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Sac1p Mediates the Adenosine Triphosphate Transport into Yeast Endoplasmic Reticulum That Is Required for Protein Translocation

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Abstract. Protein translocation into the yeast endoplasmic reticulum requires the transport of ATP into the lumen of this organelle. Microsomal ATP transport activity was reconstituted into proteoliposomes to characterize and identify the transporter protein. A polypeptide was purified whose partial amino acid sequence demonstrated its identity to the product of the *SAC1* gene. Accordingly, microsomal membranes isolated from strains harboring a deletion in the *SAC1* gene (*sac1Δ*) were found to be deficient in ATP-transporting activity as well as severely compromised in their ability to translocate nascent prepro-α-factor and preprocarboxypeptidase Y. Proteins isolated from the microsomal membranes of a *sac1Δ* strain were incapable of stimulating ATP transport when reconstituted into the in vitro assay system. When immunopurified to homogeneity and incorporated into artificial lipid vesicles, Sac1p was shown to reconstitute ATP transport activity. Consistent with the requirement for ATP in the lumen of the ER to achieve the correct folding of secretory proteins, the *sac1Δ* strain was shown to have a severe defect in transport of procarboxypeptidase Y out of the ER and into the Golgi complex in vivo. The collective data indicate an intimate role for Sac1p in the transport of ATP into the ER lumen.

SECRETION in eucaryotic cells commences with the translocation of secretory proteins into the lumen of the ER. In the yeast *Saccharomyces cerevisiae*, different sets of genes have been defined, encoding cytosolic, luminal, and ER membrane proteins involved in this multistep process (for review see Nunnari and Walter, 1992; Schekman, 1994). Factors responsible for cotranslational targeting of nascent secretory proteins to the membrane, such as the signal recognition particle and its receptor, are not crucial for cell viability (Hann and Walter, 1991; Ogg et al., 1992). In contrast, the subset of genes whose products are believed to form the translocation site on the membrane are essential. Biochemical analysis in a reconstituted system indicated that translocation across the lipid bilayer is facilitated by two membrane protein complexes that interact in a dynamic manner. A putative translocation pore is formed by Sec61p, Sss1p, and Sbh1p (Esnault et al., 1994; Panzner et al., 1995). The translocation process also requires the presence of an additional multisubunit complex consisting of Sec62p, Sec63p, Sec71p, Sec72p, and Kar2p, the yeast homologue of BiP (Brodsky and Schekman, 1993; Panzner et al., 1995). The importance of Kar2p in secretory protein translocation has also been shown genetically. In this case, a temperature-sensitive mutation in the *KAR2* gene caused the accumulation of secretory protein precursors in the cytosol at the nonpermissive temperature (Vogel et al., 1990; Nguyen et al., 1991).

Despite the rapid progress made in characterizing components required for translocation, our understanding of the driving force for the membrane passage of proteins is still limited. In a cotranslational mechanism, preproteins might be "pushed" across the membrane by elongation of the nascent chain itself. However, in yeast, where translocation can be uncoupled from translation (Toyn et al., 1988) and precursor proteins can be translocated posttranslationally into ER-derived vesicles in vitro (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986), another driving force must be operating. Using a cytosol-free assay, it was shown that the posttranslational membrane transfer of purified precursors requires only the precursor, microsomal membranes, and ATP (Sanz and Meyer, 1988, 1989). Based on the results of in vivo (Vogel et al., 1990; Nguyen et al., 1991) and in vitro studies (Sanders et al., 1992; Brodsky and Schekman, 1993), Kar2p is the only ER protein identified to date that is directly involved in translocation in yeast and that uses ATP. As a mechanism for translocation has been put forward in which preproteins are "pulled" across the mem-

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brane by ATP-dependent interactions with luminal chaperones (Simon et al., 1992; Glick, 1995), one can speculate that this represents the manifestation of the observed ATP requirement in the translocation process. Interactions of translocated chains with Kar2p are also essential for the proper folding of secretory proteins in the ER lumen and their further passage to the Golgi complex (Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Simons et al., 1995). A basic feature of such a mechanism is the continuous supply of ATP to the ER lumen. Recently a specific transport system was identified that allows efficient uptake of ATP into yeast ER (Mayinger and Meyer, 1993). When ATP uptake into microsomes was decreased below a critical level by specific inhibitors, co- as well as posttranslational translocation of preproteins was blocked.

In these studies, a reconstituted system was developed for the biochemical characterization of the ATP transporter. Using this assay, a component was purified that was shown to be the *SAC1* gene product. Loss of *SAC1* through gene deletion severely compromised translocation in vitro and subsequent intracellular transport steps in vivo. Incorporation of immunopurified Sac1p into proteoliposomes enabled the reconstitution of high levels of ATP transport. These results make a strong case for a direct involvement of Sac1p in the transport of ATP into the ER lumen, perhaps as the ATP transporter itself.

