**SNC1**, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: Genetic interactions with the RAS and CAP genes

(signal transduction/oncogenes/vesicular trafficking/cAMP)

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ABSTRACT  **SNC1**, a gene from the yeast Saccharomyces cerevisiae, encodes a homolog of vertebrate synaptic vesicle-associated membrane proteins (VAMPs) or synaptobrevins. **SNC1** was isolated by its ability to suppress the loss of CAP function in *S. cerevisiae* strains possessing an activated allele of RAS2. CAP is a component of the RAS-responsive *S. cerevisiae* adenylyl cyclase complex. The N-terminal domain of CAP is required for full cellular responsiveness to activated RAS proteins. The C-terminal domain of CAP is required for normal cellular morphology and responsiveness to nutrient extremes. Multicopy plasmids expressing **SNC1** suppress only the loss of the C-terminal functions of CAP and only in the presence of activated RAS2.

The yeast *Saccharomyces cerevisiae* contains two RAS genes that encode proteins highly homologous to mammalian RAS oncogene products (1, 2). These RAS proteins are required to activate *S. cerevisiae* adenylyl cyclase (3, 4) but may have other functions as well (5). The functions of RAS in higher organisms are not known. When expressed in *S. cerevisiae*, mammalian RAS proteins are capable of both activating adenylyl cyclase and suppressing the lethality associated with the loss of endogenous RAS function (3, 5, 6). Thus, some functions of RAS may have been conserved during the course of evolution. To explore this we have begun to characterize the *S. cerevisiae* adenylyl cyclase complex.

We previously identified a protein called CAP that copurifies with a RAS-responsive adenylyl cyclase complex (7). The gene for CAP encodes a 526-residue protein that is required for full responsiveness to activated RAS and for normal cellular morphology and responsiveness to nutrient extremes (8, 9). Deletion analysis has shown that CAP is bifunctional (10). Expression of a domain consisting of the N-terminal 168 amino acids is sufficient for full cellular responsiveness to activated RAS, while expression of the C-terminal 160 amino acids is sufficient for normal cellular responses to nutrient extremes (10). At present, it is unclear whether RAS or adenylyl cyclase influences CAP function.

To understand the function of CAP, we have isolated genes that on multicopy plasmids are capable of suppressing loss of C-terminal function. One such gene, *PFY*, encodes profilin, an actin binding protein (11). Another gene, which we have named **SNC1** (suppressor of the null allele of CAP), is described here. It encodes a protein homologous to low molecular weight proteins known as VAMPs (synaptic vesicle-associated membrane proteins) (12, 13) or synaptobrevins (14-16) that are associated with synaptic vesicles and are found in a wide variety of organisms.®

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MATERIALS AND METHODS

**Microbial Culture.** Yeast strains were grown in rich medium (YPD; yeast extract/Bactopeptone/dextrose), synthetic complete minimal medium (SC), or SC drop-out minimal medium lacking an essential amino acid or nucleotide base. Drop-out minimal medium was used to maintain selection of plasmids. Yeast extract, Bactopeptone, and yeast nitrogen base lacking ammonium sulfate and amino acids (YNB) were purchased from Difco. YPD was prepared according to Sherman et al. (17). SC minimal complete and drop-out media were prepared as described by Sherman et al. (17) and consisted of 0.7% YNB supplemented with the appropriate auxotrophic requirements and 2% glucose. Yeast medium lacking in amino acids and a nitrogen source (YNB-N) was prepared according to Toda et al. (4). Standard methods were used to introduce plasmids into the various yeast strains (17). *Escherichia coli* strains HB101 and DH5α were used for plasmid transformations and plasmid DNA preparations.

