

ER Membrane Protein Complex Required for Nuclear Fusion

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Abstract. Diploid cells of the yeast *Saccharomyces cerevisiae* form after the mating of two haploid cells of the opposite mating type. After fusion of the two plasma membranes of the mating cells, a dinucleated cell forms initially in which the two haploid nuclei then rapidly fuse to form a single diploid nucleus. This latter event, called karyogamy, can be divided into two distinct steps: the microtubule-based movement that causes the two nuclei to become closely juxtaposed and the fusion of the nuclear membranes. For the membrane fusion step, one required component, the ER luminal protein Kar2p (BiP), has been identified. For topological reasons, however, it has been unclear how Kar2p could function in this role. Kar2p is localized to the luminal (i.e., noncytoplasmic) face of the ER membrane, yet nuclear fusion must initiate from the cytosolic side of the outer nuclear membrane or the ER membrane with which it is contiguous. There is both genetic and biochemical evidence that Kar2p interacts with Sec63p, an ER membrane protein containing both luminal and cytosolic domains that is involved in

protein translocation across the membrane.

We have isolated novel *sec63* mutant alleles that display severe karyogamy defects. Disruption of the genes encoding other Sec63p-associated proteins (Sec71p and Sec72p) also results in karyogamy defects. A suppressor mutant (*sos1-1*) partially corrects the translocation defect but does not alleviate the karyogamy defect. *sec61* and *sec62* mutant alleles that cause similar or more severe protein translocation defects show no karyogamy defects. Taken together, these results suggest a direct role for Sec63p, Sec71p, and Sec72p in nuclear membrane fusion and argue against the alternative interpretation that the karyogamy defects result as an indirect consequence of the impaired membrane translocation of another component(s) required for the process. We propose that an ER/nuclear membrane protein complex composed of Sec63p, Sec71p, and Sec72p plays a central role in mediating nuclear membrane fusion and requires ER lumenally associated Kar2p for its function.

THE yeast *Saccharomyces cerevisiae*, through a cycle of mating and sporulation, can grow vegetatively as both diploid and haploid cells. In the sexual phase, cells of opposite mating types (*MATa* and *MATα*) become sensitized to the mating pheromones α -factor and α -factor if in close proximity. The binding of pheromones to their respective receptors triggers a signaling cascade in each target cell that leads to the activation of mating-specific genes. The products of these genes control an array of functions necessary to form a diploid cell. The early events include formation of mating projections, cell adhesion, localized breakdown of the cell wall, and fusion of the plasma membrane. Before cellular fusion, the nuclei migrate to the region closest to the opposing cells to be positioned for subsequent nuclear fusion (karyogamy). Normally, nuclear fusion occurs soon after fusion of the plasma membrane (32). Mutants isolated that are defec-

tive in this step have been termed *kar* (karyogamy-defective) (5, 27).

Mutations that cause defects in karyogamy have been divided into two groups: nuclear congression and nuclear fusion (18). Nuclear congression mutations prevent the movement and subsequent alignment of nuclei that precedes their fusion. These include mutations that cause defects in microtubule stability and/or morphology, such as those that affect the *TUB2* (encoding β -tubulin), *KAR4*, and *KAR9* genes (17, 18), and mutations that affect microtubule-based movement, such as those that affect the *KAR3* (encoding a kinesin-related protein) and *CIK1* (encoding a Kar3p-associated protein) genes (18, 25). Nuclear fusion mutations allow the nuclei to become closely juxtaposed but prevent their fusion so that the two nuclei remain distinct in the zygote. Mutants belonging to this group that map to the four genes *KAR2*, *KAR5*, *KAR7*, and *KAR8* have been isolated (18). Of these, only the cloning of the *KAR2* gene has been reported (33). Sequence analysis of the *KAR2* gene revealed that it is a homologue of mammalian BiP, a stress-inducible protein belonging to the Hsp70 family.

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Kar2p is a soluble, luminal resident protein of the ER, which is continuous with the nuclear envelope (23, 29, 33). Because it is the cytoplasmic faces of nuclear/ER membranes that must contact before fusion, the role that Kar2p plays in karyogamy is not understood. Furthermore, Kar2p is required for a number of basic functions in the cell: in addition to its role in nuclear fusion, Kar2p aids protein folding as a molecular chaperone (13) and promotes protein translocation across the ER membrane (42). Despite the seemingly disparate functions attributed to Kar2p, there is evidence that it plays a direct role in membrane fusion. An *in vitro* assay was recently devised to measure homotypic membrane fusion using ER and nuclear membranes (20). Membranes derived from temperature-sensitive *kar2* mutants were proficient for fusion at the permissive temperature but were defective at the nonpermissive temperature. Since there was no additional protein synthesis required in this system, the defect cannot result from an inability of mutant Kar2p to translocate or to assemble other factors required for fusion. These data point to Kar2p as playing an integral role in the nuclear membrane fusion step of karyogamy but have not yet provided further insight into its mechanism.

Additional clues regarding the function of Kar2p came from the characterization of interacting factors. Using both genetic and biochemical methods, Kar2p was shown to interact with Sec63p, an ER transmembrane protein required for posttranslational protein translocation (3, 37). Sec63p contains a large cytoplasmic domain and a luminal domain that shows similarity to the DnaJ class of chaperones that functionally interact with Hsp70 proteins (6, 11, 34).

Here we describe the isolation of mutant alleles of *SEC63* that are defective in the nuclear fusion step of karyogamy. Given the functional interaction between Kar2p and Sec63p, it is possible that the role of Kar2p in karyogamy is mediated through Sec63p. Strains bearing deletions of the nonessential Sec63p-associated proteins Sec71p and Sec72p are also defective in karyogamy. On the basis of these results, we suggest that the protein complex of Sec63p, Sec71p, Sec72p, and Kar2p plays an important role in nuclear membrane fusion. The involvement of this membrane protein complex provides the missing topological link between the ER lumen, where Kar2p resides, and the cytosolic face of the ER membrane, from where nuclear membrane recognition and fusion must be catalyzed.

Materials and Methods

Strains, Plasmids, and Antibodies

All strains used in this study are listed in Table 1. Plasmids were modified and constructed using standard protocols (36). All new plasmid constructions used in this study were based on pRS series centromeric vectors described in Sikorski and Hieter (38) unless otherwise indicated. Plasmids pCS15 (*CEN6*, *LEU2*, *SEC61*), pRD15 (*CEN6*, *LEU2*, *SEC62*), pDF26 (*CEN6*, *LEU2*, *SEC63*), pDF67 (*SEC71* in pBluescript), and pDF69 (*SEC72* in pBluescript) were gifts of David Feldheim and Randy Schekman (University of California, Berkeley, CA). pMR713 (*CEN1*, *LEU2*, *KAR2*) was a gift of Mark Rose (Princeton University, Princeton, NJ). pDN210 contains full-length *SEC63* as a 3.0 kb *StuI* (second upstream from the initiator ATG) to *HindIII* fragment cloned into pRS314 (*CEN6*, *TRP1*). pDN217 is similar to pDN210 except that the coding sequence of the *sec63-201* allele was subcloned as a *StuI* to *ClaI* insert. pDN259 is sim-

ilar to pDN210 except that the *MscI* to *ClaI* fragment is replaced with a PCR-amplified fragment of *SEC63* that results in the deletion of the COOH-terminal 27 amino acids (see below). pDN291 contains the green fluorescent protein (GFP)¹ gene (28) amplified by PCR and placed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and an actin terminator cloned into YCP50.

