SCO1 and SCO2 Act as High Copy Suppressors of a Mitochondrial Copper Recruitment Defect in Saccharomyces cerevisiae*

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D. Moira Glerum, Andrey Shtanko, and Alexander Tzagoloff§

From the Department of Biological Sciences, Columbia University, New York, New York 10027

C129/U1 is a respiratory defective mutant of Saccharomyces cerevisiae arrested in cytochrome oxidase assembly due to a mutation in COX17, a nuclear gene encoding a low molecular weight cytoplasmic protein proposed to function in mitochondrial copper recruitment. In the present study we show that the respiratory defect of C129/U1 is resuable by two multicopy suppressors, SCO1 and SCO2. SCO1 was earlier reported to code for a mitochondrial inner membrane protein with an essential function in cytochrome oxidase assembly (Buchwald, P., Krumm, G., and Rodel, G. (1991) Mol. Gen. Genet. 229, 413–420). SCO2 is a homologue of SCO1, whose product is also localized in the mitochondrial membrane but is not required for respiration. SCO1 also suppresses a cox17 null mutant, indicating that overexpression of Sco1p can compensate for the absence of Cox17p. In contrast, neither copper, COX17 on a multicopy plasmid, or a combination of the two is able to restore respiration in sco1 mutants. Rescue of cox17 mutants by Sco1p suggests that this mitochondrial protein plays a role either in mitochondrial copper transport or insertion of copper into the active site of cytochrome oxidase. Although SCO2 can also partially restore respiratory growth in the cox17 null mutant, rescue in this case requires addition of copper to the growth medium. SCO2 does not suppress a sco1 null mutant, although it is able to partially rescue a sco1 point mutant. We interpret the ability of SCO2 to restore respiration in cox17, but not in sco1 mutants, to indicate that Sco1p and Sco2p have overlapping but not identical functions.

The COX17 gene of Saccharomyces cerevisiae has been shown to code for a cytoplasmic protein that is essential for assembly of cytochrome oxidase (1). The respiratory defect of cox17 mutants is correctable by exogenous copper, indicating an insufficiency of mitochondrial copper as the basis for the assembly arrest. Cox17p is a low molecular mass protein (8 kDa) with 7 cysteine residues, of which at least one is functionally important (1). The copper deficiency in cox17 mutants appears to be confined to cytochrome oxidase. The presence in a cox17 null mutant of cytoplasmic superoxide dismutase (2) and the iron transporter encoded by FET3 (3), both of which use copper as cofactors, provides strong evidence that Cox17p targets copper to mitochondria (1).

The COX17 gene was found to be complemented by the copper deficiency of the cox17 mutant, C129/U1, with a yeast genomic plasmid (1). Transformations of this mutant yielded two other plasmids that did not contain COX17. In this communication, we show that these high copy suppressors of C129/U1 are SCO1 (4) and SCO2 (5). SCO1 has been reported to code for a cytochrome oxidase assembly factor; the product of this gene also plays a role in providing copper for cytochrome oxidase. The failure of sco1 mutants to form the mature complex is proposed to be caused either by a deficiency in mitochondrial copper uptake or by failure to insert copper during assembly of cytochrome oxidase.

MATERIALS AND METHODS

Yeast Strains and Media—The genotypes and sources of the strains of S. cerevisiae used in this study are listed in Table I. The media used for growth of yeast have been described elsewhere (1).

Cloning of SCO1 and SCO2—The SCO1 and SCO2 genes were cloned by transformation of the cox17 mutant, C129/U1, with a recombinant plasmid of yeast nuclear DNA, by the method of Schiestl and Gietz (7). The library used for the transformation was constructed from a yeast genomic library. Approximately 5 × 10⁸ cells were transformed with 100 μg of plasmid DNA. The transformation mixtures were plated on minimal glucose medium to select for plasmid-bearing colonies (approximately 10⁶ uracil-positive colonies). The minimal glucose plates were replicated on rich glycerol medium, and growth was scored after 2–5 days of incubation at 30°C. SCO1 was also cloned independently by transformation of the sco1 mutant E428/U1 with the same genomic library.

