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Mitochondrial copper metabolism in yeast: mutational analysis of Sco1p involved in the biogenesis of cytochrome *c* oxidase

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Abstract *Saccharomyces cerevisiae* Sco1p is believed to be involved in the transfer of copper from the carrier Cox17p to the mitochondrial cytochrome *c* oxidase subunits 1 and 2. We here report on the results of a mutational analysis of Sco1p. The two cysteine residues of a potential metal-binding motif (CxxxC) are essential for protein function as shown by their substitution by alanines. Chimeras consisting of Sco1p and its homolog *S. cerevisiae* Sco2p restrict the specificity of Sco1p function to the N-terminal half of the protein. A candidate region for conferring specificity on Sco1p is a stretch of hydrophobic amino acids, which act as a membrane anchor. In line with this suggestion is the result that alterations of individual amino acids within this region impair Sco1p function.

Key words Yeast Sco1p · Copper · Cytochrome*c* oxidase · Mitochondria

Introduction

In the yeast *Saccharomyces cerevisiae* copper is transported across the plasma membrane by Ctr1p and transferred to small cytosolic copper-binding proteins such as Atx1p, Lys7p or Cox17p (Valentine and Gralla 1997). Attachment of copper to the final acceptor proteins like the cytosolic CuZn superoxide dismutase (Sod1p), a multicopper oxidase in a post-Golgi compartment (Fet3p), or mitochondrial cytochrome *c* oxidase (COX), requires specific mediator proteins (Yuan et al. 1997). A candidate mediator for the attachment of copper to COX subunits 1 and 2 is the nuclearly encoded Sco1p, which is anchored by a single trans-membrane (TM) segment of about 17 amino acids to the inner mitochondrial membrane (Buchwald et al. 1991). Sco1p was originally identified as a protein required for the assembly of COX (Schulze and Rödel 1989). The two largest COX subunits, Cox1p and Cox2p, which carry heme and copper as prosthetic groups and constitute the catalytic centre of the enzyme, are rapidly degraded in the absence of a functional *SCO1* gene (Krummeck and Rödel 1990). A first clue to the function of Sco1p was obtained by Glerum et al. (1996b), who detected that overexpression of Sco1p in combination with slightly elevated concentrations of copper in the growth medium can partially suppress the deficiency of Cox17p. Cox17p is a copper-binding protein in both the cytosol (Glerum et al. 1996a) and the mitochondrial inter-membrane space (Beers et al. 1997), suggesting that Sco1p might be involved in the attachment of copper to COX (Glerum et al. 1996b). This view is supported by the presence of a potential metal-binding motif CxxxC (Glerum et al. 1996b). Such a motif, with a characteristic spacing of cysteine residues, has been shown to constitute a copper-binding site in Cox2p (Coruzzi and Tzagoloff 1979) and an iron-binding site in ferredoxins (Bruschi and Guerlesquin 1988). The CxxxC motif of Sco1p is located in the carboxy-terminal part of the protein which protrudes into the mitochondrial inter-membrane space (Krummeck 1992; Beers et al. 1997).

In the course of the yeast genome sequencing project a gene with a high degree of similarity to *SCO1* (overall aa identity of 53.8%) was detected (Smits et al. 1994) (see Fig. 2). The function of its gene product, Sco2p, is enigmatic: The protein is localized in the mitochondrial membrane (Glerum et al. 1996b) as expected from the presence of an amino-terminal sequence reminescent of mitochondrial targeting sequences and a single predicted trans-membrane segment. The position of this hydrophobic stretch and the distribution of charged flanking amino-acid residues are almost identical in Sco1p and in Sco2p, suggesting a similar topology. Like Sco1p the larger C-terminal part, which probably protrudes into the inter-membrane space, carries the potential metal-binding motif CxxxC.

