Sherman and Slonimski (27). More accurate estimation was performed by the method of low temperature (~ 196 °C) spectrophotometric recording with a modified Aviv Model 14D spectrophotometer as described by Hickey et al. (28).

Western Blotting and Immunoblotting Procedures—Samples were electrophoresed on an SDS-4-20% gradient acrylamide gel, transferred to a Hybond-ECL membrane (Amersham Biosciences), and probed with antiserum, followed by horseradish peroxidase-conjugated goat anti-
rabbit IgG (Bio-Rad). Anti-cytochrome c polyclonal antibodies were diluted 1:4000 in 0.1% Tween 20 in Tris-buffered saline (TBS; 20 mM Tris (pH 7.6) and 137 mM NaCl); anti-Myc monoclonal antibodies were diluted 1:100 in 0.1% Tween 20 in TBS; and anti-His monoclonal anti-
bodies were diluted 1:2000 in 0.1% Tween 20 in TBS. A Hybond-ECL
membrane was incubated with diluted antibodies for 1 h in 0.1% Tween 20 in TBS. The secondary antibodies were used at 1:2000 for 1 h. The membrane was washed with 0.1% Tween 20 in TBS, followed by ECL
detection (Amersham Biosciences).

Preparation of Yeast Mitochondria and the IMS of Mitochondria—
Yeast strain B-14705 with plasmid pAB2936 or pAB2937 containing the SUE1 constructs was expressed at high levels under the control of the constitutive ADH1 promoter. Mitochondrial purification and subfrac-
tionation were carried out following the method described by Zinner and Daum (29).

Detection of GFP-fused Suelp in Yeast Cells—Yeast strain B-14705 containing either pAB1931 or pAB2935 was grown overnight on syn-
thetic complete medium/Ura plates and diluted with distilled water to the appropriate concentration, followed by continuous growth in syn-
thetic complete medium/Ura overnight to A0.60 = 0.1–0.2. Cells were harvested and suspended at a concentration of 109 cells/ml in 10 mM HEPES (pH 7.4) and 5% glucose. Rhodamine B (hexyl ester; Molecular
Probes, Inc.) was added to a final concentration of 100 nM. The mixture was incubated at room temperature for 20 min. Yeast cells were har-
vested by centrifugation at 13,000 rpm for 5 s, washed, suspended in a small volume of 10 mM HEPES (pH 7.4) and 5% glucose; and visualized by confocal microscopy on a Leica TCS SP microscope equipped with argon, krypton/argon, and ultraviolet lasers. The fluorescence excita-
tion and emission maxima for rhodamine B (hexyl ester) are 555 and 579 nm, respectively.

RESULTS

Isolation of Mutants Defective in the Degradation of T78S
RDD holo-1—We used a genetic approach to identify proteins involved in the degradation of RDD holo-1 based on the prop-
properties of, for example, the following strains (Table I) containing the indicated levels of holo-1: B-12713 (pCYC1-1389), 100%; B-12714 (pCYC1-1389), 100%; B-12711 (pCYC1-1388), 100%; and B-12712 (pCYC1-1388; in which cyc1-1388 encodes RDD holo-1 with a T78S replacement), 15%. In addition, both cyc1-1388 and cyc1-1389 contain an RRASV insertion (protein kinase A phosphorylation site) and a C102A replacement at the N- and C-terminal regions, respectively (Table II). C102A holo-1 is as stable as wild-type holo-1 (16) and prevents dimerization through Cys31-disulfide bridges (30). Introducing the protein kinase phosphorylation site (RRASV) into iso-1 was designed to label holo-1 for other studies. Strain B-12712 containing cyc1-1388 shows the RDD phenotype very clearly. Therefore, the pCYC1-1388 strain was UV light-mutagenized at a dose yielding 10% survivors to screen for mutants that were able to suppress the RDD phenotype. Approximately 3 × 10⁴ colonies were screened using the benzidine staining method, in which the intensity of the blue color is proportional to the level of holo-1. A total of 832 blue colonies were subsequently examined under the microscope, and the level of holo-1 was estimated by comparison with strains containing known amounts of cytochrome c. Higher levels of RDD holo-1 in mutant colonies, designated Sue−, could be due to diminished degradation. Six Sue− mutants whose levels of RDD holo-1 were restored up to 70% of the wild-type levels in p− cells were obtained and designated Sue−/Sue− (data not shown). Meiotic analysis revealed that the Sue− mutant arose by a recessive mutation, designated sue−1, unlinked to CYC1 (data not shown).