**Yeast Strains.** cap yeast strains SKN32 (*Mata leu2 ura3 trp1 ade8 can1 cap::HIS3*) and SKN37 (*Mata leu2 ura3 trp1 ade8 can1 RAS2val9 cap::HIS3*) have been described (9). This cap::HIS3 allele lacks amino acids 78-451 of the coding region of CAP and is a null allele. cap strain SKO13 (*Mata leu2 ura3 trp1 ade8 can1 cap::HIS3*) has also been described (9). cap strain SKN50 (*Mata leu2 trp1 ade8 can1 ural::HIS3 cap::URA3*) was created by transforming the ural strain IR-1 (18) with the EcoRI fragment of the CAP disruption plasmid pUSMN2 as described (9). cap strains SKN55 and SKN56 (*Mata ade8 can1 bcy1::LEU2 tpkl2::HIS3 tpk3::TRP1 cap::URA3*) were created by transforming the bcy1 tpkl2 tpk3 strain S13-58A (19) with the EcoRI fragment of pUSMN2 CAP disruption plasmid. cap strains SKN58 and SKN59 (*Mata ade8 trp1 can1 pdel::LEU2 pdez::URA3 cap::HIS3*) were created by transforming the pdel pde2 strain DJ23-3C (20) with the EcoRI fragment of the CAP disruption plasmid pHSNP5 as described (9). HIS3+ transformants were isolated and the disruption of CAP was verified by phenotypic and Southern blot analysis. The sncl strain JG4 (*Mata leu2 trpl ade8 his3 can1 sncl::URA3*) was constructed by transforming the haploid SP1 yeast strain (*Mata leu2 ura3 trpl ade8 his3 can1*) (4) with the Sal I/Sac I fragment of the **SNC1** disruption plasmid pORF3U (see below). Integration of this construct at the **SNC1** locus results in an insertion in **SNC1**. The sncl strain JG5 (*Mata leu2 trpl ade8 his3 can1 sncl::URA3*) was created by transforming the haploid SP1 yeast strain with the Sal I/Sac I fragment of the **SNC1** disruption plasmid pNCSU (see below). Integration of this

Abbreviations: VAMP, synaptic vesicle-associated membrane protein; ORF, open reading frame.

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††The sequence reported in this paper has been deposited in the GenBank data base (accession no. M91157).
construct at the SNC1 locus results in deletion of the SNC1 gene. Diploid yeast, formed by mating DC124 (Mata leu2 ura3 trp1 ade8 his4) (19) and SP1, were also transformed with the Sal I/Sac I fragment of pORF3U. Ura+ transformants were sporulated and subjected to tetrad analysis (17). Distribution of the URA3 marker in haploid strains derived from tetrad analysis was found to be 2:2. In all the cases described above, genotypes were verified by Southern blot analysis.

DNA Manipulations. DNA restriction endonucleases, Tag polymerase, and T4 DNA ligase were used as recommended by the suppliers (New England BioLabs and Cetus). Molecular cloning, Southern blotting, and colony hybridization techniques were performed as described by Maniatis et al. (21). DNA sequencing was performed by the dideoxy nucleotide chain-termination method (22). The polymerase chain reaction (PCR) (23) and subcloning of PCR products were carried out as described (10). Oligonucleotides used to amplify SNC1 included a forward oligonucleotide bearing a Sal I site (5'-AAGCTATCTGACAACTGCTGTC-3') and a reverse oligonucleotide bearing a Sac I site (5'-CTAGGATCATGATCCATGATCC-3'). Total RNA was isolated from wild-type yeast (SP1) according to Sherman et al. (17). Isolation of poly(A)+ RNA was accomplished with a kit from Stratagene. First-strand cDNA synthesis from yeast poly(A)+ RNA, which was used as a template for PCR, was accomplished with a cDNA synthesis kit from Bethesda Research Laboratories.

Genomic DNA from a YPD+ revertant of the SKO13 cap mutant strain (9) was isolated according to Sherman et al. (17). The DNA was partially digested with the Sau3A restriction endonuclease and size-fractionated by gel electrophoresis. A library was constructed in the yeast expression plasmid YEp13M4 (20) from size-selected DNA cloned into the BamHI site of the vector.

Phenotypic Assays and Selections. cap strain SKN37 was transformed with the library and grown for 48 hr on selective medium before replica plating onto YPD plates to assay for growth on rich medium. Of 2000 Leu+ transformants, 30 were YPD+, only one of which was found to grow on rich medium in a plasmid-dependent manner. Plasmids were isolated from yeast according to standard methods (17). Candidate plasmids were then retransformed back into the cap SKN37 strain and were examined for their ability to confer growth on YPD. Assays for growth on rich medium, temperature-sensitive growth at 37°C, and sensitivity to nitrogen starvation or heat shock (55°C) were performed as described (10).

Plasmids. Plasmids used in this study included the following vectors: YEp13M4, yeast expression plasmid bearing the LEU2 selectable marker (20); pAD4, a similar plasmid bearing both the LEU2 selectable marker and the ADH1 promoter (18); and pUV2, pTV3, and pAV3, YEp-based plasmids bearing the URA3, TRP1, or ADE8 selectable markers, respectively. Previously described plasmids included the following: pADH-CAP, which expresses full-length CAP under the control of the ADH1 promoter (9); pADH-CAPΔ4, which expresses 237 amino acids of the C terminus of CAP (10); pADH-CAPΔ15, which expresses 283 amino acids of the N terminus of CAP (10); and YEpIRA2, which expresses IRA2 (R.-M. Ballester and M.W., unpublished results).