Anti-Kar2p antisera was generously provided by Mark Rose, anti-CPY antisera by Tom Stevens (University of Oregon, Eugene, OR), anti-Gas1p antisera by Howard Riezman (University of Basel, Basel, Switzerland), and affinity-purified polyclonal antiserum to Sec63p by Randy Schekman.

Genetic Selection

Details of the genetic selection for protein translocation mutants will be published elsewhere (Ng, D.T.W., J. Brown, and P. Walter, manuscript in preparation). In brief, the selection strategy depends on the expression of a reporter gene (*CU*) that is composed of *URA3* fused to the amino-proximal portion of carboxypeptidase Y (*PRC1*) containing the signal sequence. In cells deleted of *URA3*, expression of *CU* fails to rescue the uracil auxotrophy as the fusion protein is translocated into the lumen of the ER where it is inactive. Mutations (either *cis* or genomic) that cause mislocalization of the reporter to the cytosol confer uracil auxotrophy to the cell. The selection was carried out by UV mutagenesis of cells carrying the reporter gene on a plasmid, and mutants were selected on synthetic complete (SC) media lacking uracil. Mutants were characterized by complementation tests among individual isolates and with previously characterized translocation mutants. In addition, centromeric plasmids bearing previously cloned genes involved in translocation were used to screen mutants on the basis of complementation with cloned wild-type genes.

Cytoduction Assay

Defects in karyogamy were analyzed essentially as described by Rose (32). Cells of opposite mating types were grown to log phase, mixed, and placed on yeast extract/peptone/dextrose (YPD) agar. The cells were incubated at 30°C for 5 h and streaked onto YPD agar dishes. Using a micromanipulator, individual budded zygotes were picked based on their characteristic trilobed morphology and grown into colonies. The mating type of each colony was determined by crossing with tester strains. Diploids were identified by their inability to mate with tester strains and by their ability to sporulate.

Preparation of Zygotes for Fluorescence Microscopy

Cells were grown to midlog phase in selective media or in YPD, and 2 OD₆₀₀ U of each mating type were mixed and pelleted by centrifugation. Cell pellets were resuspended in 50 μ l YPD and applied to 0.8- μ m cellulose filters on YPD agar dishes. The cells were allowed to mate at 30°C for 3 h. After incubation, the cells were removed from the filters and fixed in PBS containing 5% formaldehyde for 1 h at 22°C. The fixed cells were then washed two times in Zymolyase digestion buffer (1.2 M sorbitol, 10 mM Tris, pH 7.5) and brought to a final volume of 200 μ l containing 20 μ g/ml Zymolyase 100T (Calbiochem-Novabiochem Corp., La Jolla, CA). For partial digestion of cell walls, incubation was carried out at 30°C for 30 min. *fus1*, *fus2* prezygotes were permeabilized by two freeze-thaw cycles since cell wall digestion disrupts the cell-cell junction. Permeabilized cells were then applied to 10-well glass slides treated with poly-L-lysine and allowed to adhere for 20 min on ice. The cells were then fixed for 5 min in ice-cold methanol, washed two times with PBS, replaced with PBS containing 1 μ g/ml 4',6-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO) and incubated for 10 min. The cells were again washed two times with PBS and mounted for microscopy.

Metabolic Labeling and Nonnative Immunoprecipitation

Cells grown logarithmically were harvested by centrifugation. The pellet containing 5 OD₆₀₀ U of cells was resuspended in 1 ml SC media lacking methionine. ³⁵S-labeling mix (containing 200 μ Ci [³⁵S]methionine [Amersham Corp., Arlington Heights, IL]) was added and mixed, and the cells were incubated at 30°C for 5 min. Labeling was terminated by immersion

1. Abbreviations used in this paper: CPY, carboxypeptidase Y; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; SC, synthetic complete medium; YPD, yeast extract/peptone/dextrose medium.

Table 1. Strains Used in This Study

Strain	Genotype	Source
YJL183	<i>MATa, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	J. Li (University of California, San Francisco, CA)
YJL184	<i>MATa, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	J. Li (University of California, San Francisco, CA)
DNY65	<i>MATa, sec63-201, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN106)	This study
DNY66	<i>MATa, sec63-201, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN106)	This study
DNY234	<i>MATa, sec63-201, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN210)	This study
DNY272	<i>MATa, sec63::HIS3, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN259)	This study
DNY273	<i>MATa, sec63::HIS3, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN259)	This study
DNY115	<i>MATa, sec61-101, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN106)	This study
DNY116	<i>MATa, sec61-101, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN106)	This study
DNY69	<i>MATa, sec62-101, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN106)	This study
DNY70	<i>MATa, sec62-101, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN106)	This study
RSY926	<i>MATa, sec71::LEU2, ura3-52, his3Δ200, leu2Δ1, trp1Δ63</i>	Feldheim et al., 1993
RSY1006	<i>MATa, sec72::HIS3, ura3-52, his3Δ200, leu2Δ1, trp1Δ63 lys2-801, ade2-101</i>	Feldheim and Schekman, 1994
DNY274	<i>MATa, sec71::LEU2, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY275	<i>MATa, sec71::LEU2, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY278	<i>MATa, sec72::HIS3, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY279	<i>MATa, sec72::HIS3, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY329	<i>MATa, sec63-201, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN291)	This study
DNY319	<i>MATa, sec71::LEU2, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN291)	This study
DNY320	<i>MATa, sec72::HIS3, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i> , (pDN291)	This study
IH2351	<i>MATa, fus1Δ1, fus2Δ3, ura3-52, trp1Δ1</i>	Ira Herskowitz (University of California, San Francisco, CA)
DNY368	<i>MATa, fus1Δ1, fus2Δ3, ura3-52, trp1Δ1</i> (pDN291)	This study
DNY369	<i>MATa, sec63-201, sos1-1, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY370	<i>MATa, sec63-201, sos1-1, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY371	<i>MATa, sos1-1, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study
DNY372	<i>MATa, sos1-1, ura3Δ99, his3Δ200, leu2Δ1, trp1Δ99, ade2-101^{ochre}</i>	This study

of the tube in liquid N₂. The cells were thawed in an ice-water bath and pelleted. To the pellet, 0.5 ml of cell lysis buffer (50 mM NH₄OAc, 20 mM Tris, pH 8.0, 2 mM EDTA), 0.5 ml of 20% TCA, and a 0.5 ml vol of zirconium beads (0.5 mm diameter). The cells were lysed in two 30-s cycles in a Mini Bead-beater cell disrupter (Biospec Products, Bartlesville, OK). The lysate was transferred to a microfuge tube and centrifuged for 10 min at full speed. The supernatant was discarded and replaced with 200 μl TCA resuspension buffer (3% SDS, 100 mM Tris base, 3 mM DTT). The resuspended pellet was boiled for 5 min and debris pelleted by centrifugation. A 40-μl aliquot of supernatant was combined with 560 μl of SDS dilution buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100). The appropriate antisera was added and incubated at 4°C for 2 h. Protein A Sepharose (Sigma Chemical Co.) was added and mixed by inversion for 30 min. The beads were washed three times in wash buffer (0.2% SDS, 300 mM NaCl, 1% Triton X-100, 20 mM Tris, pH 7.5) and once in PBS. A 20-μl vol of SDS sample buffer was added, boiled for 5 min, and analyzed by PAGE followed by autoradiography.