Cloning the Suppressor SUE1 Gene by Complementation with a Centromere-based Wild-type Yeast Genomic Library—The p− mutant strain B-14650 bearing sue−1 cyc1-1388 ura3-52 mutations at 55% of the wild-type holo-1 level was transformed with the YCP50-based genomic library of Rose et al. (31). Approximately 12,000 p− transformants were screened on Ura− plates by benzidine staining. A total of 61 white transformants were identified, and the level of cytochrome c was further quantified by low temperature spectroscopic examination of intact cells. Six transformants were found to have diminished levels of RDD holo-1 to nearly 15% of the wild-type level (data not shown).

Three transformants were capable of restoring the higher levels of the RDD holo-1 phenotype of sue−1 in 5-fluoroorotic acid-resistant cells that had lost the plasmids, indicating that the low levels of RDD holo-1 in these three transformants were plasmid-dependent (data not shown). Total DNA was prepared from these three transformants, and their plasmids (pAB2927, pAB2928, and pAB2929) were recovered by transformation in E. coli. Reintroduction of these three plasmids into B-14650 (cyc1-1388 sue−1) demonstrated that plasmids pAB2927 and pAB2929 (but not plasmid pAB2928) complemented the higher levels of the RDD holo-1 phenotype of sue−1 (data not shown). Sequence analysis of two plasmids (pAB2927 and pAB2929) revealed that they contain an identical segment of yeast genomic DNA from positions 821799 to 832715. (Data were obtained from the Saccharomyces Genome Database.)

To further delineate the gene responsible for restoring the SUE1 function in strain B-14650, several subclones were constructed with the YCP50 vector and transformed into strain B-14650. The levels of RDD holo-1 in these transformants were determined by low temperature (−196 °C) spectroscopic examination of intact cells. As summarized in Fig. 1, the results reveal that only the pAB2933 subclone, bearing the NCE102, YPR150w, and YPR151c genes, had a low level of RDD holo-1, as did plasmids pAB2927 and pAB2929, in comparison with the results obtained with the pAB2934 subclone, carrying the NCE102 and YPR150w genes, which retained the high level of RDD holo-1. These results indicate that the SUE1 gene is on the pAB2933 subclone and must be the YPR151c gene.

Table IV

| CYC1 derivatives | B-7889 derivatives | B-8354 derivatives | Template strains |
|------------------|--------------------|--------------------|------------------|
| Normal CYC1− | CYC1+ | CYC1+ | B-8514 |
| LDD cyc1-1120 (G61) | cyc1-1120 (G61) | Not recovered | B-8312 |
| LDD cyc1-1113 (G6A) | cyc1-1113 (G6A) | cyc1-1120 (T78S) | B-8301 |
| RDD cyc1-1209 (T78S) | cyc1-1209 (T78S) | cyc1-1205 (T78G) | B-8634 |
| RDD cyc1-1195 (T78S) | cyc1-1195 (T78S) | cyc1-1205 (T78G) | B-8513 |
| RDD cyc1-1206 (T78L) | cyc1-1206 (T78L) | cyc1-1206 (T78L) | B-8630 |
| ADD cyc1-868 | cyc1-868 | cyc1-868 | B-7734 |
| ADD cyc1-868 | cyc1-868 | cyc1-868 | B-7735 |
| ADD cyc1-868 | cyc1-868 | cyc1-868 | B-7686 |