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Table 1 Oligonucleotide primers. Underlined bases correspond to the indicated restriction sites

Primer	Sequence (5′ → 3′)	Primer position	Restriction site
#1	GTCCTCGAGCTCCCAATTGAAACTAAATTG	*SCO1*: −317 to −287	*Sac*I
#2	CTTCCAAGCTTCACATAGCCTC	*SCO1*: +1043 to +1022	*Hin*dIII
#3	GGACCCTTCCATCTAGAGGATATG	*SCO1*: +349 to +372	*Xba*I
#4	GGCACTTCTAGACAACTTCAGCAGCTTTTC	*SCO1*: +24 to −6	*Xba*I
#5	GGACAGATATCAGGAGCGTTACTAAACC	*SCO1*: +458 to +431	
#6	GGTTTAGTAACGCTCCTGATATCTGTC	*SCO1*: +431 to +457	
#7	GTTCATCAGGAGCGATATCAGGAC	*SCO1*: +466 to +443	
#8	CCTGATATCGCTCCTGATGAACTG	*SCO1*: +445 to +468	
#9	CATCAGGAGCGATATCAGGAGCGTTACTA	*SCO1*: +463 to +435	
#10	GGTTTAGTAACGCTCCTGATATCGCTCCTGATG	*SCO1*: +431 to +463	
#11	ATATATAAGCTTCTATCATGCCTCCTTCTGCTTGGC	*SCO1*: +864 to +847	*Hin*dIII
#12	ATATATAAGCTTCTATCACTGCTCTGCAGGCACATA	*SCO1*: +843 to +826	*Hin*dIII
#13	GGAATTCCCAATTGAAACT	*SCO1*: −311 to −293	*Eco*RI
#14	CTAGGACTGCAGCAATGGCCTTTC	*SCO1*: +244 to +221	*Pst*I
#15	CCACTGCAGTATTGTTGCTAAGTGG	*SCO2*: +248 to +272	*Pst*I
#16	CCCATAAGCTTGGTATCCTAGC	*SCO2*: +959 to +938	*Hin*dIII
#17	TCTGTCAAGTTCATCAGGACAGATGTCAGGACA	*SCO1*: +465 to +442	
#18	CCTGATGAACTTGACAGATTAACGTATTGGATT	*SCO2*: +484 to +507	
#19	CCTGTTGAAGAAATAAGAAAGTGCCCCACCAATGG CCTTTCCCGTGGAAAACTCGATCGA	*SCO1*: +279 to +202	
#20	CAGAGTAATACCATACGTAGAAGAGAG	*SCO1*: +522 to +496	
#21	CCACCGACTGCTAGCAATAGAGC	*SCO1*: +254 to +232	
#22	ATAAGAAGGTACCCCACCGACTG	*SCO1*: +267 to +245	
#23	GCTAGGAATAGAGCTCTGGCC	*SCO1*: +245 to +225	
#24	GGAATAGATCTATGGCCTTTCCCG	*SCO1*: +241 to +218	
#25[a]	AGGGTTTTCCCAGTCACGACGTT	pUC 18-M13 (uni-43)	
#26[a]	GAGCGGATAACAATTTCACACAGG	pUC 18-M13 (rev-49)	
#27[a]	TGTAAAACGACGGCCAGT	pUC 18-M13 (uni-21)	
#28[a]	CAGGAAACAGCTATGACC	pUC 18-M13 (rev-29)	
#29[a]	GGAGTCTCTTGCTGGATCACAAG	*SCO1*: +561 to +539	
#30[a]	GCTGAAGTTGTCAAGAAGTGCCAA	*SCO1*: +3 to +26	
#31	GTGAAGGTTTACCGTATCCTCTG	*SCO1*: +343 to +321	

[a] Indicates fluorescent-labelled sequencing primers

Despite this extraordinary similarity *SCO2* is not able to functionally complement *sco1* null-mutants. Deletion of the *SCO2* gene does not result in any obvious phenotype (Glerum et al. 1996b). Nevertheless, similar to Sco1p, overexpression of Sco2p partially suppresses *cox17* mutations, albeit less efficiently: reconstitution of growth on glycerol medium requires the addition of a higher concentration of copper. In addition it was reported that only one out of a number of *sco1* mutants (the DNA sequence of this allele has not been reported) can be partially suppressed by overexpression of Sco2p (Glerum et al. 1996b). While this observation suggests a physical interaction of Sco1p and Sco2p, experimental data so far give no indication of the existence of heterodimers (Glerum et al. 1996b).