Coimmunoprecipitation of Sec63p Complex Proteins

Cells were grown to OD₆₀₀ of 0.8/ml in SC medium lacking methionine. Cells (30 OD₆₀₀ U) were harvested and resuspended in the same medium at a density of OD₆₀₀ of 1.0/ml. 500 μCi of ³⁵S-labeling mix was added, and the cells were incubated at 30°C for 30 min with aeration. Preparation of microsomal membranes and native immunoprecipitations were performed as described (9). Immunoprecipitation of Sec63p was performed using affinity-purified polyclonal antiserum.

Results

Isolation and Characterization of a sec63 Mutant Allele Defective in Karyogamy

We devised a genetic selection to identify yeast mutants defective in posttranslational protein translocation. The scheme is a variant of the approach devised by Schekman and co-workers (7), and details will be reported elsewhere (Ng, D.T.W., J. Brown, and P. Walter, manuscript in prep-

aration). In brief, a reporter gene was constructed that encodes the signal sequence of carboxypeptidase Y (CPY) fused to *URA3* (31, 39). The *URA3* gene product, the cytoplasmic enzyme orotidine-5'-phosphate decarboxylase, is required for the de novo synthesis of uracil. In cells deleted for *URA3*, the fusion protein is exported from the cytosol to the lumen of the ER where it is inactive. Mutations that impair the export of the fusion protein support the growth on medium lacking uracil because a portion of the fusion protein is retained in the cytosol where it is active.

Isolates obtained from this genetic selection were initially placed into four complementation groups based on rescue of the mutant phenotypes by centromeric plasmids carrying known genes involved in protein translocation. These included *SEC61*, *SEC62*, *SEC63*, and *KAR2* (7, 8, 34, 42). However, when complementation tests were carried out by mating haploid isolates with the intent to score the phenotypes of diploids, crosses among three individual isolates consistently failed to generate diploids. Instead, numerous zygotes generated from these crosses and isolated by micromanipulation grew to colonies consisting of haploid cells of a single mating type or, occasionally, a mixture of both mating types but no diploids. This behavior was reminiscent of the phenotype of a class of mutants unable to undergo karyogamy (5). Interestingly, all three mutants belong to the group that can be complemented by a plasmid carrying the *SEC63* gene. We were intrigued by this observation because Sec63p has been shown to interact functionally with Kar2p, which is required for the membrane fusion step in karyogamy (18, 20). To characterize this defect in greater detail, we focused on one allele, designated *sec63-201*.

sec63-201 mutant cells were backcrossed to wild-type cells to form the heterozygous diploid. The formation of

Table II. Mating Assays of Mutant Strains

Cross No.	Strains	Relevant genotypes (<i>MATa</i> × <i>MATα</i>)	Zygotes examined	Viable zygotes	<i>MATa/α</i>	<i>MATa</i>	<i>MATα</i>	Mixture*
1	YJL183 × YJL184	wt × wt	12	12	12	0	0	0
2	DNY65 × DNY66	<i>sec63-201</i> × <i>sec63-201</i>	165	83	0	58	19	6
3	DNY × DNY234	<i>sec63-201</i> × <i>sec63-201</i> (PDN210)	24	20	20	0	0	0
4	DNY272 × DNY273	<i>sec63::HIS3</i> (PDN259) × <i>sec63::HIS3</i> (PDN259)	24	17	3	3		
5	DNY115 × DNY116	<i>sec61-101</i> × <i>sec61-101</i>	26	23	21	1	1	0
6	DNY69 × DNY70	<i>sec62-101</i> × <i>sec62-101</i>	12	11	9	1	1	0
7	DNY274 × DNY275	<i>sec71::LEU2</i> × <i>sec71::LEU2</i>	55	51	3	16	25	7
8	DNY278 × DNY279	<i>sec72::HIS3</i> × <i>sec72::HIS3</i>	50	47	14	15	10	8
9	DNY274 × YJL184	<i>sec72::HIS3</i> × wt	20	20	20	0	0	0
10	DNY278 × YJL184	<i>sec72::HIS3</i> × wt	20	20	19	0	1	0
11	DNY369 × DNY370	<i>sec63-201-201</i> , <i>sos1-1</i> × <i>sec63-201</i> , <i>sos1-1</i>	70	68	11	16		
12	DNY371 × DNY372	<i>sos1-1</i> × <i>sos1-1</i>	26	26	26	0	0	0

Matings were performed as described in *Materials and Methods*. Wild-type strains are YJL183 (*MATa*) and YJL184 (*MATα*). Mutant strains are congenic with designated wild-type strains except for mutant alleles and/or plasmids contained (see Table I and Fig. 1 for the relevant genotypes).
*Colonies grown from single zygotes containing both *MATa* and *MATα* cells.

diploid cells was efficient in this cross, indicating that a mutation in *SEC63* must be present in both parent cells (i.e., needs to be “bilaterally” expressed) to elicit the karyogamy defect. Mutant and wild-type phenotypes segregated 2:2 in 24 tetrads analyzed, suggesting that a single mutant gene was responsible for the observed phenotype. We then crossed *sec63-201 MATa* cells with *sec63-201 MATα* cells and isolated individual budded zygotes by micromanipulation based on their characteristic trilobed morphology (zygotes budding medially were analyzed in detail; laterally budded zygotes were also examined and gave identical results). Analysis of 165 zygotes revealed that mating resulted in lethality in ~50% of the zygotes examined (Table II, cross 2) whereas, as expected, matings

between control wild-type cells resulted in good viability (Table II, cross 1) (5). The poor viability of the *sec63* zygotes likely results from a defect in diploid formation because vegetatively growing *sec63-201* haploid cells show no defects in viability. Colonies grown from viable zygotes were tested for mating type using tester strains. Of 83 zygotes that grew into colonies, none tested as diploid; cells picked from each colony tested positive against mating type tester strains, and none gave rise to spores under sporulation conditions. The colonies instead consisted of pure populations of *MATa* cells or *MATα* cells, or of a mixture of both (Table II and Fig. 1, cross 2; see Table II for a display of the measured data and Fig. 1 for a mating index, calculated as percentage of diploids formed of via-

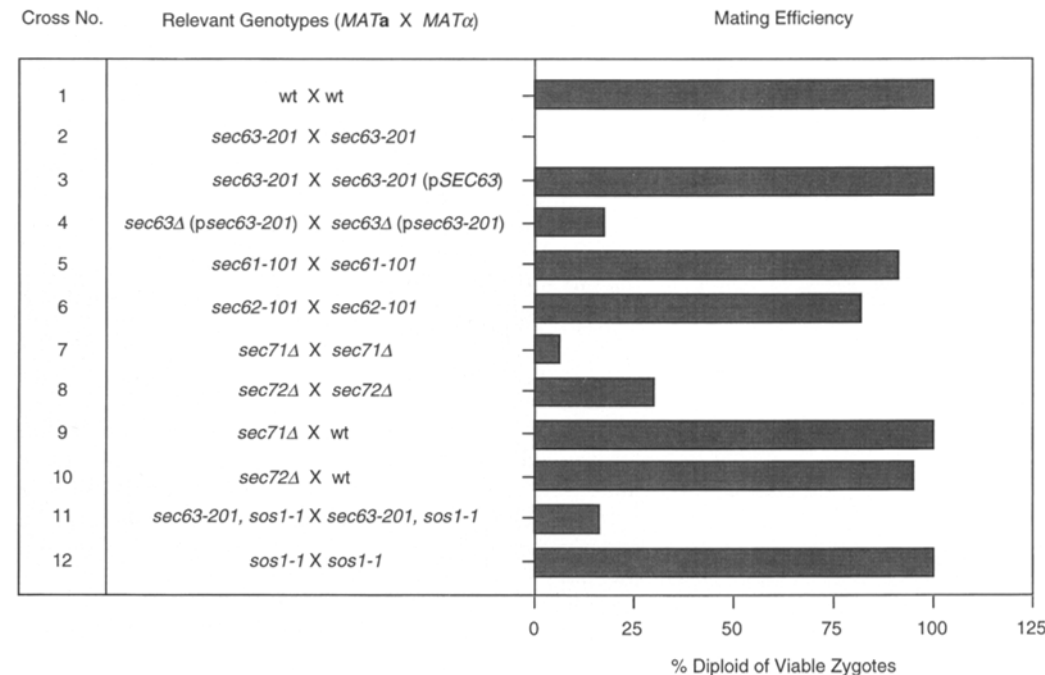


Figure 1. Frequency of diploid formation of crosses between mutant strains. The efficiency of diploid formation from crosses of wild-type and mutant strains presented in Table II. % Diploid, the fraction of viable zygotes from a given cross that resulted in diploid colonies.

