Loss of BiP/GRP78 Function Blocks Translocation of Secretory Proteins in Yeast

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Abstract. BiP/GRP78 is an essential member of the HSP70 family that resides in the lumen of the endoplasmic reticulum. In yeast, BiP/GRP78 is encoded by the *KAR2* gene. A temperature sensitive mutation was isolated in *KAR2* and found to cause a rapid block in protein secretion. Secretory precursors of a number of proteins (invertase, carboxypeptidase Y, α-factor, and BiP) accumulated that were characteristic of a block in translocation into the lumen of the ER. Protease protection experiments confirmed that the precursors accumulated on the cytoplasmic side of the ER membrane. Moreover, depletion of wild-type KAR2 protein also resulted in a block in translocation of secretory proteins. These results implicate BiP/GRP78 function in the continued translocation of proteins into the lumen of the ER.

BIP/GRP78 is a member of the heat shock protein (HSP70) family that is localized to the lumen of the ER (Munro and Pelham, 1986). BiP was first identified in mammalian cells as a result of its binding to proteins that accumulate within the lumen of the ER (Haas and Wabl, 1983). Binding of BiP/GRP78 to proteins can be detected under a variety of conditions that result in secretory proteins that are misfolded (Sharma et al., 1985; Copeland et al., 1986; Gething et al., 1986), aberrantly glycosylated (Dorner et al., 1987; Kassenbrock et al., 1988), or unassembled (Bole et al., 1986; Gething et al., 1986; Hendershot et al., 1987). Moreover, the conditions that lead to protein accumulation in the ER lead to higher levels of BiP/GRP78 (Lee, 1987; Subjeck and Shyy, 1987; Kozutsumi et al., 1988). The complexes of BiP/GRP78 and misfolded proteins can be dissociated in vitro by addition of ATP (Munro and Pelham, 1986). Recently, Flynn et al. (1989) have demonstrated a peptide dependent ATPase activity associated with BiP. These observations have led various investigators to propose that BiP/GRP78 monitors nascent secretory proteins, binds those that fail to achieve an acceptable conformation and thereby causes them to be retained in the ER (Gething et al., 1986; Pelham, 1986; Hurtley et al., 1989). On the other hand, BiP/GRP78 has also been observed to bind transiently to assembly intermediates of several secreted proteins (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1986). These results have led to the alternate suggestion that BiP/GRP78 might play an active role in the folding and assembly of nascent secretory proteins and remain bound only to those mutant variants that fail to attain a mature conformation (Bole et al., 1986; Gething et al., 1986; Pelham, 1986).

The various models for BiP/GRP78 make different predictions regarding the phenotype of mutations that result in the loss of function. In the first case, the loss of BiP/GRP78 binding would prevent ER retention and lead to increased export of misfolded proteins. In the second case, loss of an assembly function should lead to an increase in the formation of improperly assembled or folded proteins. Depending upon the actual cause of ER retention this might have different consequences for the misfolded proteins. Assuming that ER retention of aberrant proteins is not also due to binding to BiP, then loss of BiP function should lead to increased accumulation of misfolded proteins in the ER. This would result in a block in ER to Golgi transport. However, if ER retention is also due to BiP/GRP78 binding, then both models predict increased export of misfolded proteins. Neither model predicts that loss of BiP/GRP78 function would have an effect on protein translocation into the lumen of the ER.

The *Saccharomyces cerevisiae* homologue of BiP/GRP78 is encoded by the *KAR2* gene (Rose et al., 1989). The gene was originally defined by a mutation, *kar2-1*, that causes a pronounced defect in nuclear fusion at all temperatures (Polaina and Conde, 1982). The mutant grows slowly at elevated temperatures suggesting that *KAR2* might be required for some general cellular process. Gene disruption experiments determined that *KAR2* is essential for mitotic growth (Rose et al., 1989; Normington et al., 1989). In this paper, we investigate the essential function of *KAR2* by characterizing the phenotype of newly isolated conditional mutant alleles. We find that conditional mutations produce a defect in translocation that is not predicted by the previously proposed models. These findings indicate that *KAR2* function is in some way required for continued import of proteins into the lumen of the ER.

Materials and Methods

Strains and Media

Yeast strains used are listed in Table I. Yeast media were essentially as de-

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
MS10	*MATa ura3-52 leu2-3 leu2-112 ade2-101*	This study
MS17	*MATα ura3-52 ade2-101 trp1-▽1*	This study
MS87	*MATα ura3-52 leu2-3 leu2-112 ade2-101 kar2-▽L148* [pMR397]	This study
MS147	*MATa ade2-101 trp1-▽1 lys2-80 cyh^R* [ρ°]	This study
MS176	*MATα ura3-52 kar2-159*	This study
MS177	*MATα ura3-52 ade2-101 kar2-159*	This study
MS179	*MATa/α ura3-52/ura3-52 trp1-▽/+ leu2-3,leu2-112/+ ade2-101/+ kar2-159/kar2-159*	This study
MS647	*MATα ura3-52 ade2-101 pep4::URA3 kar2-159*	This study
MS648	*MATα ura3-52 ade2-101 trp1-▽1 pep4::URA3*	This study
MS777	*MATα ura3-52 leu2-3 leu2-112 kar2-▽L148::LEU2* [pMR1341]	This study
MS785	*MATα ura3-52 leu2-3 leu2-112 kar2-▽L148::LEU2* [pMR397]	This study
MY1745	*MATα ura3-52 ade2-101 his4-539 suc2-▽9*	D. Botstein
MY767	*MATα ura3-52 ade2 leu2-3 leu2-112 his4 kar2-1*	G. Fink
MY1844	*MATα ura3-52 leu2-3 leu2-112 ade2-101 trp1-▽1 sec18*	This study
MY1925	*MATα ura3-52 leu2-3 leu2-112 ade2-101 trp1-▽1 pep4::URA3 sec18*	This study
RSY151-1B	*MATα ura3-52 leu2-3 leu2-112 pep4-3 sec63*	R. Schekman
SEY5188	*MATα ura3-52 leu2-3 leu2-112 suc2-▽9 sec18*	R. Schekman
YFP329	*MATα ura3-52 leu2-3 leu2-112 his4 sec62*	R. Schekman
YFP338	*MATα ura3-52 leu2-3 leu2-112 ade2 pep4-3 sec61*	R. Schekman

scribed in Sherman et al. (1986). Yeast DNA for recovering plasmids in *Escherichia coli* was prepared by the method of Hoffman and Winston (1987). Small scale plasmid DNA preparations were made by the boiling lysis method of Holmes and Quigley (1981). Yeast genetic techniques were as described in Sherman et al. (1986).

Isolation of Temperature Sensitive KAR2 Mutations

Temperature sensitive mutations of the *KAR2* gene were obtained by hydroxylamine mutagenesis of the *LEU2* carrying centromeric plasmid pMR713 as described in Rose and Fink (1987). Mutagenized plasmids were transformed into strain MS87 by the method of Ito et al. (1983). MS87 contains a deletion of *KAR2* on the chromosome (▽L148 on plasmid pMR659, described in Rose et al., 1989) as well as an intact copy of the *KAR2* gene carried on pMR397. The "plasmid shuffle" protocol (Boeke et al., 1987) was used to identify strains in which segregation of pMR397 (selected on 5-FOA media) uncovered a temperature sensitive growth phenotype. Plasmids were recovered in *E. coli* and retested after transformation back into MS87. The candidate mutant *KAR2* genes were excised and ligated into plasmid pMR779 (YIp5 with pSC101 origin of replication). The resulting integrating plasmids were used to introduce the temperature sensitive alleles onto the chromosome in two steps as described in Rose et al. (1989).