3 Available at www.yeastgenome.org.
YPR151c genes, further confirmation of the YPR151c gene as the SUE1 gene was carried out by PCR-based site-directed mutagenesis of the YPR151c gene. The 9th base, A, in the YPR151c gene (the 147th base downstream of the stop codon of the YPR150w gene) was deleted to create a combined frame-shift and nonsense mutation on the chromosomal YPR151c gene (referred to as the sue1-2 allele) in strain B-14746 containing the cyc1-868 allele (ADD), an isogenic strain of B-7889. As described below, the cyc1-868 sue1-2 strains grew much better than the cyc1-868 SUE1 strains on medium containing a non-fermentable carbon source such as YPE or YPG, providing a convenient means for testing the functionality of Sue1p. As shown in Fig. 2, the results establish that both the cyc1-868 sue1-2 mutants have similar phenotypes. Therefore, the YPR151c gene corresponds to the SUE1 gene.

Disruption of the SUE1 Gene Leads to the sue1-Δ Phenotype in Both p+ and p− Cells—After successfully cloning the SUE1 gene, we attempted to test whether the SUE1 gene is involved in the degradation of other altered forms of holo-1 (RDD, LDD, and ADD holo-1) that are known from previous studies to be labile in the IMS of mitochondria. In addition, we wanted to know whether the SUE1 gene shows a similar phenotype in three different strain backgrounds.

Using the PCR-based one-step replacement method, we constructed three sets of isogenic mutant strains (B-7528, B-7889, and B-8354) with different RDD, LDD, and ADD cyc1 alleles (Table IV). Subsequently, the corresponding three sets of cyc1-X sue1-Δ::kanR strains were generated. To test whether the SUE1 gene is involved in the degradation of labile RDD, RDD, or ADD holo-1 in p− strains, the levels of altered holo-1 were evaluated in p− strains with the genotype of the cyc1-X SUE1 and cyc1-X sue1-Δ::kanR genes (where cyc1-X is one of alleles listed in Table IV). If the SUE1 gene is involved in the degradation of the altered holo-1, the level of altered holo-1 in the p− sue1-Δ::kanR strain will be elevated compared with that in the p− SUE1 strain. All levels of holo-1 were measured by low temperature (−196 °C) spectrophotometric examination of intact cells. Some results are shown in Tables V and VI. More accurate values were determined by low temperature (−196 °C) spectrophotometric recordings (Fig. 3). Several sue1-Δ mutant strains, derivatives of strains B-7528 and B-7889 containing either an ADD cyc1 allele (cyc1-868, cyc1-860, or cyc1-865) or an LDD cyc1 allele (cyc1-1202, G6I), had elevated levels of altered holo-1 in p− cells in comparison with the corresponding SUE1 strains. In contrast to other RDD cyc1 mutant strains (cyc1-1195, T78I, and cyc1-1206, T78L), the p− sue1-Δ cyc1-1209 (T78S) mutant strain showed elevated levels of RDD holo-1 in comparison with its SUE1 strain. These results suggest that Sue1p might be involved in the degrada-

### Table V

|            | p− B-7528 | p+ B-7889 | p+ B-8354 |
|------------|-----------|-----------|-----------|
| CYC1+ SUE1 | 90        | 55        | 100       |
| CYC1+ sue1-Δ::kanR | 100       | 60        | 100       |
| cyc1-1209 (T78S) SUE1+ | 25        | 5         | 15        |
| cyc1-1209 (T78S) sue1-Δ::kanR | 40        | 30        | 15        |
| cyc1-1195 (T78S) SUE1 | 0         | 15        | 0         |
| cyc1-1195 (T78S) sue1-Δ::kanR | 0         | 5         | 0         |
| cyc1-868 (ADD) SUE1+ | 60        | 40        | 85        |
| cyc1-868 (ADD) sue1-Δ::kanR | 80        | 60        | 70        |
| cyc1-1200 (G6I) SUE1 | 25        | 5         |           |
| cyc1-1120 (G6I) sue1-Δ::kanR | 60        | 35        |           |