Other plasmids included the following: YEpSNC1, a YEp13M4 plasmid bearing a 3.4-kilobase (kb) Sau3A partial digestion fragment of genomic SNC1; YEpTSNC1, a pTV3 plasmid bearing this gene as a Sal I/Sac I fragment; pUC-SNC, a pUC118 plasmid bearing this fragment; pADH-SNC and pADH-cSNC1, which contain a 550-base-pair (bp) fragment of genomic SNCI or a 370-bp fragment of SNC1 cDNA, cloned into the Sal I and Sac I sites of pAD4a, respectively; pADH-ASN1, which bears a 2.3-kb BamHI fragment, containing the ADH1 promoter and the SNC1 sequence from pADH-SNC1, cloned into the BamHI site of pAV3; and pADH-ACAPΔ4, which bears a 3.0-kb BamHI fragment, containing the ADH1 promoter and the CAPΔ4 allele (10) from pADH-CAPΔ4, cloned into the BamHI site of pAV3. The modified pAV3 plasmid was used in the transformation of cap strains SKN55-56 and SKN58-59.

Two plasmids derived from pUCSNC were used for disruptions of SNC1: pORF3U, which has the URA3 selectable marker cloned into the Sry I site (base pair 325 of SNC1; and pNCSU, which has URA3 cloned into the Spe I sites, which flank SNC1 (base pairs +237 and +646, respectively). For pNCSU, pUCSNC was digested with Spe I, which removes the entire SNCI coding region, and the URA3 gene was cloned into this site. Both disruption constructs were verified by restriction analysis.

RESULTS

A Yeast Gene That Suppresses Loss of the C-Terminal Functions of CAP. We have screened a yeast genomic library for multicopy suppressors of the cap null phenotype of the SKN37 strain. This strain is unable to grow on the standard amino acid-rich medium YPD. Expression of the C-terminal domain of CAP is fully able to complement this defect (10). Of 2000 transformants only one was capable of growth on YPD in a plasmid-dependent manner. A transformant containing YEpSNC1, a plasmid that was found to be as potent a suppressor of rich medium growth defects in SKN37 cells as plasmids expressing either full-length CAP or the C-terminal domain of CAP (Fig. 1). SKN37 cells display other defects associated with loss of the C-terminal function of CAP, including large cell size, temperature-sensitive growth, and inability to withstand nitrogen starvation (9). YEpSNC1 suppressed the abnormal cell size (Fig. 2) and the other growth defects as well as did plasmids expressing the C-terminal domain of CAP (data not shown). In contrast, YEpSNC1 did not suppress loss of the N-terminal functions of CAP, the loss of full RAS responsiveness. Wild-type yeast expressing the activated RAS2val19 allele are sensitive to heat shock (24). SKN37 cells, which contain the activated RAS allele, are resistant to heat shock because of the absence of the N-terminal domain of CAP (10). SKN37 cells containing the YEpSNC1 plasmid remain resistant to heat shock (data not shown).

![Fig. 1](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAgAAAAAHCAYAAABXEOjAAAAGXRFWHRTb2Z0d2FyZQBBZG9iZSBJbWFnZVJlYWR5ccllPAAAApJREFUeNrs87f8+e3QgDAwM/wAAAAABJRU5ErkJggg==)
Sequence of SNC1. The yeast SNC1 plasmid contained an insert of 3.4 kb. Deletion analysis of the plasmid localized the full suppressor activity of the yeast SNC1 to a 1-kb fragment. DNA sequencing of this fragment revealed two small open reading frames (ORFs). A search for similar DNA sequences revealed a strong homology between these ORFs, when taken together as a single ORF, and members of a class of small, highly conserved proteins [VAMPs (12, 13) or synaptobrevins (14–16)]. These proteins have been identified in a wide variety of organisms (from fish to humans). Their function is unknown, but they are suspected to play a role in vesicle fusion and neurotransmitter release. Two classes of these proteins have been identified—the type 1 and type 2 VAMP/synaptobrevins, which differ slightly in their N-terminal domains.