Here we report on the results of directed mutagenesis of the *SCO1* gene, aimed at the definition of functionally important amino-acid positions of the protein. We show that each of the two cysteine residues of the potential metal-binding motif CxxxC is essential for the protein function. We further demonstrate that the extreme carboxy-terminal tail of the protein is dispensible for its function. By constructing chimeric proteins with varying carboxy-terminal parts derived from Sco2p, we were able to show that the carboxy-terminal half of Sco1p can be replaced by the corresponding part of Sco2p. Thus, the amino-terminal half of the protein must contain the determinant(s) for Sco1p specificity.

Materials and methods

Strains and media. The *S. cerevisiae* strains employed in this study were GR20 (*MAT*α, *ura3-251, ura3-379, ura3-228, leu2-3, leu2-112, his3-11, his3-15, sco1::URA3*) (Schulze and Rödel 1988), D273-10B (ATCC 25657) and DBY 747 (ATCC 44774). Yeast media were as described by Kaiser et al. (1994). The *Escherichia coli* strains used were DH5α (BRL) and JM101 (Messing 1979). *E.coli* media were as described by Sambrook et al. (1989). Yeast transformants were selected on minimal medium (WO-plates supplemented with histidine). Growth on non-fermentable glycerol medium was tested on YPGly-plates.

Cloning of SCO1. A 1.3-kb fragment containing the promoter, the coding region and the terminator of *SCO1* (−305 bp to +1032 bp) was PCR-amplified from the genomic DNA of strain D273-10B with primers #1 and #2 (the oligonucleotides used are summarized in Table 1). The PCR-product was digested with *Sac*I and *Hin*dIII and cloned into the shuttle vector YEp351 (Hill et al. 1986). The correct sequence of the insert was verified by DNA sequencing. The resulting plasmid, YEp351/SCO1, served as a template for PCR-based in vitro mutagenesis except for the directed mutagenesis of the transmembrane segment of Sco1p.

Random mutagenesis. Mutagenic PCR of *SCO1* with primers #1 and #2 in the presence of 0.05–0.15 mM $MnCl_2$ (Shafikhani et al. 1997) was performed for 30 cycles: 1 min at 94 °C, 1 min 30 s at 50 °C and 2 min at 72 °C. After digestion with *Sac*I and *Hin*dIII the PCR-products were ligated into YEp351 and transformed into *E. coli* strain DH5α. White transformants were selected on LB-plates containing ampicillin and X-gal, pooled, and plasmid-DNA was prepared. The resulting plasmid-pool was used for the transformation of strain

GR20. Plasmids of three transformants which exhibited temperature-sensitive growth on YPGly were re-isolated and used for the transformation of *DH5α* in order to prepare plasmid-DNA for sequence analysis. Each mutant allele was analyzed in three independent clones.

Construction of the amino-terminally truncated Sco1p derivative. The *SCO1*-promoter region was PCR-amplified with primers #1 and #4, digested with *Sac*I and *Xba*I, and ligated into vector pGEM-3Z (Promega) yielding plasmid pGEM/Prom. The C-terminal part of *SCO1*, starting at the second AUG codon of the *SCO1*-ORF (bp-position +370), was obtained by PCR with primers #3 and #2. After cleavage with *Xba*I and *Hin*dIII the fragment was cloned into pGEM/Prom cut with *Xba*I and *Hin*dIII. From the resulting plasmid (pGEM/Prom/C-term) a *Sac*I/*Hin*dIII-fragment, consisting of the C-terminal part of *SCO1* behind the authentic promoter, was isolated and cloned into YEp351. The correct sequence of the insert was verified by DNA sequencing.