Preparation of Yeast Mitochondria and Enzyme Assays—Wild-type and mutant yeast were grown to stationary phase in YPGal (2% galactose, 1% yeast extract, and 2% peptone) and mitochondria were prepared by the procedure of Faye et al. (9), except that Glutamase was replaced by Zymolyase 20,000 (ICN Biomedicals, Inc.) to prepare spheroplasts. Cytochrome oxidase activity was measured by following the oxidation of ferrocytochrome c at 550 nm (10).

Construction of a SCO1–BIO Fusion Gene—To create a gene expressing Sco1p with covalently attached biotin at its carboxyl terminus (11), the termination codon of SCO1 was destroyed and replaced by a BamH1 site. A HindIII site was created 296 bp upstream of the start codon, and the amplified 1.2-kb fragment was digested with HindIII and BamHI and cloned into YEp352-Bio5. The resultant construct consisted of the entire SCO1 coding sequence fused in-frame to the 270-nucleotide fragment coding for the biotinylation signal sequence of bacterial transcaryocarbonatease (12). The fusion gene was transferred to the multicopy shuttle vector YEps1 and to the integrative plasmid YEp351 (13).

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§ To whom all correspondence should be addressed. Tel.: 212-854-2920; Fax: 212-865-8246.
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| Strain | Genotype | Source |
|--------|----------|--------|
| W303–1A | ade2–1 his3-115 leu2-3,112 trpl-1 ura3-1 | — |
| W303–1B | ade2–1 his3-115 leu2-3,112 trpl-1 ura3-1 | — |
| C129/U1 | ura3-1 cox17-1 | Ref. 1 |
| E428 | met6 sco1 | Ref. 6 |
| W303 | ade2–1 his3-115 leu2-3,112 trpl-1 ura3-1 sco1 | E428 × W303–1A |
| W303/sco1 | ade2–1 his3-115 leu2-3,112 trpl-1 ura3-1 sco1 | This study |
| W303/sco2 | ade2–1 his3-115 leu2-3,112 trpl-1 ura3-1 Δsco2::URA3 | This study |
| aw303/sco1 | ade2–1 his3-115 leu2-3,112 trpl-1 ura3-1 Δsco1::URA3 | This study |
| aw303/sco1/ST12 | ade2–1 his3-1,15 leu2-3,112 trpl-1 ura3-1 Δsco1::URA3 leu2::SCO1-BIO | This study |

* Dr. Rodney Rothstein, Department of Human Genetics, Columbia University.

Construction of W303/sco1 and W303/sco2—A null allele of SCO1 was made by cloning the 1.7-kb EcoRI fragment containing SCO1 into YEp352E. YEp352E is identical to YEp352 (13), except that the multiple cloning region of the latter plasmid is replaced with a unique EcoRI site. This plasmid was linearized at the SphI site inside SCO1 and ligated to a 1.2-kb linear SphI fragment with the yeast URA3 gene coding for a cytochrome oxidase assembly factor (SCO1) and W303 into YEp352E. YEp352E is identical to YEp352 (13), except that the linear plasmid was ligated to an SphI linker. The yeast URA3 gene (as a 1.1-kb SphI fragment) was ligated to the SphI site in the gapped gene. The disrupted sco2::URA3 allele was isolated on a linear fragment and used to transform the respiratory competent haploid strains W303-1A and W303-1B by the one-step gene replacement procedure (14). Uracil-independent transformants were selected and verified to have the disrupted alleles by Southern analysis of their chromosomal DNA (see Fig. 2).