Alignment between the translated sequence of the ORFs and those of the VAMP/synaptobrevin genes suggested that, together, these ORFs constitute the entire coding region of a yeast homolog. Several lines of evidence confirm this. First, SNC1 contains sequences that match the consensus sequences for splice donor and acceptor sites in S. cerevisiae: the 5’ splice motif (GTANGT) and the 3’ splice motif (YAG, where Y is pyrimidine) (25) are found at the candidate intron–exon junction at base pairs 101–106 and 213–215, respectively. In addition, the TACTA box, a consensus motif present in S. cerevisiae introns (26), is found at base pairs 146–152. Second, oligonucleotide-directed amplification of SNC1 from yeast cDNA by PCR generated a product ~100 bp smaller than that generated when genomic DNA was used as a template (data not shown). DNA sequencing of the PCR product generated from yeast cDNA revealed that base pairs 101–215 were absent. Finally, expression of the PCR-amplified cDNA clone was able to suppress the CAP-deficient phenotypes of SKN37 cells (data not shown). These studies demonstrate not only the existence of an intron but also that we have identified the correct ORF with suppressor activity.

The ATG codon starting at nucleotide 1 is preceded by multiple in-frame stop codons. Given this, we conclude that SNC1 encodes a protein of 117 amino acids and has a predicted molecular mass of ~13 kDa. A 115-bp intron sequence connects the region encoding the first 33 amino acids to that encoding the last 84 amino acids.

Homology Between SNC1 and Mammalian VAMPs. The yeast SNC1 protein is highly homologous to both the type 1 and type 2 mammalian VAMP/synaptobrevins (Fig. 4). The amino acid sequence of SNC1 shows ~40% identity and ~50% overall homology to either type of protein. The VAMP/synaptobrevin protein has been structurally divided into four domains: a 20–25 kDa hydrophobic region that interacts with phospholipid bilayers, an ~13 kDa hydrophilic region that extends into the cytosol (13). The first 20 residues of the N terminus of both the type 1 and type 2 proteins are highly diverged (12, 14). This is the region with the least homology to yeast SNC1.

In summary, we have identified a synaptic vesicle fusion protein homologous to mammalian VAMPs in yeast. While the function of these proteins in other systems is unknown, we propose that the SNC1 protein is involved in synaptic vesicle fusion.

Fig. 3. Sequence of the SNC1 gene and its encoded product. Coordinates for nucleotides and codons are indicated on the left. The single-letter amino acid code for the SNC1 protein is given below each 3-bp codon of the ORFs. The consensus sequences for intron splicing in S. cerevisiae are indicated in boldface type. The stop codon is indicated with an asterisk.
CAP, SKN32 or SKN34 cells transformed with YEpSNC1 remained incapable of growth on rich medium or on synthetic minimal medium at 37°C and retained their abnormal morphology and sensitivity to nitrogen starvation. These results suggest that activation of the RAS pathway is required for SNC1 suppressor activity.

To rule out the possibility that undetected genetic differences between the cap RAS2 strains (SKN32 and SKN34) and the cap RAS2' strain (SKN37) might be affecting the suppressor activity of SNC1, we created another cap null strain, SKN50 (see Materials and Methods). This strain bears the RAS2' allele but also has a disrupted IRA1 locus. The yeast IRA1 gene is a functional homolog of the mammalian GTPase-activating protein (GAP) (18, 28). An ical CAP strain shows all the phenotypes associated with persistent activation of the RAS pathway (3, 24, 28), including heat shock sensitivity, presumably due to perpetuation of RAS in its activated GTP-bound state (29). SKN50 cells, however, are resistant to heat shock because of loss of the N-terminal functions of CAP and also show the phenotypes associated with loss of the C-terminal functions of CAP (data not shown). The typical ical phenotypes can be restored to SKN50 cells upon expression of full-length CAP. In contrast to the results obtained with the SKN32 and SKN34 strains, but like our results with the SKN37 strain, YEpSNC1 was able to confer robust growth on rich medium in SKN50 cells (Fig. 5A). In addition, the expression of SNC1 was able to correct other defects associated with loss of the C-terminal functions of CAP—namely, growth at 37°C (Fig. 5B) and resistance to nitrogen starvation.

To verify further that the functional activity of SNC1 results from activation of RAS, we coexpressed the yeast IRA2 gene from a multicopy plasmid containing the LEU2 selectable marker YEpIRA2 and SNC1 from a multicopy plasmid containing the TRP1 selectable marker YEpT5NC1 in SKN50 cells. The IRA2 gene encodes a second GAP-like protein and is able to functionally complement the loss of IRA1 (30). SKN50 cells transformed with both SNC1 and IRA2 plasmids were found to grow slowly on YPD and were unable to grow altogether at 37°C under conditions selective for the plasmids (Fig. 5B). In contrast, SKN50 cells transformed with the SNC1 plasmid and a control plasmid bearing the LEU2 marker YEp13M4 were capable of strong growth either on YPD or on synthetic medium at 37°C. Thus, the overexpression of IRA2 appears to inhibit the ability of SNC1 to suppress the loss of CAP functions in SKN50 cells. This conclusion was further confirmed by plasmid segregation analysis (data not shown).