Fluorescent Staining

DAPI staining of yeast cells was performed by modification of the methods of Adams et al. (1984) and Kilmartin et al. (1984) as described in Rose and Fink (1987).

Radiolabeling and Immunoprecipitation

Labeling of cells and immunoprecipitation of proteins were performed essentially as described by Rose et al. (1989). For most experiments, cells were grown at a permissive temperature and shifted to the restrictive temperature for 30 min to 1 h before a 30-min labeling with [^{35}S]SO_4. Before pulse labeling invertase, cells were derepressed in low glucose medium for 30 min, pelleted, and resuspended in prewarmed SO_4-free medium. The cells were then incubated at 35°C for varying intervals before addition of 250 μCi of TRAN^{35} S-LABEL (ICN Radiochemicals, Irvine, CA). Labeling was terminated after 5 min by addition of NaN_3 to 10 mM and cycloheximide to 100 μg/ml on ice. Cell extracts were prepared as above except that 1 mg of cold protein extract from a *MATa* strain or a *suc2* deletion strain was added to the α-factor and invertase immunoprecipitations, respectively. Antibodies to *KAR2* (0.5 μl/OD_{600}), invertase (1.5 μl/OD_{600}), alpha-factor (1.0 μl/OD_{600}), or CPY (0.6 μl/OD_{600}) were added, incubated overnight at 4°C and precipitated by addition of protein A sepharose as described previously (Rose et al., 1989). *KAR2* protein, CPY, and invertase were fractionated by electrophoresis on 7.5% SDS-polyacrylamide gels. Alpha factor was fractionated either using 12.5% SDS-polyacrylamide gels or 18% polyacrylamide gels containing 4 M urea as indicated.

Protease Protection

Protease protection was performed essentially as described by Deshaies et al. (1987). Cells (25 OD_{600} U) were preshifted for 30 min and labeled for 30 min with 5 mCi [^{35}S]SO_4 at 33°C. Labeling was terminated by addition of NaN_3 to 10 mM and cycloheximide to 100 μg/ml. Cells were pelleted, and washed in 0.1 M Tris-SO_4 (pH 9.4), 10 mM DTT for 10 min at room temperature. Cells were then washed in spheroplast buffer (1.4 M sorbitol, 60 mM 2-mercaptoethanol, 25 mM MOPS [pH 7.4], 5 mM $MgCl_2$, 10 mM NaN_3) and resuspended in 1.5 ml of same. Cell walls were removed by digestion with 0.45 mg Zymolyase-100T (ICN Radiochemicals) for 45 min at 30°C. Spheroplasts were purified by centrifugation in a rotor (HB4 Sorvall Co., Dupont, Wilmington, DE) at 5 K rpm, for 5 min, through 10 ml of spheroplasting buffer made up to 1.9 M sorbitol. The pellet was resuspended in 0.73 ml lysis buffer (0.3 M mannitol, 0.1 M KCl, 50 mM Tris-Cl [pH 7.5], 1 mM EGTA). Spheroplasts were lysed gently on ice in a motor driven Potter-Elvejem homogenizer (3 cycles of 1-min homogenization and 1-min cooling). Unbroken cells were removed by centrifugation (2 K rpm, 4 min). The supernatant was divided into two portions, one of which received Triton X-100 to 0.4%. Proteinase-K was added to 0.3 mg/ml and aliquots were removed after various times of incubation on ice. Digestion was terminated by dilution into 0.54 ml 20% TCA. Precipitates were collected by centrifugation, washed with −20°C acetone, and resuspended in Laemmli sample buffer. Alpha factor precursors were immunoprecipitated and analyzed as described above.

Depletion of KAR2

Strains MS777 and MS785 were grown to mid-log phase (80 Klett U) in YEP liquid with 2% galactose. At the initiation of the experiment, glucose was added to 2%, 4,000 Klett U of cells were harvested for preparation of protein extracts, and the remainder of the cultures were diluted to 40 Klett U with YEP containing 2% glucose. At each doubling (120 min for the first doubling and 90 min thereafter), 4,000 Klett U of each culture were harvested for protein, and the remainder was diluted to a density of 40 Klett U as above. After the mutant culture (MS777) slowed in growth rate (>8 h), each time point represented a generation of the wild-type strain. Protein extracts were prepared by the method of Ohashi et al. (1982), fractionated on polyacrylamide gels, electrophoretically transferred to nitrocellulose (Burnette, 1981; Towbin et al., 1979) and probed with antibody to alpha factor or *KAR2* protein. Antibody binding was visualized by incubation with ^{125}I-protein A (Amersham Corp., Arlington Heights, VA) followed by autoradiography.

Results

Isolation of Temperature Sensitive Alleles of KAR2

The essential function of *KAR2* was investigated by isolation of temperature sensitive conditional alleles by the "plasmid shuffle" method (Boeke et al., 1987). A haploid strain (MS87) was constructed that contained an internal deletion (*kar2*-▽L148) on the chromosome and a wild-type *KAR2* gene on a *URA3* marked centromere plasmid (pMR397). The *KAR2* gene on a *LEU2* marked centromere plasmid (pMR713) was mutagenized with hydroxylamine in vitro and transformed into the deletion strain. Transformants were then replica plated to 5-fluoro-orotic acid (5-FOA)[1] plates to select for cells that had lost the *URA3*-based plasmid. The 5-FOA resistant segregants that also became temperature sensitive for growth were picked and retested to determine whether the mutation was in *KAR2*. 12 temperature sensitive mutations in *KAR2* were isolated by this procedure. One mutation, *kar2-159*, which conferred the strongest block to cell growth, was selected for further study. The *kar2-159* mutation was then integrated into the chromosome by homologous recombination to replace the wild-type allele.

The *kar2-159* mutant was initially characterized by examination of the plating efficiency at the restrictive temperature. Less than 1×10^{-7} cells were able to form colonies at 37°C. Shifting the growth temperature of a liquid culture from 23°C to 37°C led to a rapid arrest of cell growth (Fig. 1). After 25 min at 37°C, <10% of the cells were capable of forming colonies upon plating at 23°C. Thus, the *kar2-159* mutation leads to a precipitous and irreversible loss of viability.

KAR2 Is Required for Bud Emergence and Growth

Cells that had been shifted to the nonpermissive temperature were examined by immunofluorescent staining to discern effects of the *kar2-159* mutation on the cell cycle or cell morphology. Two unusual classes of cells appeared after shifting the culture to the nonpermissive temperature. One class consisted of binucleate cells with very small buds (Fig. 2, *C* and *D*). The second class consisted of exceptionally small cells (*C*, *small arrow*), apparently derived from small buds in which both nuclear division and cytokinesis had been completed. In contrast, nuclear division and cytokinesis are not completed in wild-type cells until the bud is almost the size of the mother cell. Thus, bud growth appeared to be defective in the *kar2-159* mutant.