Site-directed mutagenesis of the CxxxC motif. Sco1p derivatives Sco1p(C148A), Sco1p(C152A) and Sco1p(C148A; C152A) were created by the PCR-based overlap extension technique (Pogulis et al. 1996). The mutagenic primers used were #5 and #6 for Sco1p(C148A), #7 and #8 for Sco1p(C152A), #9 and #10 for Sco1p(C148A;C152A). First, the N-terminal part, including the CxxxC motif, was PCR-amplified with primers #5, #7 and #9 respectively, in combination with primer #1. The C-terminal part, including the CxxxC motif, was obtained in a second PCR using primers #6, #8 and #10 respectively, in combination with primer #2. PCR-products of both reactions were mixed in a 1:1 ratio and used as a template for a third PCR with primers #1 and #2. The resulting products were digested with *Sac*I and *Hin*dIII and cloned into YEp351. The sequences of the mutant alleles were confirmed by DNA sequencing.

Construction of carboxy-terminally truncated Sco1p derivatives. For the generation of Sco1pΔ289-295 and Sco1pΔ282-295 PCRs were performed using primer #1 in combination with primers #11 and #12 respectively. The products were cleaved with *Sac*I and *Hin*dIII and ligated into YEp351. The DNA sequences of the truncated ORFs were verified by sequence analysis.

Construction of Sco1p/Sco2p chimeric proteins. For the creation of the chimeras their two different parts were separately PCR-amplified. The following primer combinations were employed: #13 and #14 for Sco1p(1–91), #15 and #16 for Sco2p(83–301), #1 and #17 for Sco1p(1–155) as well as #18 and #16 for Sco2p(161–301). The overlap extension technique with primers #1 and #16 was used to construct Sco1p(1–155)/Sco2p(161–301). Sco1p(1–91)/Sco2p(83–301) was generated as follows: the *SCO1*-fragment (1–91) was digested with *Eco*RI and *Pst*I and subcloned into pGEM-3Z yielding plasmid pGEM/C. The *SCO2*-fragment (83–301) was cleaved with *Pst*I and *Hin*dIII and fused in-frame to the *SCO1*-part of pGEM/C cut with *Pst*I and *Hin*dIII. (Due to the introduction of the *Pst*I site, the first amino acid of the *SCO2*-part is a valine instead of the original leucine.) From the resulting plasmid, pGEM/CD, an *Eco*RI/*Hin*dIII-fragment was isolated. Both chimeras were cloned into YEp351 and their correct sequence was confirmed by DNA sequencing.

Directed mutagenesis of the trans-membrane segment of Sco1p. Oligonucleotide-directed mutagenesis, based on the method described by Kunkel (1985), was carried out with the "Muta-Gene M13 in vitro mutagenesis kit" (Bio-Rad, Richmond) according to the instructions of the manufacturer. A 1.7-kb *Eco*RI-fragment, including the *SCO1*-promoter, coding region and terminator derived from plasmid pB10E (Schulze and Rödel 1988), was cloned into the vector M13mp18 (Messing 1983). The resulting plasmid (M13mp18B10E) served as a template for the construction of the *sco1* alleles described below: Sco1pΔ77–82: deletion of nucleotides +229 to +247 (corresponding to aa residues 77–82) was performed by using primer #19. The resulting plasmid (M13mp18/del6) was cleaved with *Eco*RI. The 1.7-kb fragment produced was subcloned into pBS-Bluescript (Stratagene), re-isolated as a *Xba*I/*Hin*dIII-fragment and subsequently cloned into YEp351 and YIp351 (Hill et al. 1986) respectively. This cloning procedure was used for all constructions mentioned below; mutations were verified by DNA sequencing.

Sco1p(F80L): primer #21 was used to change the nucleotide C^{+240} to G.

Sco1p(AL85/86VP): oligonucleotide #22 was used to replace the nucleotides C^{+257} and T^{+260} by T and C respectively.

Sco1p(I77R): the nucleotides T^{+230} and T^{+231} were changed to G and A respectively by using primer #23.

Sco1p(A78D): primer #24 was used in order to replace the nucleotides T^{+231} and C^{+233} by As.