ble zygotes analyzed). If the *sec63-201* mutation in one mating partner was complemented by wild-type Sec63p expressed from a plasmid, mating efficiency approached 100% (Table II and Fig. 1, cross 3), as was observed in crosses between wild-type haploid cells (Table II and Fig. 1, cross 1).

Two known classes of mutants could explain our observations: *fus* and *kar*. Both classes cause mating deficiencies, but the mutations affect different stages of diploid formation. *fus* mutants are blocked in zygote formation after agglutination but before plasma membrane fusion, whereas *kar* mutants are blocked at steps downstream of plasma membrane fusion (5, 41). *fus* mutants can be distinguished from *kar* mutants because the cytoplasm of the mating haploid cells fail to mix. We devised a visual assay to differentiate the two possibilities. This assay takes advantage of the properties of the GFP, a soluble protein that emits green light when exposed to near UV light (4). After expressing GFP as a marker in the cytosol of only one mating type, we monitored cytosol mixing by direct fluorescence microscopy. To this end, GFP was expressed constitutively in wild-type, MAT α ; in *fus1*, *fus2*, MAT α ; and in *sec63-201* MAT α cells (see *Materials and Methods*). When GFP-labeled wild-type MAT α cells mated to unlabeled wild-type MAT α cells, the resulting zygotes showed an even distribution of GFP throughout their cytosol as expected when cell fusion is complete, and therefore, no barriers exist to prevent GFP diffusion (Fig. 2 C). In contrast, when a similar cross was performed with cells bearing mutations in *FUS* genes, GFP failed to diffuse from the parent cell (Fig. 2 O). Examination of zygotes derived from mating *sec63-201* MAT α and *sec63-201* MAT α mutant cells showed an even distribution of GFP throughout the cytosol of the zygote (Fig. 2 F). Thus, we conclude that during mating, the plasma membranes of the *sec63-201* mutant cells fuse. The defect in these mutant cells is therefore a *kar*-type mutation.

To determine unequivocally that the mutant gene responsible for the karyogamy defect is indeed *sec63*, we cloned the *sec63-201* allele from genomic DNA of *sec63-201* mutant cells by PCR using the high fidelity Vent polymerase (New England Biolabs, Inc., Beverly, MA). A DNA restriction fragment containing the *sec63-201* coding sequences was used to replace the corresponding wild-type sequences in plasmid pDN210 (see *Materials and Methods*). The resulting plasmid, pDN217, was transformed into a *sec63::HIS3/SEC63* diploid strain that was sporulated to generate a haploid *sec63::HIS3* strain complemented by the plasmid-born *sec63-201* allele. The resulting strain displayed growth, protein translocation, and karyogamy phenotypes similar to the original *sec63-201* mutant strain, suggesting that the mutation was contained within the cloned sequence. Sequence analysis of cloned *sec63-201* revealed a single point mutation that introduces a stop codon in *SEC63*, resulting in the truncation of the COOH-terminal 27 amino acids (see Fig. 6 A). This truncation eliminates a large portion of the highly acidic cytoplasmic domain of Sec63p (11).

To confirm that truncation of Sec63p is responsible for the observed karyogamy defect, we generated a 27-amino acid truncated allele of *SEC63* by PCR amplification using wild-type *SEC63* as template. The COOH-terminal por-

tion of *SEC63* in pDN210 was replaced with the corresponding sequences from the amplified DNA to create the plasmid pDN259. As with pDN217, pDN259 was used to complement the lethality of a *sec63::HIS3* null allele. In a self-cross of this strain, a severe karyogamy defect was observed, thereby confirming that the karyogamy defect of *sec63-201* is caused by a COOH-terminal truncation of Sec63p (Table II and Fig. 1, cross 4). The severity of the phenotype was slightly less dramatic than that of the original mutant. From 17 viable zygotes, we obtained three diploid colonies. This increased leakiness in the karyogamy-defective phenotype may be due to limited suppression caused by an increased expression of the mutant allele because yeast centromeric plasmids are usually present in several copies per cell.

sec61 and sec62 Mutants Display Similar Protein Translocation Defects but Are Karyogamy Proficient

Because the *sec63-201* mutant was selected on the basis of defective protein translocation, we considered the possibility that the karyogamy defect could result indirectly as a consequence of this defect. No karyogamy defects were observed, however, in cells bearing alleles of previously isolated translocation mutants *sec61*, *sec62*, and *sec63* (42). Similarly, a new mutant allele of *SEC61* (*sec61-101*) and a new allele of *SEC62* (*sec62-101*) that were isolated in this selection as translocation-defective mutants showed no apparent karyogamy defects in self-crosses (Table II and Fig. 1, crosses 5 and 6). Three additional new mutant alleles of *SEC62* behaved indistinguishable from the *sec62-101* allele shown (not shown).

To ask directly whether the severity of translocation defects in these strains correlates with the differences in mating competence, we monitored the translocation efficiency of endogenous proteins. To this end, cells were pulse labeled with ³⁵S-labeled amino acids. Cell lysates were immunoprecipitated with specific antisera and then analyzed by gel electrophoresis followed by autoradiography. Translocation of CPY and Gas1p can be monitored by gel mobility shifts caused by acquisition of NH₂-linked glycosylation upon reaching the ER lumen, and translocation of Kar2p can be monitored by signal sequence cleavage (16, 24). As shown in Fig. 3, the degrees of translocation defects for CPY, Kar2p, and Gas1p were similar in *sec63-201*, *sec61-101*, and *sec62-102* cells (Fig. 3, compare lanes 2, 5, and 6). In addition, examination of five additional substrates (dipeptidyl amino peptidase B, Och1p, Pho8p, prepro- α -factor, and protein disulfide isomerase) showed that *sec63-201* and *sec62-101* cells have indistinguishable translocation defects for each protein (Ng, D.T.W., J. Brown, and P. Walter, manuscript in preparation).