Bud emergence also failed at the nonpermissive temperature. At 23°C both wild-type and *kar2-159* cultures contained 37% unbudded cells (Fig. 2, *A* and *B*). After growth at 37°C the wild-type culture contained 42% unbudded cells whereas the mutant culture contained 84 and 94% unbudded cells after 1.5 and 3 h. Prolonged incubation caused the mutant cells to appear swollen (Fig. 2, *E* and *F*). In some cells, nuclear division proceeded without cell division giving rise to binucleate unbudded cells (8%). Thus, the *KAR2* gene is apparently required for both bud growth and bud emergence. As bud growth entails the localized secretion of new cell surface components, these data are consistent with a requirement for *KAR2* in the secretory pathway.

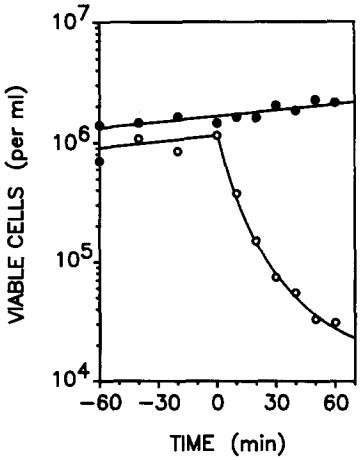

Figure 1. Viability of the *kar2-159* mutant at nonpermissive temperature. A *kar2-159* strain (MS177) was pregrown in YPD at 23°C. At time zero the culture was split in half. One portion was incubated at 23°C (*solid circles*). The remaining part was added to an equal volume of prewarmed media to shift rapidly the temperature to 37°C (*open circles*). Samples were removed every 10 min and plated on YPD at 23°C.

kar2-159 Blocks Export of Secretory Proteins

To determine whether the *kar2-159* mutant exhibited a defect in secretion, we examined the export of several well-characterized proteins that are transported via the secretory pathway: invertase, carboxypeptidase Y (CPY), alpha factor, and KAR2 itself.

Invertase is the product of the *SUC2* gene and is synthesized in two forms, a constitutively expressed cytoplasmic form and an inducible, highly glycosylated secreted form (Esmon et al., 1981). The two species differ only by the inclusion of a signal sequence at the amino terminus of the secreted form (Carlson and Botstein, 1982). Proteins from wild-type and *kar2-159* strains were labeled with $^{35}S[SO_4]$ at the permissive temperature (23°C) or the restrictive temperature (37°C). Cell extracts were prepared and invertase was immunoprecipitated. Wild-type cells contained three expected species of invertase: the constitutively expressed cytoplasmic form, core-glycosylated forms in transit through the ER, and heterogeneous highly glycosylated mature invertase (Fig. 3 *A*, lanes *1* and *2*). In the wild-type strain, tunicamycin treatment blocked appearance of the glycosylated forms (Olden et al., 1979) and resulted in accumulation of a processed precursor that had the same mobility as the constitutive, cytoplasmic form (Fig. 3 *A*, lane *3*). The *sec18* mutation blocks export from the ER (Novick et al., 1981) causing core-glycosylated species to accumulate (Fig. 3 *A*, lane *7*). Invertase secretion was normal in the *kar2-159* strain at the permissive temperature (Fig. 3 *A*, lane *4*). At 37°C the *kar2-159* mutant accumulated a novel species that ran with reduced mobility (62 kD) relative to the cytoplasmic form (60 kD). The novel form is not N-linked glycosylated as its mobility was unaffected by the addition of tunicamycin (Fig. 3 *A*, lane *6*). The novel form co-migrated with the precursor that accumulated in a *sec61* strain (compare lanes *5* and *8*

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; 5-FOA, 5-fluoro-orotic acid.