Miscellaneous procedures. Standard DNA techniques were as described (Sambrook et al. 1989). DNA fragments were isolated from agarose gels using Qiaex columns (Qiagen). Preparation of yeast genomic DNA was performed as described by Kaiser et al. (1994). Yeast cells were transformed by the lithium-acetate method described by Schiestl and Gietz (1989). For the re-isolation of plasmid DNA from yeast cells a modified procedure of Ling et al. (1995) was used. Sequence analysis of the constructs Sco1pΔ77–82, Sco1p(F80L), Sco1p(AL85/86VP), Sco1p(I77R) and Sco1p(A78D) was carried out with the "T7 DNA-sequencing kit" (Pharmacia) according to the instructions of the manufacturer using α-[^{35}S] dATP and primer #31. A "Thermo sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Pharmacia Biotech) was employed for sequencing of the remaining constructs. IRD 800-labelled sequencing primers were provided by MWG.

Results and discussion

Random mutagenesis of the *SCO1* gene

In an attempt to identify *sco1* alleles with an impaired function, we amplified the *SCO1* gene by PCR in the presence of manganese (see Materials and methods). PCR products were cloned in plasmid YEp351, amplified in *E. coli* and used for the transformation of strain GR20. Leu^+ transformants were tested for temperature-sensitive (ts) growth on non-fermentable medium by replica-plating on YPGly plates and incubation at 25 °C, 30 °C, and 37 °C respectively. Three transformants exhibiting a ts-phenotype (see Table 2 and Fig. 1) were further analyzed. To confirm that plasmid-borne mutations are responsible for the phenotype, plasmids were re-isolated from the yeast cells and subsequently used for a further transformation of GR20. All transformants exhibited a ts phenotype similar to that initially observed, i.e. clones K132ochre and K63ochre showed reduced growth rates at 25 °C and 37 °C, while clone ΔC^{+47} exhibited a reduced growth rate at 25 °C and 30 °C. The optimal growth temperature of clone ΔC^{+47} was 37 °C. The results of the sequence analyses of the plasmids are summarized in Table 2. The alleles K132ochre and K63ochre result from point mutations at bp-positions +394 and +187, respectively, both of which create premature termination codons in the *SCO1*-ORF. In addition, both alleles contain one neutral base-substitution which does not alter the primary protein sequence. The mutant allele ΔC^{+47} exhibits deletion of a C at bp-position +47, resulting in a –1 frameshift. The new reading frame leads to a termination codon after 19 codons. In addition the ΔC^{+47} allele contains two further base-substitutions (see Table 2) within the original *SCO1*-ORF, but outside of the newly created ORF.

Re-initiation of translation at the second AUG codon of the *SCO1*-ORF at bp-position +370 could account for the

Table 2 Compilation of the *sco1*-mutant alleles created by random mutagenesis

Mutant allele	Mutations	Protein sequence	Growth on YPGly[a] at		
			25 °C	30 °C	37 °C
K132ochre	A → T (394)	K → ochre (stop)	+	+++	++
	T → C (456)	Neutral			
K63ochre	A → T (187)	K → ochre (stop)	+	+++	+
	G → A (219)	Neutral			
ΔC^{+47}	Deletion of C (47)	–1 frame shift	+	+	+++
	T → A (98)	Neutral in original *SCO1*-ORF			
	A → T (166)	Neutral in original *SCO1*-ORF			

[a] ++++ wild type level; +++ slightly reduced growth; ++ reduced growth; + strongly reduced growth

observed phenotype. The resulting Sco1p derivative (Sco1pΔ-123) would lack the mitochondrial import sequence and the TM anchor region (Fig. 1). However, the previous observation that deletion of the Sco1p-TM segment results in a mitochondrial matrix localization and renders the protein non-functional (Buchwald et al. 1991), makes this interpretation very unlikely. Nevertheless, we tested whether the carboxy-terminal part of Sco1p, starting at the second AUG codon and expressed from the authentic *SCO1* promoter, is able to complement the respiratory deficiency of strain GR20. As expected, this construct proved to be non-functional. Based on this result, other explanations, e.g. leaky read-through translation of the termination codons as well as translational frame-shifting, must account for the observed complementation behaviour of the *sco1* mutant alleles.