Preparation of Antibodies to Sco2p—In order to generate antibodies to the SCO2 gene product, the gene was amplified by PCR, with primers which created a BamHI site at amino acid residue 11 and a HindIII site 120-bp downstream of the stop codon. This fragment was ligated into the expression vector pATH20 (15), creating an in-frame fusion with the Escherichia coli trpE gene. The fusion protein expressed from the trpE fusion constituted most of the insoluble protein fraction of the E. coli cells. This fraction was dissolved in a 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 buffer, containing 2% SDS, 5 mM β-mercaptoethanol, and 20 μg/ml phenylmethylsulfonylfluoride, and the Sco2 fusion protein was further purified on a Bio-Gel A0.5 column developed with a buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, and 5 mM β-mercaptoethanol. Fractions containing primarily the fusion protein were pooled, concentrated by acetone precipitation, and used to raise antibodies in rabbits.

Miscellaneous Procedures—Standard procedures were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from E. coli (16). The preparation of yeast nuclear DNA and the conditions for the Southern hybridizations were as described by Myers et al. (17). DNA probes were labeled by random priming (18), and DNA was sequenced by the method of Maxam and Gilbert (19). Proteins were separated by polyacrylamide gel electrophoresis in the buffer system of Laemmli (20), and Western blots were treated with antibodies against Sco2p followed by a second reaction with peroxidase-conjugated goat anti-rabbit antibodies. The peroxidase conjugate was visualized with peroxidase conjugated to avidin (1). Protein concentrations were determined by the method of Lowry et al. (22).

Isolation of Suppressors of a cox17 Mutant—C129 is a cytochrome oxidase defective mutant of S. cerevisiae with a single point mutation in COX17 (17). The COX17 gene was cloned by complementation of C129/U1 with a yeast genomic library (16). Some of the respiratory competent clones obtained from the transformations, however, were found to have plasmids with genomic fragments unrelated to one another or to COX17. The physical maps of two such plasmids indicated the presence of SCO1, a gene coding for a cytochrome oxidase assembly factor (4, 23). The identity of SCO1 as the suppressor was corroborated by the ability of pG41/T2 and pG41/ST8 to confer respiration to C129/U1. pG41/T2 was cloned independently by transformation of a sco1 mutant. pG41/ST8 is a subclone of pG41/T2 containing only SCO1 (Fig. 1).

SCO1 codes for a constituent of the yeast mitochondrial inner membrane and is essential for the expression of cytochrome oxidase (4, 23). Mutations in SCO1 induce a specific deficiency in cytochrome oxidase but do not substantially affect other enzymes of the respiratory chain or of the ATPase (4, 23). The ability of sco1 mutants to synthesize the mitochondrial encoded subunits of cytochrome oxidase (23) and to accumulate the nuclear gene products has led to the suggestion that Sco1p promotes some late post-translational step in the assembly pathway (4, 23), although its precise function has not been clarified.

Two other plasmids (pSG74/T1 and T2) obtained by transformation of C129/U1 had overlapping inserts unrelated to either COX17 or SCO1 (Fig. 1). Partial sequencing of the insert in pSG74/T1 revealed that it contained a fragment of yeast chromosome II with SCO2, a homologue of SCO1 (5). Restoration of respiration in C129/U1 by pSG74/T4, but not by pSG74/T5 (with a 450-bp deletion in the SCO2-coding region), confirmed SCO2 to be a second high copy suppressor. The function of the SCO2 product is not known. The phenotype of a mutant with a null allele of the gene (see below) precludes a

![Restriction maps of pG41/T2, pSG74/T1, and subclones.](image-url)
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Some 4 kDa are lost during processing of the precursor. The 30-kDa protein detected by the antibody is much more abundant in mitochondria from a transformant harboring SCO2 on a high copy plasmid and is absent in W303△SCO2. The absence of a signal in the null mutant confirms that this mitochondrial constituent is not required for respiration. Mitochondria from the other strains with the same antibodies are compared with W303△SCO2△ST12 expressing a biotinylated Sco1p fusion protein (WT, ST4, ΔSCO1, ΔSCO2, ΔSCO1/ST12). Approximately 30 μg of total mitochondrial protein were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with a antibody against the SCO2-TRP fusion protein. The cross-reacting bands are visualized with 125I-protein A. The migration of molecular mass standards is indicated in the left-hand margin. Panel A, mitochondria were prepared from the wild-type strain W303-1B (WT), the SCO2-disrupted strain (ΔSCO2), C129ΔU1 transformed with SCO2 on a multicopy plasmid (ST4), W303△SCO1 with a disrupted copy of SCO1 (ΔSCO1), and W303△SCO1/ST12 expressing a biotinylated Sco1p fusion protein (ΔSCO1/ST12). Approximately 30 μg of total mitochondrial protein were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody against the SCO2-TRP fusion protein. The cross-reacting bands are visualized with 125I-protein A. The migration of molecular mass standards is indicated in the left-hand margin.