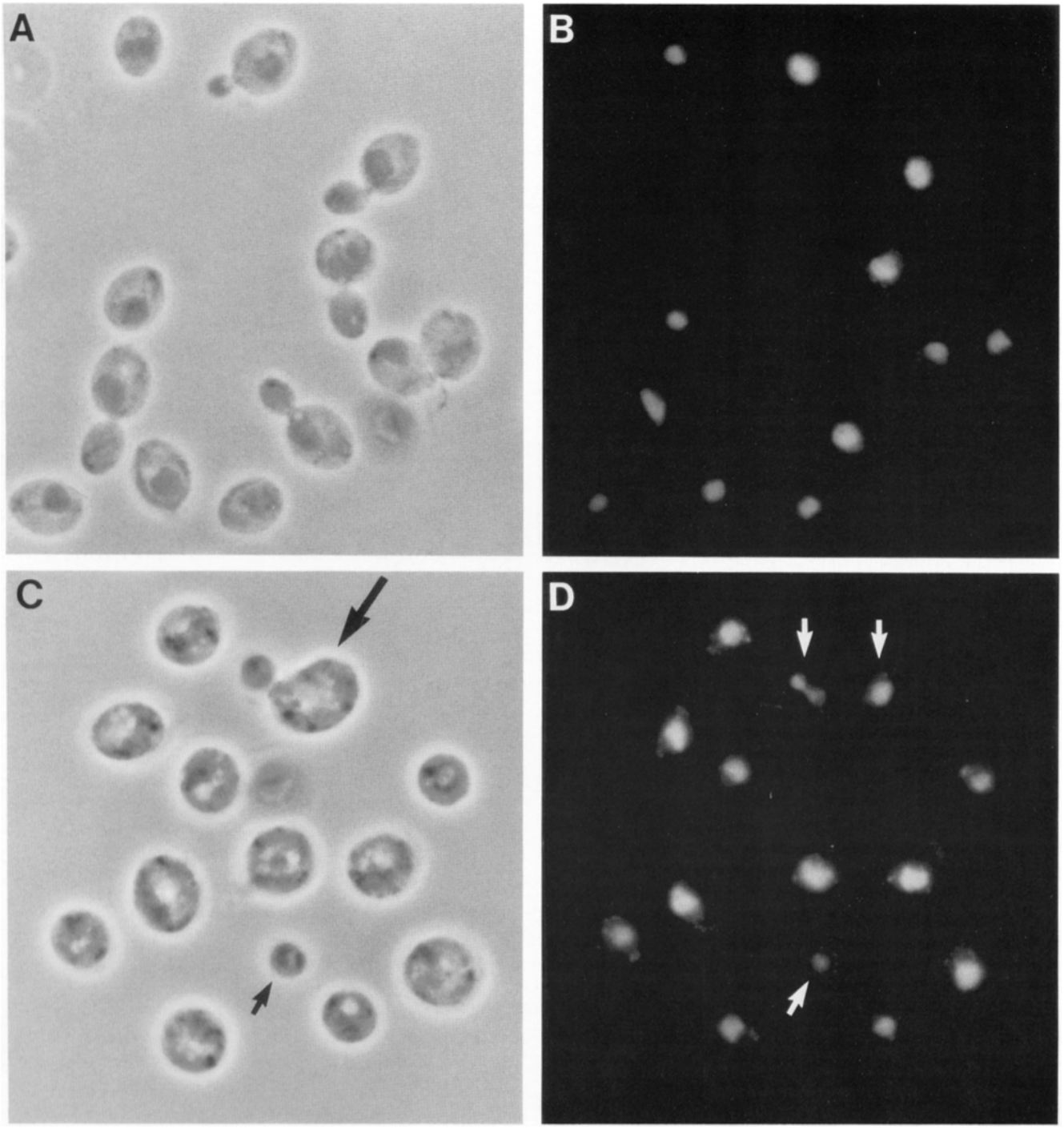

Figure 2. The *kar2-159* mutant arrests as unbudded cells at nonpermissive temperature. A *kar2-159* homozygous diploid (MS179) was grown at 23°C and shifted to 37°C. Cells were harvested at the time of temperature shift (*A* and *B*), after 1.5 h at 37°C (*C* and *D*), and after 3 h at 37°C (*E* and *F*) and prepared for immunofluorescent staining. *A*, *C*, and *E* show phase-contrast images of the cells. *B*, *D*, and *F* show DAPI staining of DNA to localize the position of the nuclei. In *C*, the large arrow indicates a cell with a small bud that contains 2 nuclei. These are also shown by the arrows in *D*. The small arrow in *C* points to one of the exceptionally small cells. The arrows in *E* and *F* indicate binucleate unbudded cells.

in Fig. 3 *A*). The *sec61* mutation partially blocks translocation of proteins into the lumen of the ER and causes the accumulation of the precursors in the cytoplasm (Deshaies and Schekman, 1987). The mobilities of the precursors of secreted proteins seen in the *sec61* mutant have been shown to be identical to their primary translation products. The reduced synthesis of invertase seen in the *kar2-159* mutant at 37°C is not understood but was only observed after prolonged incubation at the high temperature. In other experiments using shorter incubations and lower temperatures, normal levels of invertase were observed (cf., Fig. 3 *B*).

To examine how quickly the *kar2-159* mutation causes the accumulation of the invertase precursor, cells were shifted to 35°C for varying amounts of time and pulse labeled for

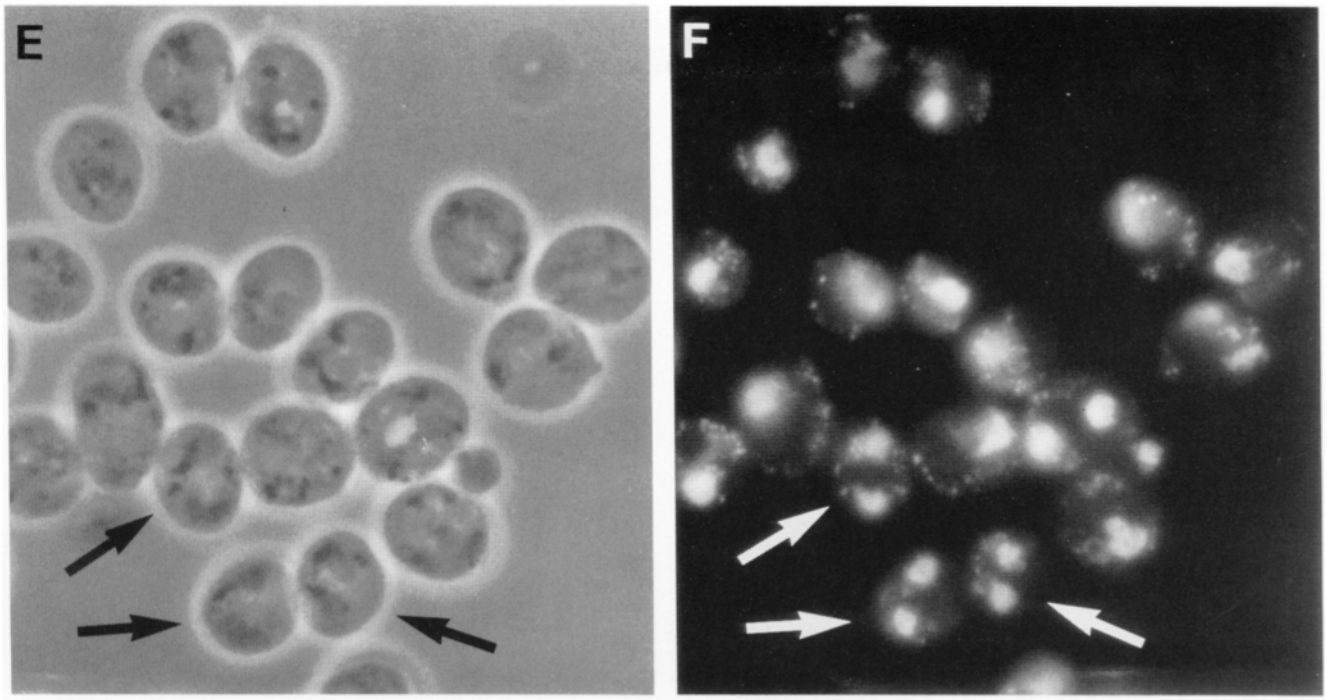

Figure 2. (Continued).

5 min at the restrictive temperature. Accumulation of the novel species of invertase was seen within the first 5 min after the temperature shift (Fig. 3 *B*). The block was >50% complete between 5 and 10 min and was essentially complete between 10 and 20 min. These results correlate well with the kinetics of the loss of viability. The rapidity with which the defect is expressed suggests it is a direct effect of the loss of *KAR2* function.

CPY is a vacuolar protease that enters the secretory pathway via translocation into the ER (Stevens et al., 1982). Signal sequence cleavage and addition of core glycosylation in the ER gives rise to the p1 form. Transit through the Golgi complex allows further modification to the p2 form, and the protein is then sorted to the vacuole. In the vacuole of wild-type cells, CPY is cleaved to the fully mature form by the product of the *PEP4* gene (Hemmings et al., 1981). To simplify the results, *pep4* mutant strains were used to prevent the final cleavage. In the Kar$^+$, Pep4$^-$ strain, CPY was found as a mixture of the p1 and p2 forms (Fig. 4 *A*, lanes *2* and *3*). The *sec18* mutant accumulated only the ER-derived p1 form (Fig. 4 *A*, lane *8*) whereas the *sec61* mutant accumulated the secretory precursor with the signal sequence intact (Fig. 4 *A*, lane *9*). Addition of tunicamycin to wild type cells (Fig. 4 *A*, lane *4*) caused the accumulation of a nonglycosylated precursor with a faster mobility than the *sec61* form consistent with signal sequence cleavage. The *kar2-159* strain showed normal formation of p2 CPY at permissive temperature (Fig. 4 *A*, lane *5*) but accumulated a novel form of CPY at the nonpermissive temperature (Fig. 4 *A*, lane *6*). The novel form was unaffected by tunicamycin indicating that it lacked N-linked glycosylation (Fig. 4 *A*, lane *7*). The novel form of CPY co-migrated with the precursor that accumulated in the *sec61* strain (Fig. 4 *A*, lane *9*), suggesting that the signal sequence was retained.