### Table VI

|            | p− B-7528 | p+ B-7889 | p+ B-8354 |
|------------|-----------|-----------|-----------|
| CYC1+ SUE1+ | 100       | 100       | 100       |
| CYC1+ sue1-Δ::kanR | 100       | 100       | 100       |
| cyc1-1209 (T78S) SUE1+ | 80       | 100       | 100       |
| cyc1-1209 (T78S) sue1-Δ::kanR | 80       | 90        | 90        |
| cyc1-1195 (T78S) SUE1 | 65        | 85        | 85        |
| cyc1-1195 (T78S) sue1-Δ::kanR | 35        | 90        | 90        |
| cyc1-868 (ADD) SUE1+ | 30       | 15        | 10        |
| cyc1-868 (ADD) sue1-Δ::kanR | 15       | 15        | 15        |
| cyc1-1120 (G6I) SUE1 | 30        | 20        |           |
| cyc1-1120 (G6I) sue1-Δ::kanR | 35       | 15        |           |
Degradation of Labile Yeast Cytochromes c

Fig. 3. Low temperature (−196°C) spectrophotometric recordings of intact cells from a series of isogenic ρ− and ρ+ strains derived from strain B-7528. Left panel, recordings for ρ− strains; right panel, recordings for ρ+ strains. Cells were grown on YPS plates for 3 days at 30°C. Trace A, CYC1 sue1-Δ:kan6; trace B, CYC1 SUE1; trace C, cyc1-1209 (T78S) sue1-Δ:kan6; trace D, cyc1-1209 (T78S) SUE1; trace E, cyc1-1120 (G61) sue1-Δ:kan6; trace F, cyc1-1120 (G61) SUE1. The α-peaks of cytochromes aa2, b, c1, and c are located at 602.5, 558.5, 553.3, and 547.3 nm, respectively. The peak at ~575 nm is due to the presence of Zn-coproporphyrin.

Fig. 4. Nfs+ phenotype. Better growth on YPE medium was observed in the ρ+ sue1-Δ strains containing either the cyc1-1120 (G61 LDD) or cyc1-868 (ADD) allele. All strains are isogenic strains of B-7528. Cultures of wild-type CYC1, cyc1-1195 (T78I RDD), cyc1-1209 (T78S RDD), cyc1-868 (ADD), and cyc1-1120 (G61 LDD) with or without the SUE1 gene were grown on YPD medium. Serial dilutions (10-fold) of logarithmically growing cultures were spotted onto YPD or YPE plates and incubated at 30°C for 2–3 days. Panel I, row A, CYC1 SUE1; row B, CYC1 sue1-Δ; row C, cyc1-1195 (T78I) SUE1; row D, cyc1-1195 (T78I) sue1-Δ; row E, cyc1-1209 (T78S) SUE1; row F, cyc1-1209 (T78S) sue1-Δ. Panel II: row A, CYC1 SUE1; row B, CYC1 sue1-Δ; row C, cyc1-31 SUE1; row D, cyc1-868 SUE1; row E, cyc1-868 sue1-Δ. Panel III: row A, CYC1 SUE1; row B, CYC1 sue1-Δ; row C, cyc1-31 SUE1; row D, cyc1-1120 (G61) SUE1; row E, cyc1-1120 (G61) sue1-Δ.

green fluorescence throughout the cell, excluding the vacuole. These results were obtained by confocal microscopy performed using a Leica TCS SP microscope equipped with argon, krypton/argon, and ultraviolet lasers. Images were processed using Adobe Photoshop Version 5.5 and are shown in Fig. 5. This indicates that Sue1p resides within the mitochondria and that the target signal for mitochondria may not be located at the C-terminal end of Sue1p.