The Sco2p antibody was used to determine whether this protein is a mitochondrial membrane protein like Sco1p. Mitochondria prepared from the respiratory competent strain W303-1B were disrupted by sonication and the membrane and soluble fractions were probed for Sco2p. The Western blot, shown in Fig. 3B, indicates Sco2p to be present exclusively in the submitochondrial membrane fraction. Solubilization of Sco2p requires extraction of mitochondria with 0.2–0.5% deoxycholate and 0.5M NaCl, further confirming the hydrophobic

Fig. 2. Southern analysis of W303△SCO2 genomic DNA. The construction of the sco2::ura3 null allele is shown in the lower part of the figure. The 450-bp AflII fragment was removed from pSG74/ST4 (see Fig. 1) and replaced by an SpII linker. After transfer of the insert to pUC8, the construct was linearized with SpII and ligated to the URA3 gene. The delineating Smal (Sm) and PstI (P) sites of pSG74/ST4, as well as the two AflII (A) sites are indicated on the map. aw303△SCO2 is a ura3 prototroph obtained by transformation of W303-1A with the linear fragment containing the disrupted gene. Chromosomal DNA from both the SCO2-disrupted and wild-type strains was digested with PstI and Smal, separated on a 1% agarose gel, and transferred to nitrocellulose. The blot was hybridized with the entire pSG74/ST4 insert. The probe detects the homologous 1.9-kb band in wild-type genomic DNA from both W303-1A and a 1.1-kb band in the mutant (ΔSCO2). The novel 1.1-kb band consists of the upstream and coding region of SCO2 and 200 bp of the URA3 fragment up to the PstI site. Not seen on the photograph is a second hybridizing band of approximately 0.6 kb representing the 3’-untranslated region of SCO2 and 60 bp of the URA3 disruptor. The migration of DNA size standards is shown in the left hand margin.

requirement for the protein in respiration.

Phenotype of a sco2 Mutant and Localization of the Product—To facilitate further studies on the relationship of Cox17p to Sco1p and Sco2p, null alleles of SCO1 or SCO2 were constructed as described under “Materials and Methods.” Mutants with a disrupted chromosomal copy of SCO1 (aw303△SCO1) were constructed by microprojecting the coding region of SCO1 into an auxotrophic strain. aw303△SCO1/ST12 expressing a biotinylated Sco1p fusion protein, approximately 7 kDa larger than the native protein. The absence of a cross-reacting protein of 37 kDa in mitochondria from W303△SCO1 ST12 and the lack of an effect of the sco1 mutation on the strength of the signal indicates that the antibody specifically recognizes Sco2p.

The Sco2p antibody was used to determine whether this protein is a mitochondrial membrane protein like Sco1p. Mitochondria prepared from the respiratory competent strain W303-1B were disrupted by sonication and the membrane and soluble fractions were probed for Sco2p. The Western blot, shown in Fig. 3B, indicates Sco2p to be present exclusively in the submitochondrial membrane fraction. Solubilization of Sco2p requires extraction of mitochondria with 0.2–0.5% deoxycholate and 0.5M NaCl, further confirming the hydrophobic
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Fig. 4. Suppression of a cox17 null mutant by overexpression of SCO1 and SCO2. The respiratory competent strain W303-1B (W303), the cox17 null mutant W303ΔCOX17 (ΔCOX17), and W303ΔCOX17 transformed with CTR1 (ΔCOX17/CTR1), COX17 (ΔCOX17/COX17), SCO2 (ΔCOX17/SCO2), and SCO1 (ΔCOX17/SCO1) were streaked on rich glucose medium and replica-plated onto rich ethanol/glycerol (top plate), and rich ethanol/glycerol supplemented with either 0.1% CuSO4 (middle plate) or 0.4% CuSO4 (bottom plate). The replica plates were incubated for 3–4 days at 30 °C.

nature of this protein (Fig. 3C).