Mutagenesis of the CxxxC motif

As outlined in the Introduction, the presence of a sequence motif CxxxC was quoted to support the idea that Sco1p might be involved in transferring copper to COX. To test the importance of the two cysteine residues, we changed them into alanine residues by directed mutagenesis (see Fig. 1). All mutant proteins, including the single-mutants Sco1p(C148A) and Sco1p(C152A) and the double-mutant Sco1p(C148A; C152A), failed to restore the respiratory competence of the *sco1* null-mutant GR20. This result clearly demonstrates that both cysteine residues are absolutely required for Sco1p function and is in agreement with its proposed function in metal-binding. Expression of the *sco1* mutant alleles in a wild-type strain (DBY 747) does not impair growth on YPGly-plates, i.e. the mutations have no dominant negative effects.

According to topological data on Sco1p (see Introduction) this motif is exposed in the mitochondrial inter-membrane space. As Cox17p, which seems to act as a copper transporter for the mitochondrial compartment, as well as major parts of COX, including the copper-binding region of Cox2p, are positioned in the same compartment, the postulated transfer from Cox17p to COX via Sco1p might occur in the IMS. However, transfer of copper to the mitochondrial matrix and subsequent attachment of the metal ion to the COX subunit prior to its assembly into the membrane is not excluded.

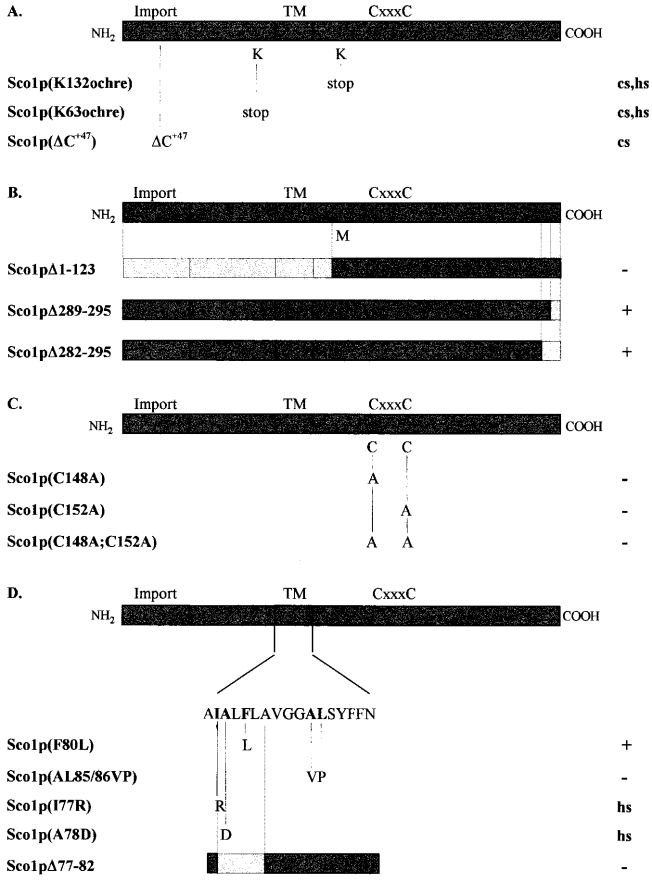

Fig. 1A–D Schematic presentation of the mutant alleles of *SCO1*. *Import* indicates the mitochondrial pre-sequence, *TM* the putative transmembrane domain, and *CxxxC* the potential metal-binding site. Deleted protein parts are shown in *light grey* Amino-acid substitutions are given in the one letter code. ΔC^{+47} represents the –1 frame-shift mutation due to the deletion of the cytosine at bp-position +47 in the *SCO1* gene. Growth of GR20-transformants on YPGly are shown on the right side: + = growth; – = no growth; *cs* cold-sensitive growth; *hs* heat-sensitive growth