The *kar2-159* mutant was also defective for the secretion of mating pheromone alpha factor. Alpha factor is a 13 amino acid peptide encoded by two genes *MFα1* and *MFα2* (Kurjan and Herskowitz, 1982; Singh et al., 1983). The primary translation products of these two genes are identical polypeptides of 21 kD containing four copies of the alpha factor peptide. After translocation of the 21-kD precursor into the ER, the signal sequence is cleaved giving rise to a 19-kD protein that is rapidly glycosylated at three sites to form a 26-kD protein (Julius et al., 1984; Waters et al., 1988). This species is then transported to the Golgi apparatus where further processing and cleavage produces the mature peptide, which is secreted from the cell. In wild-type cells, alpha factor is rapidly processed and released into the media. Therefore, no precursors were observed in wild-type strains (Fig. 4 *B*, lanes *2* and *3*). Addition of tunicamycin to the wild-type strain caused the nonglycosylated 19-kD ER precursor to accumulate (Fig. 4 *B*, lane *4*), whereas the *sec18* mutation caused the glycosylated 26-kD precursor to accumulate (Fig. 4 *B*, lane *8*). In the *kar2-159* mutant, a nonglycosylated protein accumulated (Fig. 4 *B*, lanes *6* and *7*) that had the same mobility as the precursor that accumulated in the *sec61* strain (Fig. 4 *B*, lane *9*).

The KAR2 protein is itself translocated into the ER via a *SEC61* dependent pathway (Rose et al., 1989). It was of interest to determine whether *kar2-159* caused a block in processing of KAR2 protein itself. In Fig. 5, cells were labeled during incubation at various temperatures. The *kar2-159* protein was found to accumulate a secretory precursor at the same temperatures at which alpha factor precursor accumulated. The defect was found to be extremely temperature dependent with 23°C being fully permissive and 35°C being almost completely defective. The block was not due to an inherent difficulty in the translocation or processing of the mu-

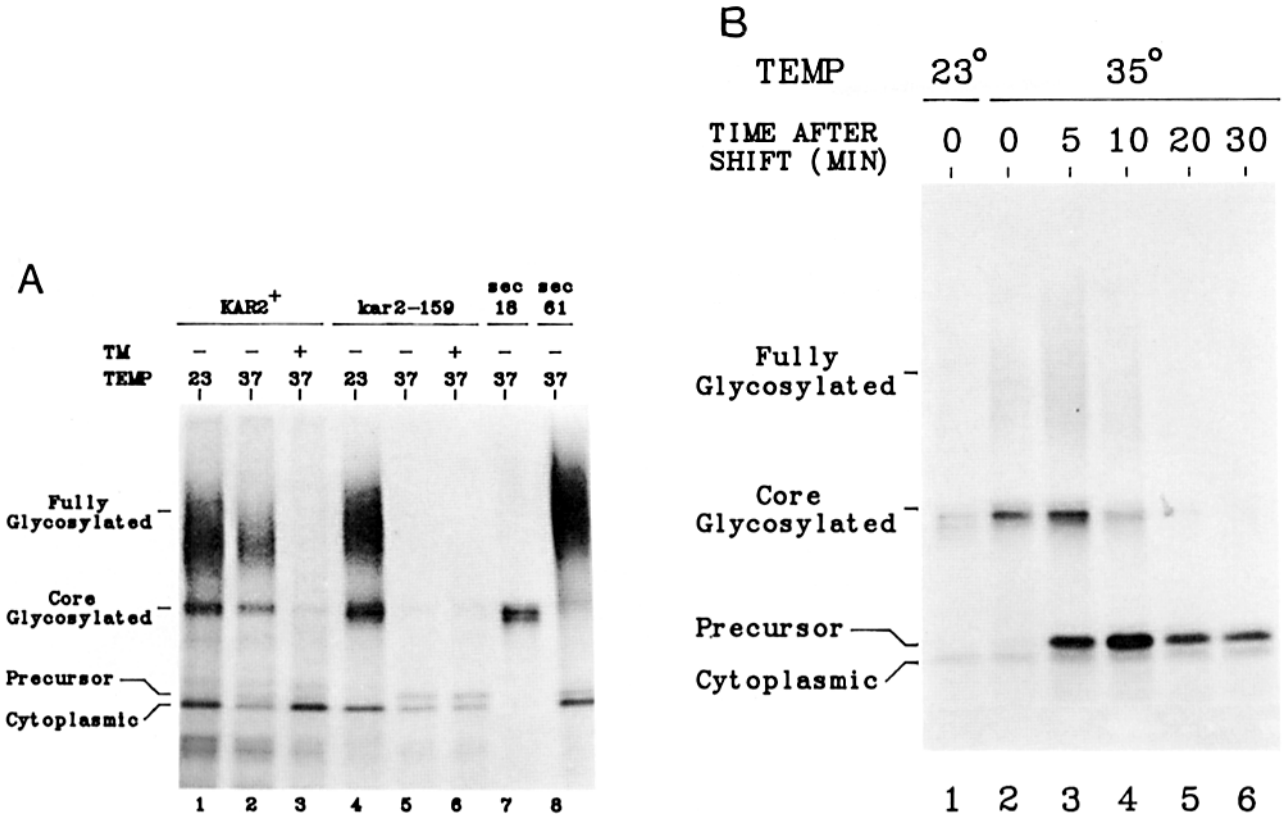

Figure 3. kar2-159 blocks formation of secreted invertase. (*A*) Immunoprecipitation of invertase. Lanes *1–3* show immunoprecipitates of invertase synthesized in a wild-type strain (MS17) while lanes *4–6* show a *kar2-159* strain (MS177). Lanes *7* and *8* show a *sec18* strain (MY1844) and a *sec61* strain (YFP338), respectively. Cells were derepressed in low glucose for 30 min at 23°C, shifted to the indicated temperature for 1 h, and then labeled for 30 min with [^{35}S]SO_4 at that temperature. Where indicated, tunicamycin was added to cultures 15 min after the start of the temperature shift. (*B*) Kinetics of appearance of the *kar2-159* block. A *kar2-159* strain (MS177) was shifted to the indicated temperatures for varying amounts of time. Cells were then labeled for 5 min at that temperature. Labeled invertase was immunoprecipitated as in *A*.