The results show that the observed protein translocation defects do not correlate with the observed karyogamy defect, thereby suggesting that the karyogamy defect does not result indirectly from defects in protein translocation.

sec71 and sec72 Mutants Also Show Karyogamy Defects

We next tested strains carrying mutant alleles of the *SEC71* and *SEC72* genes (10, 12, 14), because their respective wild-type gene products, like Sec62p, can be found in a stable complex with Sec63p (9, 12). In contrast to *SEC61*,

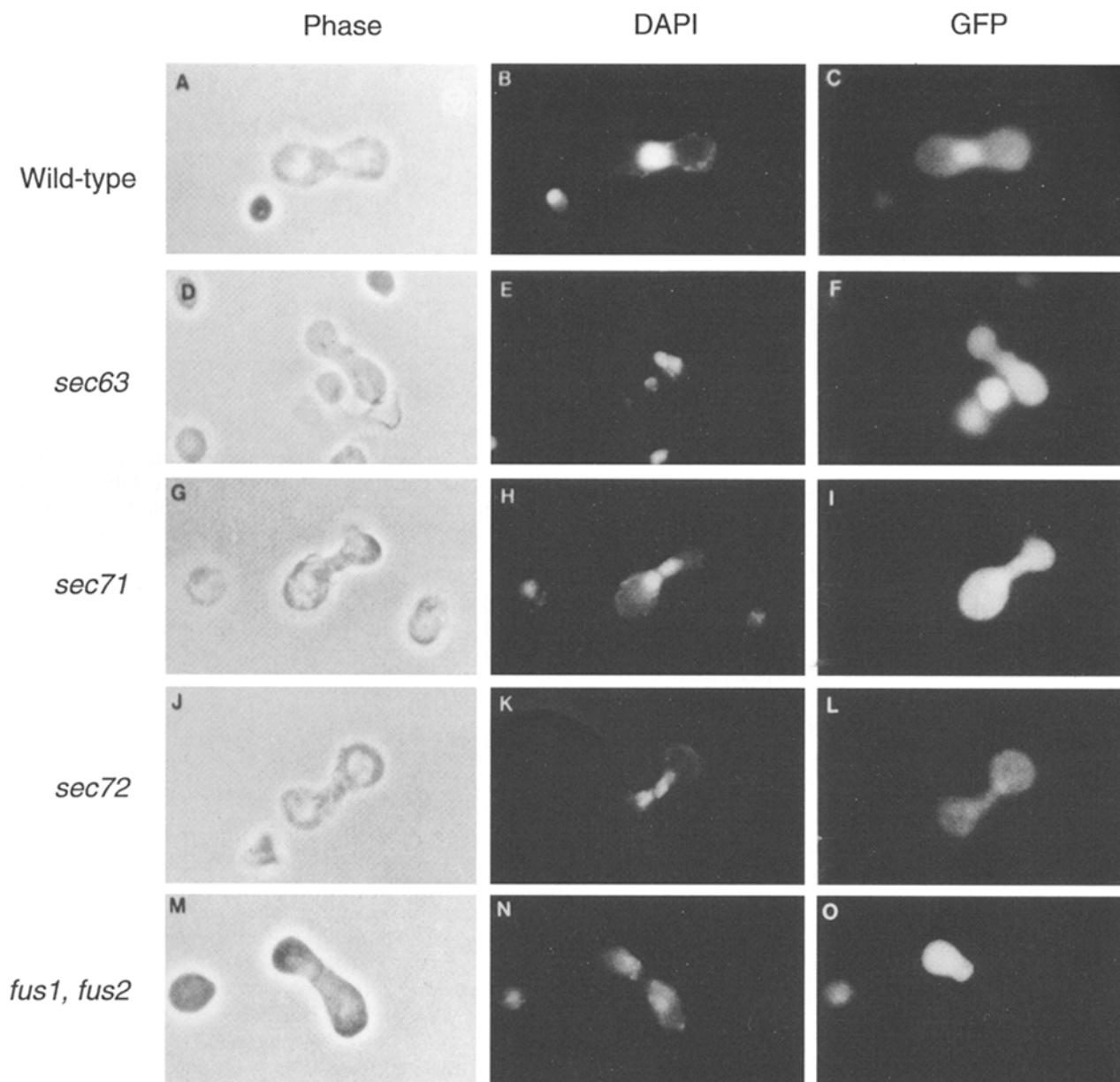


Figure 2. Microscopy of karyogamy mutant zygotes. Cells of opposite mating type grown separately to logarithmic phase were collected, mixed, and applied to filter membranes placed on YPD agar. The cells were incubated at 30°C for 3 h and harvested. The cells were then fixed in formaldehyde and stained with DAPI to visualize nuclei. (A–C) A zygote from the wild-type cross YJL183 × YJL184 (pDN106); (C–E) *sec63*, DNY65 × DNY329; (G–I) *sec71Δ*, DNY274 × DNY319; (J–L) *sec72Δ*, DNY278 × DNY320. The GFP expression vector pDN291 was carried by the MAT α cells in each cross. Micrographs were taken under phase-contrast, DAPI, and GFP (fluorescein channel) optics as indicated.

SEC62, and *SEC63*, however, *SEC71* and *SEC72* are not essential for cell viability, so that their null alleles could be examined. Interestingly, strains deleted for the *SEC71* or *SEC72* genes also showed significant defects in diploid formation. The *sec71Δ* mutant was the more severe, yielding only three diploids from 51 viable zygotes derived from bilateral crosses (Table II and Fig. 1, cross 7). The *sec72Δ* mutant yielded 14 diploids from 47 viable zygotes (Table II and Fig. 1, cross 8).

Using GFP as a cytoplasmic marker, we determined that, as for *sec63-201* cells, the defect in diploid formation of *sec71Δ* and *sec72Δ* cells is at the level of karyogamy because the GFP expressed in one parent freely diffused

throughout the zygotes isolated after mating (Fig. 2, *I* and *L*). Furthermore, as seen with the *sec63-201* strain, the *sec71Δ* and *sec72Δ* strains showed bilateral karyogamy phenotypes: crosses with wild-type cells yielded diploids efficiently (Table II and Fig. 1, crosses 9 and 10). As shown in Fig. 3, *sec71Δ* cells displayed translocation defects for CPY, Kar2p, and Gas1p. Translocation in *sec72Δ* cells was only mildly affected, showing only a modest defect for CPY and Gas1p and no defect for Kar2p (Fig. 3, lane 4). Note, however, that for each of the three substrate proteins analyzed, the magnitudes of translocation defects in *sec71Δ* and *sec72Δ* cells, which are karyogamy defective, were less severe than those observed in *sec61-101* and

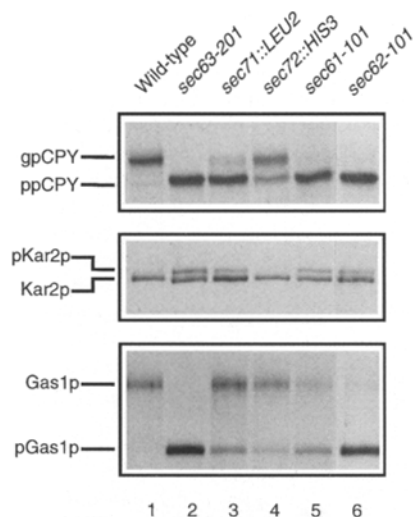


Figure 3. Translocation defects of karyogamy mutants. Wild-type (YJL184), *sec63-201* (DNY66), *sec71Δ* (DNY275), *sec72Δ* (DNY279), *sec61-101* (DNY116), and *sec62-101* (DNY70) cells were labeled metabolically with ^{35}S -amino acids for 5 min, and lysates were prepared for immunoprecipitation with antiserum specific for CPY and Kar2p. Immunoprecipitated proteins were analyzed by gel electrophoresis and autoradiography. Positions of translocated forms of CPY and Kar2p, gpCPY (glycosylated pro-CPY), Kar2p (mature, signal peptide-cleaved Kar2p), and Gas1p. Positions of untranslocated proteins, ppCPY (preproCPY), pKar2p (preKar2p), and pGas1p (preGas1p).

sec62-101 cells, which were mating competent (Fig. 3, compare lanes 3 and 4 with lanes 5 and 6). These results provide further support for the notion that the observed karyogamy defects do not result from defects in protein translocation.