Truncation of the carboxy-terminus of Sco1p

A prominent feature of the extreme carboxy-terminal end of Sco1p is a motif of hydrophobic amino acids (WYSFLF) preceded by nested charged amino acids (see Fig. 2). Comparison of the amino-acid sequence of Sco1p with that of homologs (*S. cerevisiae* Sco2p, ScSco2p; *S. pombe* Sco1p,

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SCO1p MLKLSR----SANLRLVQLPAARLSGNGAKLLTQRGFFTVTRLWQSNGKKPLSRVPVG----GTPIKDNGKVREGSIEFSTG
SCO2p MLNSSRKYACRSLFRQANVSIKGLFYNGG--AYRRGFSTGCCLRSDNKESPSARQPLDRLQLGDEINEPEPIRTRFFQFSRW
                 TM
SCO1p KAIALFLAVGGALSYFFNREKRRLETQKEAEANRGYGKPSLGGPFHLEDMYGNEFTEKNLLGKFSIIYFGFSNCPDICPDEL
SCO2p KATIALLLLSGGTYAYLSRKRRLLETEKEADANRAYGSVALGGPFNLTDFNGKPFTEENLKGKFSILYFGFSHCPDICPEEL

SCO1p DKLGLWLNTLSSKYGITLQPLFITCDPARDSPAVLKEYLSDFHPSILGLTGTFDEVKNACKKYRVYFSTPPNVKPGQDYLVD
SCO2p DRLTYWISELDDKDHIKIQPLFISCDPARDTPDVLKEYLSDFHPAIIGLTGTYDQVKSVCKKYKVYFSTPRDVKPNQDYLVD

SCO1p HSIFFYLMDPEGQFVDALGRNYDEKTGVDKIVEHVKSYVPAEQRAKQKEAWYSFLFK                (295 aa)
SCO2p HSIFFYLIDPEGQFIDALGRNYDEQSGLEKIREQIQAYVPKEERERRSKKWYSFIFN                (301 aa)
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Fig. 2 Alignment of *S. cerevisiae* Sco1p and Sco2p. Identical amino acids are given in *bold letters*, gaps as *lines*. Predicted trans-membrane segments (*TM*) and the conserved potential metal-binding motifs are indicated by *grey areas*. The identity of Sco1p to Sco2p is 53.8%. Alignment was performed using ClustalW

SpSco1p; and human Sco1p, hsSco1p) reveals that such a signature is conserved in Sco1p and Sco2p but is not present in the other homologs. The *S. pombe* homolog lacks a hydrophobic stretch at the extreme C-terminus but ends with a cluster of positively charged amino-acid residues, which are conserved with respect to ScSco1p. To test the functional importance of the carboxy-terminus, we constructed two truncated versions of Sco1p, one lacking the last seven amino acids (ScSco1pΔ289–295), the other lacking both the hydrophobic and the charged amino-acid cluster (ScSco1pΔ282–295). Interestingly, both constructs proved to be functional when transformed into the *sco1* null mutant GR20 (see Fig. 1). This result shows that the carboxy-terminal tail of Sco1p is dispensable for the biological function of this protein.

Sco1p/Sco2p chimeric proteins

As outlined in the Introduction, Sco2p is not able to substitute for Sco1p. To define the portion of Sco1p which confers this specificity, we constructed chimeric proteins. First we combined the amino-terminal part of Sco1p, including the first three amino acids of the membrane anchor, with the carboxy terminal part of Sco2p, including 14 amino acids of the TM region (Sco1p(1–91)/Sco2p(83–301)). This chimeric protein failed to complement strain GR20, even when present in high-copy number plasmids (Fig. 3). This result shows that the amino-terminal 91 amino acids of Sco1p are not sufficient to provide Sco1p-specificity to the chimera.

In a second construct the junction of the two donor proteins was placed within the region of the conserved CxxxC motif (position 148–152 in Sco1p): the fusion protein consisted of the 155 N-terminal amino acids of Sco1p and the 126 C-terminal amino acids of Sco2p(Sco1p(1–155)/Sco2p(161–301)). This chimeric protein proved to functionally complement strain GR20 (see Fig. 3).