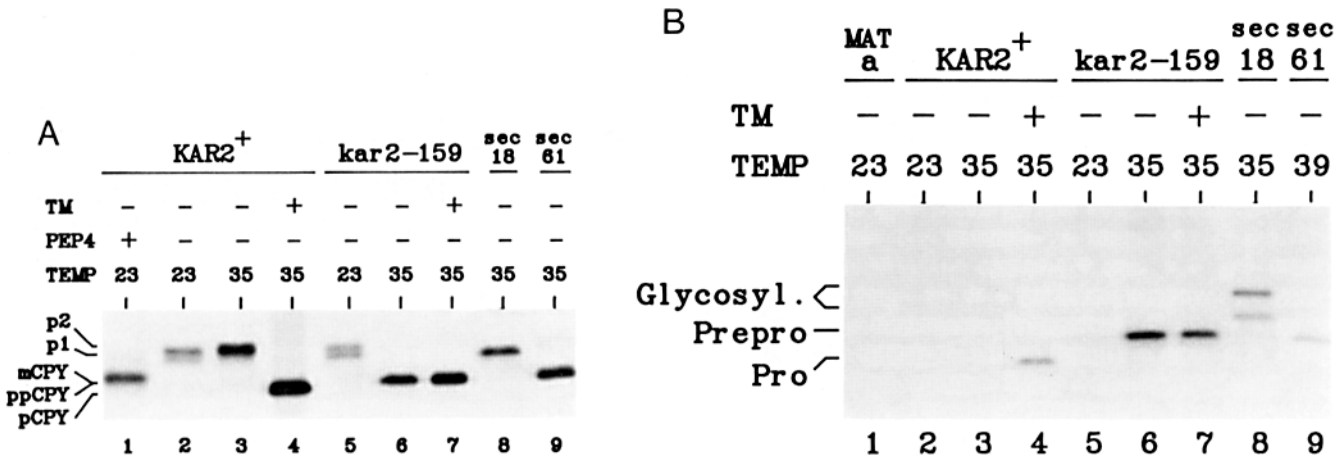

Figure 4. kar2-159 blocks the secretion of vacuolar CPY and the peptide mating pheromone, alpha factor. (*A*) Immunoprecipitation of CPY. Cells were labeled with [^{35}S]SO_4 for 30 min after a 30-min incubation at the indicated temperature. CPY synthesized at the indicated temperatures in a wild-type strain (MS648) is shown in lanes *2–4*, in a *kar2-159* strain (MS647) in lane *5–7*, in a *sec18* strain (MY1925) in lane *8*, and in a *sec61* (YFP338) strain in lane *9*. Lane *1* shows CPY synthesized in a *PEP4*$^+$ strain (MS17). (*B*) Immunoprecipitation of alpha factor precursors. Cells were labeled with TRAN^{35}S-LABEL for 30 min after a 30-min incubation at the indicated temperature. Alpha factor precursors synthesized in a wild-type *MATα* strain (MS17) are shown in lanes *2–4*, in a *kar2-159* strain (MS177) in lanes *5–7*, a *sec18* strain (SEY5188) in lane *8*, and in a *sec61* strain (YFP338) in lane *9*. Lane *1* shows a *MATa* strain (MS10) as a control.

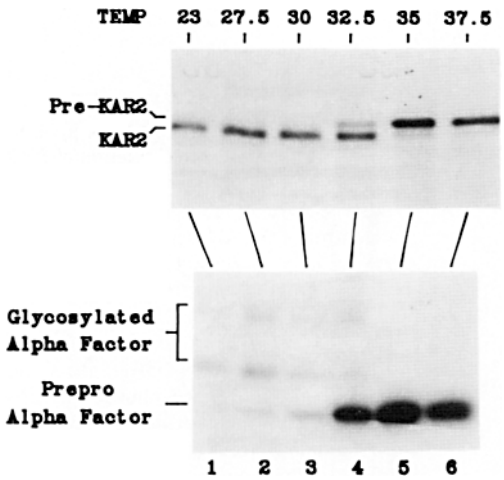

Figure 5. Accumulation of KAR2 protein precursors in *kar2-159*. A *kar2-159* strain (MS177) was shifted to various temperatures for 30 min before a 30-min labeling with [^{35}S]SO_4 at that temperature. Extracts were split and immunoprecipitated either with anti-KAR2 or anti-alpha factor antibodies.

tant protein as the precursor did not accumulate if wild-type KAR2 protein was provided in trans (data not shown). Moreover, the *kar2-159* mutation led to a block in import and glycosylation of a *KAR2-lacZ* hybrid protein (our unpublished observation). Thus, KAR2 is dependent upon its own function for attaining its mature form.

These results demonstrate that the *kar2-159* mutation causes a severe block in the secretion of several different proteins. For each protein examined, a nonglycosylated precursor accumulated that had the same electrophoretic mobility as the precursor found in the translocation defective *sec61* mutant. These data suggest that KAR2 function is required for an early step in the secretory pathway.

kar2-159 Blocks Translocation into the Lumen of the ER

The accumulation of nonglycosylated forms of secretory proteins that appear to be identical to the primary translation products could be the result of two fundamentally different defects. First, the translocation of secretory precursors into the lumen of the ER might be blocked. This would prevent exposure of the precursors to both signal peptidase and glycosyl-transferase. Alternatively, translocation might occur, but the activities of both the signal peptidase and the glycosyl-transferase would be dependent upon wild-type KAR2 function. The former model predicts that the precursors would remain exposed on the cytoplasmic side of the ER membrane. In contrast, the latter model predicts that the precursors would be located within a membranous compartment somewhere within the secretory pathway.

To differentiate between these two models, the intracellular location of the accumulated precursors was determined using a protease protection assay. The *kar2-159* strain was labeled at an intermediate temperature (33°C) to allow some secretory precursors to enter the ER. These served as internal controls for the integrity of the ER membranes. Cell extracts were prepared and treated with proteinase K. At various times, the reactions were terminated and the alpha factor precursors were immunoprecipitated. As expected for proteins in the lumen of the ER, the glycosylated alpha factor precursors were found to be resistant to proteolysis (Fig. 6, lanes *1–6*). However, if the membranes were first disrupted with Triton X-100 before protease addition, then these species were very rapidly degraded (Fig. 6, lanes *7–12*). In contrast, the alpha factor precursor that accumulated due to the *kar2-159* mutation was degraded by the protease regardless of the addition of detergent. Thus, the *kar2-159* dependent precursor remained exposed on the cytoplasmic side of the ER membrane. Similar results were also found for both the KAR2 and CPY precursors (data not shown). From these data, we conclude that the *kar2-159* mutation blocks translocation of proteins into the lumen of the ER.

Depletion of Wild-Type KAR2 Protein Causes a Translocation Defect

BiP/GRP78 is an abundant protein that is itself translocated into the lumen of the ER. One explanation for the defect in translocation is that the mutant protein might be incapable of being translocated, leading to "jamming" of the secretory machinery. This is unlikely as both the lethal phenotype and the translocation phenotype are completely recessive to the wild type in heterozygous diploids (J. Vogel, unpublished data). However, to eliminate this possibility by an independent test, we chose to examine the phenotype of cells in which the synthesis of the wild-type protein could be eliminated in a regulated manner. The *KAR2* gene was placed under the control of the *GAL1* promoter that is highly expressed in cells grown on galactose but is repressed more than 1,000-fold in the presence of glucose. A strain (MS777) containing this plasmid construct as the only copy of the *KAR2* gene was found to be viable on galactose media, but failed to grow on

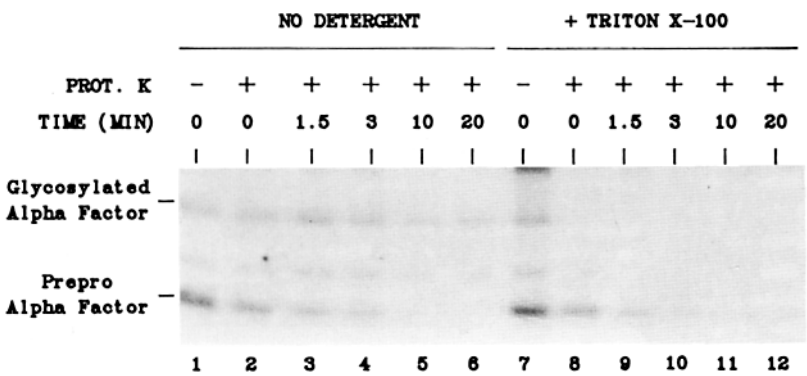

Figure 6. Protease sensitivity of alpha factor precursors. Proteins synthesized in a *kar2-159* strain (MS177) at semipermissive temperature (33°C) were labeled for 30 min. Cell lysates were prepared and digested for various times with proteinase K with or without prior addition of Triton X-100 as described in Materials and Methods. Alpha factor precursors were immunoprecipitated from aliquots of the digested samples.