Suppression of a cox17 Null Mutant by SCO1 and SCO2—C129/U1 has a point mutation in COX17 resulting in the substitution of a tyrosine for the carboxyl-proximal cysteine in the protein (1). To test if overexpression of SCO1 and SCO2 can also suppress a cox17 null mutant, W303ΔCOX17, harboring a disrupted copy of COX17, was transformed with pG41/ST8 (SCO1), pSG74/ST4 (SCO2), and as a control, also with pG74/ST8, a multicopy plasmid containing COX17. The respiratory competent parental strain, W303-1B, and the different transformants were replicated on rich glycerol/ethanol medium (YEPG) and scored for growth after 3 days of incubation at 30 °C (Fig. 4, top plate). The null mutant was partially suppressed by SCO1, but not by SCO2 (Fig. 4, top plate). The generation time of the mutant transformed with SCO1 was approximately double that of the wild-type strain.

Growth of C129/U1 and W303ΔCOX17 on mitochondrial substrates is rescued by addition of exogenous copper to the growth medium (1). The effect of copper supplementation was also tested on the cox17 mutants transformed with SCO1 and SCO2. The somewhat leaky growth phenotype of C129/U1 made it difficult to score differences in the growth of this strain as a function of the different plasmids and the copper supplement. In the case of W303ΔCOX17, copper supplementation clearly enhanced the suppressor activity of SCO1 with the null mutant. Addition of copper to the medium also allowed SCO2 to partially suppress the respiratory defect in the null mutant (Fig. 4, middle plate), at a concentration lower than that required to rescue the null mutant itself (Fig. 4, bottom plate).

sco1 Mutants Are Not Rescued by Copper Supplementation and by Overexpression of COX17 and CTR1—Suppression by SCO1, and to a lesser degree by SCO2, of the cytochrome oxidase deficiency of cox17 mutations suggested that the products of the two genes might be involved in mitochondrial copper metabolism. Unlike cox17 mutants, neither the sco1 null mutant, nor seven independent sco1 point mutants were rescued by inclusion of 0.01–0.8% copper in the medium. Neither were the mutants rescued with high copy numbers of either COX17 or CTR1 (the structural gene for the plasma membrane copper pump) (25), either in the presence or absence of added copper. In previous studies, the concentration of copper needed to restore respiratory growth of the cox17 null mutant could be significantly lowered (>10-fold) when it was transformed with CTR1 on a high copy plasmid (1). Overexpression of the plasma

Fig. 5. Alignment of yeast Sco1p and Sco2p with other Sco-like proteins and with the copper binding domain of cytochrome oxidase subunit 2. The copper binding domain of subunit 2 (Cox2p) of yeast cytochrome oxidase (26) is aligned with a domain containing two conserved cysteine residues present in all known members of the SCO family. The amino acid residues are numbered in the left-hand margin and the sources of the proteins are indicated in the right-hand margin. The references for the different sequences are: Yeast Sco1p (4), Yeast Sco2p (5), Rhodobacter (capsulatus) (27), Anaplasma (marginale) (28), Pseudomonas (stutzeri) (29), and Cowdria (ruminantium) (30). Identical and conserved residues in Cox2p and the Sco homologues are marked by the asterisks. Four of the residues in Cox2p that make contact with the two copper atoms at the Cuα site of subunit 2 are indicated by the arrows. The contact with glutamic acid is at the carbonyl of the peptide backbone (31).
membrane copper pump was presumed to increase internal copper pools, thereby allowing rescue to occur in the presence of lower exogenous copper (1).