Although we cannot exclude that the failure of the first fusion protein (Sco1p(1–91)/Sco2p(83–301)) to complement strain GR20 is caused by improper folding, we suggest that an important determinant of Sco1p specificity must be localized in the segment between amino-acid positions 92 and 155, i.e. either in the TM segment and/or its 3′-flanking region up to the CxxxC motif. As the latter stretch is almost identical in Sco1p and Sco2p, the determinant which confers Sco1p-specificity may be represented by the TM segment itself.

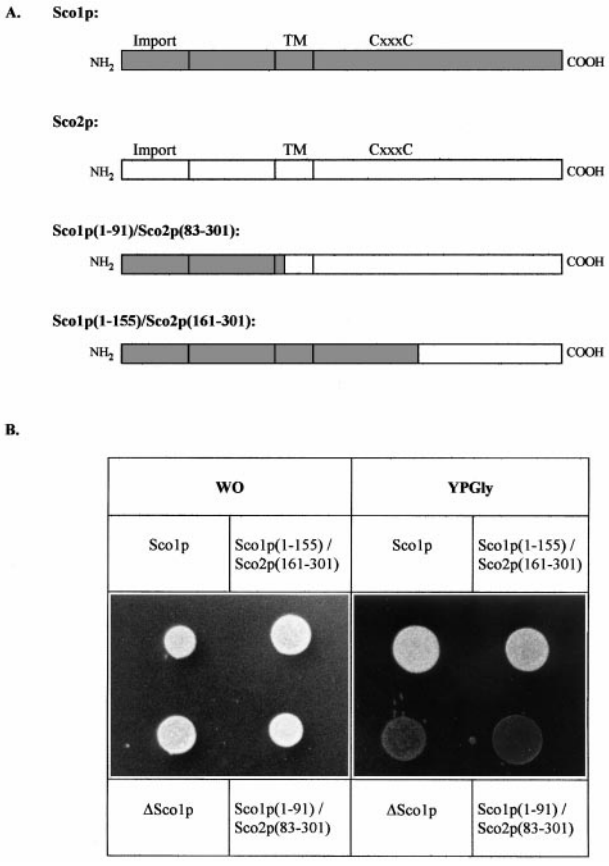

Fig. 3A, B Complementation behaviour of Sco1p/Sco2p chimeras. **A** schematic presentation of Sco1p, Sco2p and the two chimeras. Abbreviations are as in Fig. 2. **B** growth of transformants of GR20 at 30 °C on minimal medium (*WO*) and glycerol medium (*YPGly*)

Directed mutagenesis of the trans-membrane segment of Sco1p

The importance of the Sco1p TM region has been documented by the demonstration that deletion of 18 amino acids (Δ75–91) results in a non-functional matrix-localized Sco1p derivative (Buchwald et al. 1991). To test whether truncation (instead of deletion) of the TM segment, abolishes function too, we deleted the segment between amino-acid positions 77 and 82 (see Fig. 1). The resulting mutant protein (Sco1pΔ77–82) with a hydrophobic stretch of only 10–11 amino acids is not able to confer respiratory competence to strain GR20. The creation of a hydrophobic

stretch of about 20 amino-acid residues by in vitro mutagenesis at another position of Sco1p (amino acid position 159–179) is not sufficient for a functional protein (Krummeck 1992).

The importance of the TM sequence was further tested by directed mutagenesis of various amino acids. A compilation of these *sco1* mutant alleles and their effects on function is given in Fig. 1. While replacement of Phe^{80} by Leu (F80L) does not affect function, the introduction of a Pro at position 86 (AL85/86VP) inactivates function completely, in line with its known effect to disrupt helical segments. Interestingly the effect of charged residues, introduced at the amino-terminal border of the TM segment (I77R and A78D, respectively), depends on the copy number of the mutant construct. When present in a single copy (YIp vector) the mutant alleles are unable to complement strain GR20. By contrast, in high copy (YEp vector), GR20 transformants show reduced ts growth on YPGly medium.

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