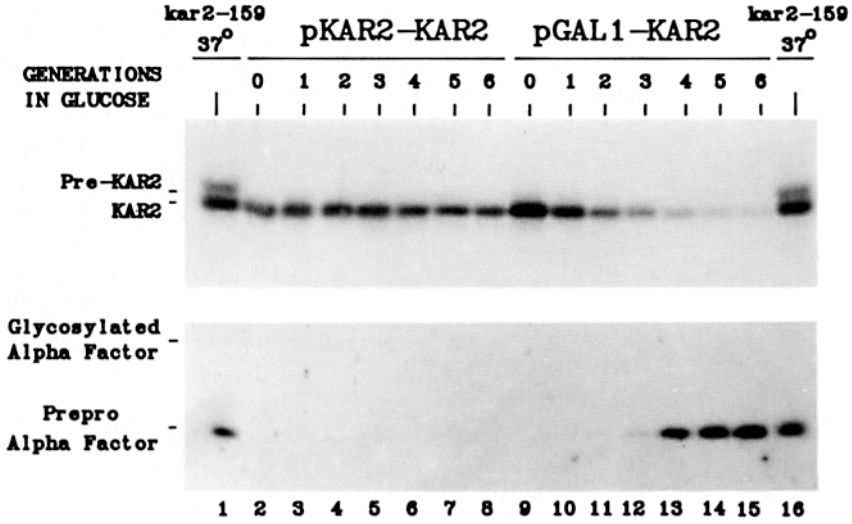

Figure 7. Depletion of *KAR2* protein causes accumulation of secretory precursors. Strains containing a chromosomal deletion of *KAR2* plus an autonomous plasmid bearing either wild-type *KAR2* (MS785) or *KAR2* under the control of the *GAL1* promoter (MS777) were initially grown on galactose. At the beginning of the experiment glucose was added to repress the *GAL1* promoter. Samples were taken at various time points and alpha factor precursors (*lower panel*) and *KAR2* protein (*upper panel*) were examined by Western immunoblotting. For comparison, samples from the *kar2-159* mutant shifted to the nonpermissive temperature are displayed in lanes *1* and *16*.

glucose. We examined the formation of alpha factor in this strain at various times after shift to glucose. As a control, an isogenic strain (MS785) containing *KAR2* under the control of its own promoter was examined in parallel.

Expression of *KAR2* from the *GAL1* promoter led to slightly elevated levels of KAR2 protein (Fig. 7, compare lane *9* with lane 2). With each subsequent generation after addition of glucose, the level of KAR2 protein was reduced by approximately one half. After four generations, the level of KAR2 protein was reduced to <10% of wild-type levels at which time cell growth slowed considerably and cell viability was reduced to ~10%. In contrast, the level of the KAR2 protein in the wild-type strain was unaffected by the change in carbon source. Concurrent with cell growth arrest in the experimental strain, the alpha factor precursor began to accumulate. The precursor accumulated to the same level observed for the *kar2-159* mutant. Thus, the phenotype observed for the temperature sensitive mutation is identical to that seen for depletion of KAR2 protein. As the two different genotypes result in the same conditional phenotype, the temperature sensitive defect in translocation must be the result of the loss of KAR2 function.

Nuclear Fusion and Translocation

The finding that *KAR2* is required for both nuclear fusion and translocation prompted us to ask whether there is a direct connection between these two apparently diverse phenotypes. We examined the efficiency of nuclear fusion of the *kar2-159* mutant as well as various translocation defective *sec* mutants at various temperatures. The original karyogamy defective *kar2-1* mutant was defective for nuclear fusion at all temperatures (Table II). The *kar2-159* mutation, in contrast, conferred a temperature sensitive nuclear fusion defect. Although not resulting in as strong a defect as *kar2-1*, the *kar2-159* mutation blocked nuclear fusion by >50% at 30°C. In contrast, the defect in translocation was barely detectable at 30°C (cf., Fig. 5, lane *3*). Thus, in the *kar2-159* strain, the karyogamy defect appeared at a lower temperature than the translocation defect. The *sec61*, *sec62*, and *sec63* mutants all manifest an appreciable defect in translocation at 30°C (Deshaies and Schekman, 1987; Rothblatt et al., 1989). However, these mutant strains were not at all deficient in nuclear fusion. Finally, the *kar2-1* mutant shows no defect in translocation at any temperature (J. Vogel, unpublished observations). Taken together, these data establish that a general defect in translocation does not cause a defect in nuclear fusion.

Discussion

KAR2, the yeast homologue of mammalian BiP/GRP78, is essential for cell viability as well as for nuclear fusion during mating. We have found that a temperature sensitive mutation in *KAR2* resulted in a strong conditional block in the translocation of several secretory proteins. The secretory precursors remained exposed on the cytoplasmic side of the ER membrane as determined by protease protection experiments. The phenotype of the temperature sensitive mutation was mimicked by depletion of the wild-type KAR2 protein as expressed from a regulated *GAL1* promoter. Therefore, the block in translocation resulted from loss of KAR2 function and was not due to interference by the defective *kar2-159* protein. The rapidity with which the defect is expressed in the temperature sensitive mutant suggests that the translocation block is a direct result of the loss of KAR2 function.

Table II. Nuclear Fusion in Translocation Defective Secretion Mutants

Strain	Relevant genotype	23°C	30°C
MS17	*KAR2+*	0.002	0.0004
MY767	*kar2-1*	3.3	5.6
MS176	*kar2-159*	0.008	0.92
YFP338	*sec61*	0.004	0.002
YFP329	*sec62*	0.004	0.005
RSY151-1B	*sec63*	0.001	0.006

Strains were mated to a *MAT*a *KAR2*$^+$ [rho°] *cyh*^{R} strain (MS147) at the indicated temperatures for 5 h, and aliquots were plated on different selective media to measure the frequency of diploid and cytoductant formation. The ratio of haploid cytoductants to prototrophic diploids is reported above. Cytoductants are the haploid [rho$^+$], *cyh*^{R} cells that arose from zygotes in which nuclear fusion failed. A larger cytoductant to diploid ratio indicates a stronger defect in nuclear fusion.