Suppression of *sec63-201* Growth and Translocation Defects but Not Karyogamy Defect

As a first step to dissect the roles of *SEC63* in protein translocation and nuclear membrane fusion, we sought to isolate extragenic suppressors that alleviate defects caused by the *sec63-201* allele. *sec63-201* cells grow at a reduced rate as compared with wild-type cells. We exploited this phenotype to isolate extragenic suppressor mutants in the *sec63-201* background. When *sec63-201* cells were grown nonselectively (i.e., on media containing uracil), faster growing colonies appeared spontaneously. Some colonies, however, still grew at slower rates than wild-type cells, suggesting that they were due to suppressors rather than due to reversion of the *sec63-201* mutation. Cells derived from one such colony that exhibited faster growth than *sec63-201* cells were examined further.

To show that we had isolated a suppressor mutation that is unlinked to the *SEC63* locus, the strain was backcrossed to wild-type cells. The resulting diploids were sporulated. 70 tetrads that gave rise to four viable spores were scored by colony size as either parental ditype (wild-type [two spores], *sec63-201*, suppressor mutant [two spores]; 13 occurrences), nonparental ditype (*sec63-201* [two spores], suppressor mutant [two spores]; 12 occurrences), and tetratype (wild-type [one spore], *sec63-201* [one spore], suppressor mutant [one spore], *sec63-201*, suppressor mutant

[one spore]; 45 occurrences). The results of the tetrad analysis are consistent with the ratio of 1:1:4 for parental ditype:nonparental ditype:tetratype, indicating that the suppression results from a single mutation that is unlinked to the *sec63-201* locus (37a). We designate the suppressor allele as *sos1-1* for suppressor of *sec63-201*.

We next determined the karyogamy phenotype of *sec63-201* in the presence of the *sos1-1* mutation. *sec63-201*, *sos1-1* cells were mated in a self-cross. Zygotes were isolated, grown into colonies, and tested for mating type. Of 68 viable zygotes, 57 failed to generate diploids but instead gave rise to colonies containing pure populations of MATa, MATα, or a mixture of both (Fig. 1 and Table II, cross 11). These results show that in the presence of *sos1-1*, the karyogamy phenotype of *sec63-201* remains severe, although it is somewhat less restrictive than that of *sec63-201* cells. In contrast, in self-crosses performed with *sos1-1* cells, 26 of 26 zygotes isolated formed diploids, showing that the *sos1-1* mutation alone does not exhibit a karyogamy-defective phenotype (Fig. 1 and Table II, cross 12).

Next, we examined the translocation phenotype of the *sec63-201*, *sos1-1* strain using the immunoprecipitation assay described above. As shown in Fig. 4, *sec63-201*, *sos1-1* cells show significantly reduced translocation defects for CPY, Kar2p, and Gas1p (Fig. 4, lane 2) when compared with *sec63-201* cells (Fig. 3, lane 2). Most importantly, the translocation defects in the karyogamy-defective *sec63-201*, *sos1-1* cells are less severe than those found for the karyogamy-proficient *sec62-101* (Fig. 4, compare lanes 2 and 3; Fig. 1, compare crosses 11 and 6). Thus, the analyses of the *sos1-1* suppressor strains provide a third line of evidence that the *sec63* karyogamy defect is not a consequence of its defects in protein translocation.

Karyogamy Is Blocked at the Level of Nuclear Membrane Fusion

Karyogamy can be divided into the two stages of nuclear congression and nuclear membrane fusion. Mutants in the fusion step produce zygotes that localize unfused nuclei in close proximity at the former cell-cell boundary, whereas nuclei in congression mutants remain well separated (18). We examined the positions of the nuclei in zygotes produced in bilateral crosses of *sec63-201*, *sec71Δ*, and *sec72Δ* mutant strains. Zygotes were stained with the DNA-inter-

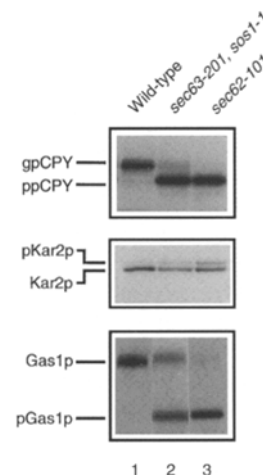


Figure 4. Partial suppression of the *sec63-201* translocation phenotype by *sos1-1*. Wild-type, *sec63-201*, *sos1-1*, and *sec62-101* cells were pulse labeled, and proteins were immunoprecipitated as in Fig. 3. Translocated forms, gpCPY (glycosylated pro-CPY), Kar2p, and Gas1p. Untranslocated precursor forms, ppCPY (prepro-CPY), pKar2p (preKar2p), and pGas1p (preGas1p).

calating dye DAPI to visualize the nuclei, and the cytosol of the *MATa* cells was marked with GFP as described above. In *sec63-201*, *sec71Δ*, and *sec72Δ* bilateral crosses, all analyzed zygotes contained two closely juxtaposed but clearly separate and unfused nuclei (Fig. 2, *E*, *H*, and *K*). In contrast, zygotes derived from crosses of control wild-type cells contained a single, fused nucleus (Fig. 2 *B*). Thus, we conclude that the analyzed mutant alleles of *SEC63*, *SEC71*, and *SEC72* do not affect nuclear congression but, as *KAR2* mutants, block the nuclear fusion step.

Stability of the Sec63p-associated Proteins

Our results show that in addition to *SEC63*, both *SEC71* and *SEC72* are also important for karyogamy. The products of these two genes form a membrane protein complex with Sec63p (10). It seemed possible that the truncation of the Sec63p carboxyl-terminal domain in the *sec63-201* strain may lead to the disruption of this complex, which in turn may destabilize the individual proteins. We examined the composition of the complex directly after immunoprecipitation using monospecific antibodies against Sec63p (9). To this end, wild-type and *sec63-201* cells were metabolically labeled with ³⁵S-labeled amino acids. A microsomal membrane fraction was prepared from the labeled cells and solubilized in 1% Triton X-100. Affinity-purified anti-Sec63p antibodies were added to the extract under non-denaturing conditions. Sec63p complexes were immunoprecipitated and examined by SDS-PAGE and fluorography. As shown in Fig. 5, Sec63-201p is stably expressed in the mutant cells: Sec63p and mutant Sec63-201p were recovered in similar amounts from wild-type and *sec63-201* mutant cells, respectively (Fig. 5, lanes 1 and 2). Sec63-201p shows an increase in electrophoretic mobility consistent with the loss of 27 amino acids that is predicted from nucleotide sequencing of the mutant allele. In addition, the characteristic pattern of Sec62p, Sec71p, and Sec72p were coprecipitated from wild-type cell extracts as a complex with Sec63p, as previously shown (9, 12). Similar amounts of radiolabeled Sec71p and Sec72p were associated with mutant Sec63-201p (Fig. 5, lane 2), suggesting that a gross disruption or degradation of this complex is not responsible for the karyogamy phenotype. In contrast, the amount of a coprecipitated protein migrating at the position of Sec62p was reduced in the *sec63-201* strain.

Discussion

We have identified three genes, *SEC63*, *SEC71*, and *SEC72* whose functions are required for karyogamy. Mutant zygotes from bilateral crosses show a dinucleate morphology consistent with a defect at the level of nuclear membrane fusion. In an elegant genetic screen recently devised by Rose and colleagues to isolate bilateral karyogamy mutants, three of five new complementation groups were found to affect the nuclear fusion step (18). It is possible that the genes we have characterized may be represented among the mutants obtained by this screen. However, as the screen has not yet been exhausted, additional genes will likely be identified through the screen to aid in elucidating the mechanisms of homotypic membrane fusion during karyogamy.