Genetic Interactions Between SNC1 and Genes Encoding Components of the CAMP Pathway. Evidently expression of SNC1 suppresses the phenotype resulting from loss of C-terminal CAP function only in cells that have activated RAS. One possible explanation for this phenomenon is that activation of RAS stimulates the cAMP effector pathway even in the absence of CAP. We therefore sought to determine whether the suppressor activity of SNC1 was dependent on stimulation of the cAMP effector pathway. We created four additional cap mutant strains: two, SKN58 and -59, lacked the cAMP phosphodiesterases encoded by PDE1 and PDE2 (20, 24); and two, SKN55 and -56, lacked the regulatory subunit of the cAMP-dependent protein kinase encoded by the Bcy1 gene (31) and two of the three catalytic subunit isoforms encoded by the TPK2 and TPK3 genes (32). Wild-type yeast lacking the PDE genes are unable to hydrolyze cAMP and are sensitive to heat shock (20). Yeast lacking Bcy1, but expressing at least one of the TPK genes, are also heat shock sensitive (31). As expected, disruption of CAP in these yeast strains had no effect on their sensitivity to heat shock, because the N-terminal function of CAP is not required in these strains. Like other cap disruption strains, SKN55, -56, -58, and -59 were unable to grow on rich medium and displayed the other defects associated with loss of the C-terminal functions of CAP. Overexpression of SNC1 was unable to confer growth on rich medium in these four cap mutant strains. On the basis of these experiments, it appears that the functional activity of SNC1 in cap mutant strains is dependent on the activated state of RAS but not on the level of cAMP or cAMP-dependent protein kinase activity.

**DISCUSSION**

The SNC1 gene encodes a protein of 117 amino acids that is highly homologous to members of the VAMP/synaptobrevin family (12–16), which, because of their subcellular localization, are presumed to be involved in targeting and fusion of synaptic vesicles with the presynaptic membrane (12–16).
These proteins have a distinct structure, composed of a C-terminal transmembrane domain and a small cytoplasmic N-terminal domain. Other proteins of similar overall structure but with larger cytoplasmic domains are known to function in the secretory pathway of S. cerevisiae. SNC1 is very weakly homologous to these other yeast proteins [e.g., SLY12/BET1 (33, 34), SLY2 (33), and BOS1 (35)].

A phenotypic assay for the VAMP/synaptobrevin proteins in yeast would aid in evaluation of their function. Unfortunately, despite the high degree of conservation between SNC1 and the VAMP/synaptobrevin, expression of either the type 1 or type 2 rat VAMP genes does not complement defects seen in cap cells (data not shown). Suppression of the loss of SNC1 function could in principle provide an assay for VAMP/synaptobrevin function. Unfortunately, disruption of the SNC1 locus does not cause a discernible phenotype. Recently, we have found clear evidence for a second VAMP/synaptobrevin homolog in yeast. Disruption of both homologs do not cause a discernible phenotype. Recently, we have found clear evidence for a second VAMP/synaptobrevin homolog in yeast. Disruption of both homologs do not understand how profilin suppresses the loss of the C-terminal functions of CAP. The first of these, PFY (11), encodes profilin, an actin/phospholipid binding protein that is involved in cytoskeletal organization (36) but that we are unable to resolve this problem without understanding the normal function of SNC1.

We and others (8, 9) have shown that yeast with a disrupted CAP locus is at least partly uncoupled from activated RAS proteins: cells without CAP function do not display the phenotypes normally associated with the expression of activated RAS. Our experiments with SNC1 suggest that cap strains are in fact at least partly responsive to activated RAS, although apparently in a manner independent of the RAS-adenyl cyclase interaction. SNC1 is capable of suppressing defects associated with loss of the C-terminal function of CAP only in strains with constitutively activated RAS2 protein. SNC1 is unable to confer suppressor activity in cap strains bearing the RAS24 allele, even when other negative regulators of the cAMP effector pathway are absent. Thus, the suppressor activity of SNC1 appears to be independent of the presently known downstream effectors of RAS in yeast. This result confirms that RAS proteins in the yeast S. cerevisiae have functions in addition to regulation of adenyl cyclase (5). Moreover, these functions do not depend on CAP.

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