Allele-specific Suppression of a sco1 Mutation by SCO2—The primary sequence homology of Sco1p and Sco2p, and their activity as suppressors of the cox17 allele in C129/1U, suggested that they might have similar or overlapping functions. Transformation of W303△SCO1 with SCO2 (pSG74/ST4), however, failed to restore respiratory growth in the sco1 null mutant, indicating that the two proteins are not exchangeable and therefore cannot be functionally equivalent. When tested for suppression of point mutations, SCO2 was found to partially rescue one of the seven sco1 mutants tested (W30).

The allele-specific suppression of a sco1 mutant by SCO2 could indicate that a physical interaction of Sco1p and Sco2p is necessary for function. This is contradicted by the observation that the sco2 null mutant has no phenotype on nonfermentable carbon sources. Attempts to detect a physical complex of the two proteins also were unsuccessful. Extraction of Sco1p-Bio and purification of the biotinylated protein by affinity chromatography on a monomeric avidin column failed to disclose co-purification of Sco2p. Secondly, although both Sco1p and Sco2p sediment with apparent molecular masses of approximately 60 kDa, the sedimentation behavior of Sco2p was the same in a strain with a sco2 null mutation (data not shown). Finally, suppression by overexpression of Sco1p or Sco2p alone is difficult to rationalize if the two proteins are subunits of a stoichiometric complex.

**DISCUSSION**

The cytochrome oxidase deficiency of cox17 mutants was previously shown to be corrected by exogenous copper (1). This observation together with the cytoplasmic localization of Cox17p led us to propose a role for this low molecular weight protein in targeting copper to mitochondria (1). In the present study we demonstrate rescue of cox17 mutants, including a strain with a null allele of the gene, by overexpression of Sco1p, a constituent of the mitochondrial inner membrane, encoded by SCO1 (23). The suppressor activity of SCO1 suggests an involvement of the protein, Sco1p, in mitochondrial copper metabolism. Since sco1 mutants are not rescued by COX17, Sco1p is likely to act downstream of Cox17p, either prior to or during the copper maturation step. An alignment of yeast Sco1p with several homologues from eucaryotic and procaryotic sources reveals a potential copper binding domain characterized by the presence of two conserved cysteines whose spacing and proximity to a short conserved hydrophobic domain is reminiscent of the copper binding domain of subunit 2 of cytochrome oxidase (Fig. 5).

Sco1p could be a mitochondrial copper carrier, accepting copper from Cox17p and translocating it to the mitochondrial matrix. This model implies that copper addition to subunits 1 and 2 of the enzyme occurs on the matrix side of the inner membrane (Fig. 6, scheme A), perhaps before subunits 1 and 2 of cytochrome oxidase are fully integrated in the lipid bilayer. Excess Sco1p may help to correct lesions in Cox17p by increasing the efficiency of copper uptake from alternate cellular pools. Sco1p could also be a mitochondrial copper storage and/or transfer protein more directly involved in addition of copper to the cytochrome oxidase precursor (Fig. 6, scheme B). Copper need not be transferred across the inner membrane if addition occurs in the intermembrane space where the active site of the mature enzyme resides (31). According to this model also, overexpression of Sco1p could compensate for the absence of the mitochondrial copper targeting protein by allowing for a more efficient uptake or transfer of copper during maturation of the enzyme.

Overexpression of Sco2p can partially suppress the respiratory defect of a cox17 point mutant and a strain with a null allele, the latter in the presence of added copper. The activity of SCO2 on a high copy plasmid as an allele-specific suppressor of sco1 mutations could mean that Sco1p and Sco2p exist and function as a complex. Even though the molecular weight of Sco1p and Sco2p is two times the monomer size, no evidence could be obtained for a complex of the two proteins. The dependence of function on such a complex, even if it exists, is also unlikely in view of the absence of a discernable phenotype in the sco2 deletion mutant. An alternative explanation for allele-specific suppression of a sco1 mutation by SCO2 is that the product of this gene is able to provide one of the Sco1p functions lost in the mutant. A redundancy in one of the activities of these two proteins would explain the lack of a phenotype in the sco2-disrupted strain.

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