To further investigate whether Sue1p is located in the IMS of mitochondria, we constructed the C-terminal (Myc)3-fused SUE1 gene in the yeast 2μ-based expression vector pBEVY under the control of the constitutive ADH1 promoter (pAB2936). Concomitantly, we also constructed the SUE1 gene in the same vector without the Myc tag (pAB2937) as a control. These two vectors (pAB2936 and pAB2937) were then transformed into strain B-14705, an isogenic strain of B-7528 containing the CYC1 and sue1-Δ:kan6 genes. Using anti-c-Myc oncoprotein Ab-2 (a mouse monoclonal antiserum; NeoMarkers), (Myc)3-tagged Sue1p was detected in the mitochondria purified on a 20–50% sucrose gradient (Fig. 6). This result confirms that Sue1p is a mitochondrial protein. Disruption of the mitochondrial outer membrane by osmotic swelling did not result in the release of (Myc)3-tagged Sue1p from the IMS fraction, and (Myc)3-tagged Sue1p was detected in the mitoplast fraction (Fig. 7). As a control, cytochrome b6, a soluble IMS protein, was visualized in the IMS fraction using cytochrome b6-specific antiserum (Fig. 7). Porin, an outer membrane protein, also served as a control and was visualized in the mitoplast fraction using monoclonal antiserum against porin (Fig. 7). The mitoplast fraction consists of inner and outer membrane proteins and matrix proteins. The membrane potential across the inner membrane is directly involved in importing preproteins into the matrix (33). Because Sue1p may play a role in the degradation of some labile holo-1 in ρ− strains, these results suggest that Sue1p might reside in mitochondria associated with its membranes.

Primary Sequence Analysis of SUE1—As indicated above, Sue1p plays an important role in the degradation of some altered holo-1. However, it does not have proteolytic activity in substrates such as purified T78S RDD holo-1 or the casein that was tested in vitro (data not shown). To determine the function of Sue1p, we searched sequence data bases using the sequence of Sue1p. The SUE1 gene (YPR151c) encodes a protein of 206 amino acids. So far, the function of Sue1p is still unknown, and
there is no evidence in the data bases that any protein interacts with Sue1p. The most closely related protein is another yeast protein that is encoded by the \textit{PET20} gene (YPL159c). These two proteins share 27% amino acid identity (Yeast Protein Database). Analysis with MitoProt II suggests that both Sue1p and Pet20p have a 98% probability of being imported into mitochondria. To test whether the protein encoded by the \textit{PET20} gene is functionally comparable with Sue1p, the \textit{pet20-Δ1::kan} mutant strains were constructed in the set of isogenic mutant strains that originated from B-7528 and that contained...
Degradation of Labile Yeast Cytochromes c

30457

various cyc1 alleles (Table IV). The pet20-Δ mutants did not show the similar phenotype that was observed in both ρ− and ρ− sue1-Δ mutants. In addition, unlike the ρ− CYC1 sue1-Δ mutants, the ρ− CYC1 pet20-Δ mutants exhibited diminished growth on YPG medium at 37 and 15 °C. Therefore, we conclude that Pet20p is not functionally equivalent to Sue1p.

DISCUSSION

In this study, we have isolated and characterized mutations that partially prevented the degradation of RDD holo-1 in a ρ− strain, and we have demonstrated that one of these mutations corresponds to SUE1. The degradation of three classes of altered holo-1 (RDD, ADD, and ADD holo-1) was investigated in sue1-Δ cells. Elevated levels of T78S RDD, ADD, and G6I LDD holo-1 were observed in ρ− sue1-Δ strains compared with the corresponding ρ− SUE1 strains. Although sue1-Δ did not detectably elevate the levels of ADD and ADD holo-1 in ρ− strains, interestingly, the growth of ρ− sue1-Δ strains (compared with that of ρ− SUE1 strains) containing G6I LDD and ADD holo-1 was enhanced on medium containing a non-fermentable carbon source.