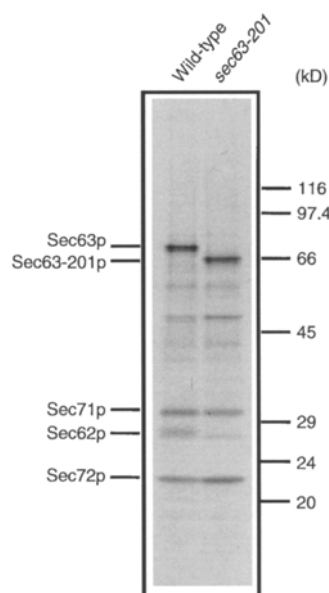


Figure 5. Stability of Sec63p-associated protein complex. Wild-type and *sec63-201* cells were labeled with [³⁵S]methionine for 30 min, homogenized, and subjected to cell fractionation. Crude membrane fractions were solubilized in a buffer containing 1% Triton X-100, and the detergent extracts were used for immunoprecipitation with affinity-purified anti-Sec63p antibodies and protein A Sepharose. Immunoprecipitated protein complexes were separated by electrophoresis using 10–15% PAGE and visualized by autoradiography. The positions of Sec63p-associated proteins are indicated. Assignments are made based on the reproducible banding pattern described in previous publications (7, 12).

Sec63p, Sec71p, and Sec72p form a multisubunit complex with Sec61p, Sec62p, and Kar2p. This complex has a well-established role in mediating protein translocation across the ER membrane (3). Protein translocation across the ER membrane is thought to occur through a protein tunnel composed of the Sec61 complex, consisting of Sec61p and two associated membrane subunits, Sbh1p and Sss1p. Sec63p also interacts with additional membrane proteins, Sec62p, Sec71p, and Sec72p. Together with Kar2p, the Sec62/63/71/72p complex interacts with the Sec61 complex. This interaction is thought to specialize the translocation tunnel formed by the Sec61 complex to receive and to translocate a specific subset of proteins that cross the ER membrane posttranslationally. (26, and Ng, D.T.W., J. Brown, and P. Walter, manuscript in preparation). The possibility therefore arises that the observed karyogamy defects result indirectly from defects in translocation or membrane integration of some protein(s) that, in turn, is required directly for karyogamy. We consider this possibility unlikely, however, in light of our data that show that no correlation between translocation and karyogamy phenotypes was found. In particular, mating-competent *sec61* and *sec62* cells (*a*) displayed similar translocation defects to those observed in karyogamy-defective *sec63-201* cells, (*b*) displayed more severe translocation defects than those observed in karyogamy-defective *sec71Δ* and *sec72Δ* cells, and (*c*) displayed more severe translocation defects than those observed in karyogamy-defective *sec63-201* cells in the presence of the *sos1-1* suppressor mutation. These arguments are particularly compelling if one considers that all substrate proteins analyzed to date show an indistinguishable dependence for their translocation on Sec62p and Sec63p. Thus taken together, these results suggest a direct role for Sec63p, Sec71p, and Sec72p in nuclear membrane fusion and argue against the alternative interpretation that the karyogamy defects result as an indirect

consequence of the impaired membrane integration or translocation of another component(s) required for the process. Formally, we cannot exclude the possibility that translocation or membrane integration of yet unidentified protein(s) is more severely impaired in the karyogamy defective strains. In the absence of evidence for such a hypothetical protein, however, a direct role for the Sec63/71/72p complex in nuclear membrane fusion is presently the simplest plausible explanation for the presented results.

Our model suggesting a direct involvement of the Sec63/71/72p complex in karyogamy is strengthened further by recent evidence that Kar2p, which also binds directly to Sec63p, plays a direct role in membrane fusion. Membranes derived from temperature-sensitive *kar2* mutants are proficient for fusion at the permissive temperature but are defective at the nonpermissive temperature in *in vitro* membrane fusion assays (20). Since there was no additional protein synthesis required in this assay system, the defect cannot result from an inability of mutant Kar2p to translocate or to assemble other factors required for fusion. These data therefore point to Kar2p as playing an integral role in the membrane fusion step. Furthermore, ER vesicles derived from strains bearing deletions of the *SEC71* and *SEC72* genes display temperature-dependent fusion defects *in vitro* (M. Latterich, personal communication). Corresponding studies for *SEC63*, because it is an essential gene, will have to await the isolation of suitable conditional alleles.

Although Sec61p, Sec62p, Sec63p, Sec71p, Sec72p, and Kar2p can form a complex, karyogamy functions would not necessarily require all these components. In cross-linking and coimmunoprecipitation experiments, Sec61p appears as a more loosely associated component of the complex (9). In addition, Sec62p is present in approximately fivefold lower abundance than Sec63p (9), such that the excess Sec63p could form a separate subcomplex with Sec71p and Sec72p (whose abundance is yet unknown) and Kar2p (which is present in great excess). It is possible that a Sec63p subcomplex lacking Sec61p and Sec62p functions to facilitate nuclear membrane fusion. We cannot rule out the alternative possibility, however, that Sec61p and/or Sec62p also participate in karyogamy, but that karyogamy-defective alleles of these genes were not isolated in our screen.

The involvement of ER proteins in nuclear membrane fusion is not surprising given the continuity of the nuclear envelope with the ER membrane. Moreover, the involvement of ER membrane proteins is an appealing result because it provides a topological link between Kar2p, which resides as a soluble protein in the ER lumen (and which was originally identified because of its role in nuclear membrane fusion) and the cytosolic face of the ER membrane. After congression of the two nuclei, membrane fusion has to initiate from the cytosolic side of the ER or outer nuclear membrane (Fig. 6 B, Step I). That step must be followed by a second fusion event (Fig. 6 B, Step II) that initiates from the periplasmic side of the inner nuclear membrane, thereby allowing the two nuclear contents to intermix. Because the membranes of the two juxtaposed nuclei are not fused in the zygotes derived from our mutant strains (see Fig. 2) or from *kar2* mutant strains (18), we consider it likely that nuclear fusion in all of these mu-

nants is blocked at Step I. Membrane fusion activity that may be related to these events has recently been reconstituted *in vitro* between ER-derived vesicles and shown to depend on functions required for nuclear membrane fusion *in vivo* (18, 20). These studies may provide a powerful tool for a functional biochemical dissection of the process. In such assays, membranes treated with protease were fusion defective, arguing for an involvement of membrane-associated proteins exposed to the cytosol. Sec63p, Sec71p, and Sec72p have all or large portions of their polypeptides exposed to the cytoplasmic face of the ER, and their proteolysis could account, at least in part, for the observed loss in fusion activity.

Membrane fusion in a variety of other systems also requires the functions of integral membrane proteins. The most extensively studied examples are the fusion proteins of enveloped viruses (43). Fusion of the viral membrane to the host can be divided into two steps. The first step requires an interaction between a viral receptor-binding protein and a cellular membrane protein (or the carbohydrate moiety thereof) to bring the two membranes into close proximity. The membranes are fused in the second step mediated by a viral fusion protein; the fusion activity may be a second function of the receptor-binding protein or reside in another viral protein. Viral fusion proteins generally contain a hydrophobic domain that partitions into the target membrane. The protein-lipid interaction is thought to mediate fusion by providing a path for the flow of lipids at the fusion site. A cellular counterpart of viral fusion proteins is the PH-30 complex that mediates sperm-egg fusion during fertilization (2). Intracellular membrane fusion, which is an essential step in all vesicular trafficking events in the secretory pathway, is more complex. A number of soluble and membrane components have been identified, including those that are required to provide specificity for the docking of the correct membrane surfaces and those that are required to catalyze lipid bilayer fusion (19). The molecular mechanism that leads to lipid bilayer fusion is still unknown, however.