Possible Roles for KAR2 in Translocation

The observation that loss of yeast BiP/GRP78 function in vivo causes a block in the import of secretory proteins is surprising in that a protein within the lumen of the ER would appear to be in the wrong place to be required for translocation. Several recent observations concerning the functions of the cytoplasmic HSP70 proteins offer possible explanations for our findings. The cytoplasmic HSP70 proteins have been demonstrated to play a role in the import of secretory proteins in yeast, apparently by maintaining secretory precursors in an unfolded state (Chirico et al., 1988; Deshaies et al., 1988). Depletion of the cytoplasmic HSP70 proteins results in a translocation block in vivo and in vitro (Chirico et al., 1988; Deshaies et al., 1988). A related activity for the cytoplasmic HSP70s is clathrin-uncoating ATPase (Ungewickell, 1985; Chappell et al., 1986), characterized by the dissociation of clathrin chains and the "uncoating" of clathrin-coated vesicles in vitro (Schlossman et al., 1984). In yeast, the same cytoplasmic HSP70s that are required for import into the ER appear to have clathrin-uncoating ATPase activity (Chappell et al., 1986). Moreover, dissociation of tightly bound complexes may be a general feature of HSP70s (Pelham, 1986).

These observations lead us to propose two related models for the involvement of BiP/GRP78 in translocation. In both models absence of KAR2 function results in the inactivation of the translocation machinery. The first model is analogous to the role of the HSP70s in maintaining the conformation of secretory precursors. We hypothesize that during translocation, certain proteins must be maintained in an unfolded conformation on both sides of the ER membrane. BiP/GRP78 would act to maintain the unfolded conformation on the lumenal side by binding transiently to various nascent proteins during translocation. After translocation, ATP hydrolysis and the completion of protein folding or oligomerization would allow dissociation of the complex. Mutant proteins failing to achieve a mature conformation would remain stably bound to BiP/GRP78. In the absence of BiP/GRP78, wild-type proteins might fold prematurely, or become aggregated via exposed hydrophobic surfaces. Partially folded precursors or aggregates would cause inactivation of the secretory machinery. The jamming might arise if unfolded precursors (perhaps only partially translocated) remain stably associated with the secretory machinery. By preventing the jamming, BiP/GRP78 would appear to catalyze both the import of secretory proteins as well as their folding and assembly.

The second model is analogous to the ability of clathrin-uncoating ATPase to dissociate stably bound complexes. We hypothesize that some component of the secretory machinery becomes inactivated during each round of translocation. The inactive state might be caused by formation of a stable complex involving the subunit proteins of the translocation machinery, possibly including the newly translocated protein. BiP/GRP78 would be required to dissociate the stable complex and recycle the components of the secretory machinery for each new round of import. In this model, interaction of BiP/GRP78 with secretory proteins would occur after translocation is completed.

Neither model implies that BiP/GRP78 must be an integral component of the translocation machinery. Rather, the models attempt to explain the demonstrated requirement for KAR2 function by proposing a role in the maintenance of the translocation machinery. A variation on the first model is based on the idea that loss of BiP/GRP78 function would cause the accumulation of misfolded proteins in the ER that would then lead to a nonspecific block in translocation. Contrary to this view, we note that tunicamycin and certain *sec* mutations do cause the ER accumulation of misfolded proteins but do not cause a block in translocation. Furthermore, at least for invertase, the block in translocation arises without any obvious prior accumulation of ER precursors. This fact, together with the rapidity with which the block occurs, would imply that the translocation apparatus is extremely sensitive to the presence of misfolded protein. Although we think this model is unlikely, we currently have no data to rule out this possibility. Nevertheless, this model, like the two previous models, essentially proposes that one role for BiP/GRP78 would be to prevent the lethal inactivation of the secretory machinery.

With regard to the first model, BiP/GRP78 has not been observed to bind to nascent secretory proteins. In a coupled in vitro translation and translocation system, Kassenbrock et al. (1988) were able to observe stable BiP/GRP78 complexes only with completely translocated, incorrectly disulfide-bonded prolactin. As expected, complexes could only be observed after rigorous depletion of ATP. Given the requirements for ATP in the translation and translocation reactions, and the presumed presence of ATP in the lumen of the ER, it is unclear whether transient complexes with nascent proteins could have been preserved in these studies.

In contrast, Flynn et al. (1989) have recently demonstrated a peptide dependent ATPase activity associated with BiP/GRP78. This activity is consistent with models in which BiP interacts with the unfolded secretory precursors. Different peptides were found to show very different activities in this assay. The stable binding observed with some mutant proteins may therefore reflect the presence of particularly strong binding sites.

There appears to be no absolute requirement for BiP/GRP78 or other lumenal ER proteins during in vitro translocation using mammalian extracts (Bulleid and Freedman, 1988). It is possible that this discrepancy reflects a fundamental difference in the mechanism of import between yeast and mammalian systems. Experiments are currently in progress to precisely define the function of BiP/GRP78 in a yeast in vitro system. We think it more likely that the discrepancy lies with differences between what is measured in in vitro and in vivo import. The in vitro translocation systems monitor the functions most directly required for transport across the membrane. Proteins that have not been released into the lumen of the ER would appear to have been translocated by standard criteria (protease protection). Likewise, a protein required for recycling of secretory components would not be essential in an in vitro system capable of only a single round of import. Finally, various proteins may have substantially different requirements for BiP/GRP78 function during import. A general defect in the translocation of most, if not all, proteins could occur in vivo because of the toxic jamming caused by only proteins that do require BiP/GRP78. Thus, the lack of BiP/GRP78 dependence in vitro may reflect only the narrow scope of the current in vitro translocation assays.

Role of KAR2 in Nuclear Fusion

The *KAR2* gene was originally identified by a mutation, *kar2-1*, whose most striking phenotype was a nonconditional defect in nuclear fusion. We have found that *kar2-159* (as well as the other temperature sensitive alleles) also has an associated defect in nuclear fusion. Thus, a common feature of all known mutations in *KAR2* is that they impair karyogamy. The requirement for BiP/GRP78 in this process is puzzling. A simple model is that a protein required for nuclear fusion must be transported to the nuclear envelope via the lumen of the ER. A translocation defect would interfere with the localization of this protein. Three findings argue against a general requirement for translocation in nuclear fusion. First, other translocation defective secretory mutants show no defect in nuclear fusion even under conditions where translocation is clearly impaired. Second, the temperature sensitive mutation, *kar2-159*, exhibits a defect in nuclear fusion that is manifest at a lower temperature than the translocation defect. This implies that the function required for nuclear fusion can become limiting before the function required for translocation. Finally, *kar2-1* is defective for nuclear fusion at all temperatures but shows no defect in translocation at any temperature.

As *kar2-1* mutants grow slowly at high temperature, the implication is that KAR2 function can become limiting for cell growth without generally blocking translocation. It is possible that a class of proteins exists whose translocation is exquisitely sensitive to KAR2 function but not dependent upon the function of the other translocation *SEC* genes. However, a large quantity of data already exists that implicates mammalian BiP/GRP78 as playing a critical role in protein assembly and export from the ER. We think it more likely that nuclear fusion is dependent upon such later steps in the secretory pathway and that these steps are specifically affected by the *kar2-1* mutation. As *kar2-159* blocks the secretion pathway at the earliest step in which KAR2 is required, any defects in subsequent steps would be concealed. Characterization of *kar2-1* and the other alleles of *KAR2* should clarify the nature of the manifold functions of BiP/GRP78.

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