Yeast cells grown on a non-fermentable carbon source such as glycerol (YPG) or ethanol (YPE) require a functional cytochrome c; however, mutants are able to grow on plates with a non-fermentable carbon source (Nfs−) if they contain as low as 5% of the normal amount of holo-1. In some genetic backgrounds, mutants exhibit nearly normal growth on a non-fermentable carbon source containing as low as 10% of the normal amount of holo-1 (32, 34, 35). In cases of cyc1 mutants with a threshold level of holo-1, just below the level required for growth on non-fermentable media, growth can occur by marginal increases in holo-1 levels. For example, cyc1-1120 (G6I) SUE1 and cyc1-1120 (G6I) sue1-Δ strains are Nfs− and Nfs+, respectively. Although the levels of holo-1 in cyc1-1120 SUE1 and cyc1-1120 sue1-Δ strains grown on YPD medium are nearly the same, the level of holo-1 in the cyc1-1120 sue1-Δ strain grown on YPG medium is slightly higher than when grown onYPD medium. Thus, the difference in growth on YPG medium due to sue1-Δ is easily observed only with strains that have threshold levels of holo-1. In contrast, growth is indistinguishable in SUE1 and sue1-Δ with high levels of holo-1. Therefore, the growth difference on YPG medium between sue1-Δ and SUE1 strains with certain ADD or LDD cyc1 mutations appear to reflect simply the differences in holo-1 levels. The results obtained with the holo-1 levels in RDD ρ− strains and those obtained with ADD and LDD ρ− strains grown on YPG, etc., media collectively suggest that the SUE1 gene plays an important role in the degradation of altered holo-1 in mitochondria.

To determine the function of Sue1p, we carried out a computer analysis of the S. cerevisiae genome data base using the full-length Sue1p sequence. Pet20p, the most closely related protein to Sue1p, is not functionally equivalent to Sue1p. However, a segment of Sue1p from residues 37 to 109 has 28% identity and 50% similarity to a segment of the YFR041c protein from residues 30 to 97. The YFR041c protein is considered to be a molecular chaperone containing the characteristic J-domain from residues 42 to 110. J-domain proteins and Hsp70 are two molecular chaperones that work together to bind non-native proteins and to promote the folding process (36, 37). Considerable evidence now indicates that Hsp70 and J-domain proteins are also involved in protein degradation (38, 39). They may be the components of a protein quality control system, targeting misfolded or unfolded proteins to the degradation machinery for destruction (40, 41). Through studying a model substrate in E. coli, a non-secreted alkaline phosphatase mu-

4 B. Pulevoda, J. Wei, S. Brown, and F. Sherman, unpublished data.
Degradation of Labile Yeast Cytochromes c

34, 7103–7112

36. Bukau, B., and Horwich, A. L. (1998) Cell 92, 351–366
37. Lu, Z., and Cyr, D. M. (1998) J. Biol. Chem. 273, 27824–27830
38. Lee, D. H., Sherman, M. Y., and Goldberg, A. L. (1996) Mol. Cell. Biol. 16, 4773–4781
39. Zhang, Y., Nijbroek, G., Sullivan, M. L., McCracken, A. A., Watkins, S. C., Michaelis, S., and Brodsky, J. L. (2001) Mol. Biol. Cell 12, 1303–1314
40. Hohfeld, J., Cyr, D. M., and Patterson, C. (2001) EMBO Rep. 2, 885–890
41. Huang, H. C., Sherman, M. Y., Kandror, O., and Goldberg, A. L. (2001) J. Biol. Chem. 276, 3920–3928

31. Rose, M. D., Novick, P., Thomas, J. H., Botstein, D., and Fink, G. R. (1987) Gene (Amst.) 60, 237–243
32. Sherman, F., Stewart, J. W., Jackson, M., Gilmore, R. A., and Parker, J. H. (1974) Genetics 77, 255–284
33. Rehling, P., Wiedemann, N., Pfanner, N., and Truscott, K. N. (2001) Crit. Rev. Biochem. Mol. Biol. 36, 291–336
34. Linske-O’Connell, L. I., Sherman, F., and McLendon, G. (1995) Biochemistry 34, 7094–7102
35. Linske-O’Connell, L. I., Sherman, F., and McLendon, G. (1995) Biochemistry
Sue1p Is Required for Degradation of Labile Forms of Altered Cytochromes c in Yeast Mitochondria
Jun Wei and Fred Sherman

J. Biol. Chem. 2004, 279:30449-30458. doi: 10.1074/jbc.M403742200 originally published online April 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403742200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 40 references, 13 of which can be accessed free at http://www.jbc.org/content/279/29/30449.full.html#ref-list-1