The Sec63p/Sec71p/Sec72p/Kar2p complex could function either to provide specificity in membrane recognition and/or to catalyze lipid bilayer fusion. At present, we cannot predict any specific roles for Sec63p, Sec71p, Sec72p, or Kar2p: there are no sequences in their cytoplasmic domains that resemble viral fusion peptides, nor is there any significant sequence homology to any of the proteins implicated in intracellular membrane fusion. The *sec63-201* mutation truncates a significant portion of the acidic COOH-terminal domain of Sec63p. On the basis of this unusual structural feature, it was previously speculated that this domain may be involved in electrostatic protein-protein interactions (11). It is also possible that the acidic tail plays a role in Ca^{++} binding that may be required for nuclear membrane fusion. It has been suggested that a local release of Ca^{++} ions from the ER is required for the membrane fusion events that lead to the reassembly of nuclear envelopes after mitosis in higher eukaryotic cells (40).

Domains of Sec63p along with Sec71p and Sec72p may interact with their counterparts or with other proteins in the opposing membrane during nuclear fusion to mediate homotypic membrane recognition, lipid bilayer fusion, or both. In the *sec63-201* mutant strains, Sec63-201p remains

A

Sec63p --SEEDDEYSTDDDETSDDESADSYTDIDTDTEAEDDESPE-COOH
 Sec63-201p --SEEDDEYSTDDDET-COOH

B

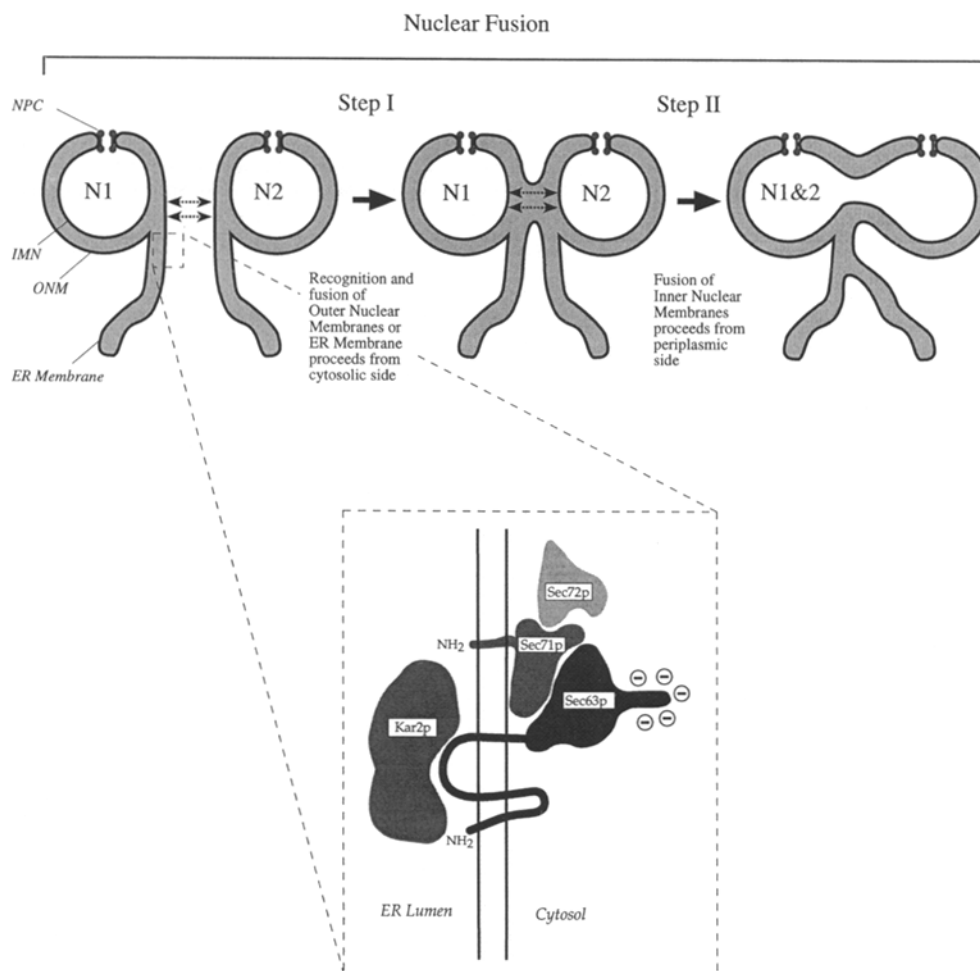


Figure 6. Model for the involvement of Sec63p in nuclear membrane fusion. (A) Deduced carboxyl-terminal sequences of wild-type Sec63p and mutant Sec63-201p. (B) The predicted steps in nuclear membrane fusion, the predicted membrane topology, and interactions of polypeptides implicated in nuclear membrane fusion are depicted schematically. The Sec63p/Sec71p/Sec72p/Kar2p complex is suggested to function in mediating the homotypic interaction of the two juxtaposed outer nuclear/ER membranes, possibly recruiting other components that then mediate the lipid bilayer fusion event. The acidic carboxyl terminus of Sec63p (minus signs) is essential for this function.

stably expressed and assembled with its Sec71p and Sec72p subunits. Interestingly, however, the interaction with Sec62p appears to be impaired. The observed defect in this interaction could, in principle, be the cause for the observed defects in protein translocation and/or karyogamy. If an interaction of the Sec63p/Sec71p/Sec72p/Kar2p complex with Sec62p is required for karyogamy, it should be possible to isolate karyogamy mutants in *SEC62*.

Recently, using an *in vitro* assay, Latterich and Schekman have shown that the product of a cell cycle-related gene Cdc48p is required for ER membrane fusion (21). In independent studies, the mammalian Cdc48p homologue, p97, has also been found to be required for the reassembly of Golgi membranes (1, 30). These results suggest that

Cdc48p may function as a general factor for intracellular homotypic membrane fusion. During nuclear/ER membrane fusion during karyogamy, the Sec63p complex may provide both specificity for homotypic membrane recognition and a nucleation site for the assembly of a fusion complex containing Cdc48p and/or other cytosolic components. This scenario is similar to the paradigm put forth by Rothman and co-workers describing membrane fusion in vesicular transport (19). The Sec63p complex would be analogous to SNARE proteins found on membranes of transport vesicle and their targets. The interaction of SNAREs brings prefusion membranes into close proximity and mediates the assembly of a fusion machine.

The results presented here demonstrate a function of

Sec63p in nuclear membrane fusion. This is the third distinct function that has been attributed to this protein. The other two functions of Sec63p are ER protein translocation and nuclear import. A number of the alleles defective in nuclear import show only minor defects in ER protein translocation (35). Unlike the cases of ER protein translocation and karyogamy, the associated Sec71p and Sec72p proteins are not required for the nuclear import function of Sec63p, indicating that these two functions can also be uncoupled from one another (19). Presently, it is not known how Sec63p carries out each seemingly disparate function mechanistically. Extragenic suppressors have been isolated, for example, that suppress *sec63-101*, an allele that primarily affects nuclear localization (22), or that partially suppress the translocation defect of *sec63-201* (*sos1-1*, this study). Thus, clues about the different functions of SEC63 may be forthcoming from analyses that characterize interacting genes associated with each function.

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