Materials and Methods

Reconstitution of ATP Transport into Proteoliposomes

Yeast microsomes were prepared according to Rothblatt and Meyer (1986). The membranes were resuspended in a buffer containing 30 mg/ml Triton X-100, 150 mM Na_2SO_4 and 10 mM Tris-HCl, pH 7.4, at a concentration of 10 mg protein/ml. After 10 min incubation at 4°C the mixture was centrifuged at 100,000 *g*. The detergent concentration of the supernatant was adjusted to 60 mg/ml by the addition of Triton X-100. A sonicated mixture of phosphatidylcholine and cholesterol (10:1) was added to this solution resulting in a final lipid concentration of 24 mg/ml. After the solution became translucent it was supplemented with nucleotide counter substrates at a final concentration of 10 mM and the mixture was incubated for 30 min at 0°C. Liposomes were generated by removal of detergent accomplished by repeated passage over Bio Beads SM2 columns (Bio-Rad Laboratories, Hercules, CA) (1 g SM2 beads/30 mg Triton X-100). The proteoliposomes were separated from nonreconstituted material and solutes by gel filtration on a Sephadex G-100 column (Sigma Chemical Co., St. Louis, MO).

ATP Transport Assay

ATP uptake into yeast microsomes was measured as described previously (Mayinger and Meyer, 1993). Nucleotide exchange in proteoliposomes was determined as follows: 300 μl liposome suspension containing a suitable counter substrate (usually 10 mM ATP or ADP) was rapidly mixed with 25 μM [^{14}C]ATP at 25°C. The exchange was stopped at different time points by rapid filtration of 50-μl aliquots using 100 mg wet Dowex (Sigma Chemical Co.) (8, chloride form, 100–200 mesh) in small glass columns. Free ATP is bound tightly by the strong anion exchange resin, while liposomes are not retained. The columns were washed once with 100 μl 100 mM MOPS, pH 7.2. Radioactivity in the combined eluate and wash was quantified by scintillation counting. Nonspecific uptake was determined either by treatment of liposomes with 4,4′-diisothiocyano-2,2′-stibene disulfonic acid (DIDS),[1] an effective inhibitor of ATP transport, or by conducting the transport assay at 0°C. Both methods gave approximately the same level of background. All transport assays were performed in the presence of carboxyatractyloside, a specific inhibitor of mitochondrial ATP transport, to rule out any influence of mitochondrial contamination.

Purification of ATP Transport Activity

Membranes (10 mg/ml final concentration) were extracted with 3% Triton X-100 and 150 mM Na_2SO_4 and 10 mM Tris-HCl, pH 7.4. The extract was mixed with a pasty suspension of hydroxyapatite (100 mg/mg protein). After centrifugation the supernatant was applied to an ATP agarose column (2.5 ml/ml supernatant). The column was washed with 10 ml buffer A (500 mM NaCl, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4) and with 10 ml buffer B (50 mM NaCl 1% Triton X-100, 10 mM Tris-HCl, pH 7.4). Then the column was eluted with 10 ml buffer B supplemented with 3 mM ATP. The eluate was concentrated on a centricon10 spin column (Amicon, Beverly, MA). The concentrated fractions were analyzed by SDS-PAGE or reconstituted into proteoliposomes and assayed for ATP transport as described above.

Microsequence Analysis

The ATP agarose eluate was analyzed by SDS-PAGE using 10% polyacrylamide gels. After staining and destaining, bands of interest were cut out and the gel pieces were washed twice for 20 min with 150 μl 50% acetonitrile to dehydrate the gel. The shrunken gel pieces were placed on parafilm and dried for an additional 15–25 min. The dried pieces were incubated overnight in 100 μl 80% formic acid containing 2 mg cyanogen bromide. To remove formic acid and excess cyanogen bromide the gel pieces were treated by three 20-min washes in 1 ml water followed by lyophilization. Then the pieces were incubated in SDS-PAGE sample buffer for 20 min and placed on top of a high resolution gel appropriate for separating small peptides (Schägger and von Jagow, 1987). The separated peptides were transferred to polyvinylene difluoride membranes. After staining and destaining bands were cut out and subjected to microsequence analysis.

Assays for Translocation

Prepro-α-factor (pp-αF) mRNA lacking glycosylation sites was prepared as described previously (Mayinger and Meyer, 1993). In vitro transcription, translation, and translocation reactions were performed as outlined in Rothblatt and Meyer (1986). An equal aliquot of translocation reactions were treated with proteinase K. The protease digestion was carried out at 0°C for 60 min at a final concentration of 0.5 mg/ml. Proteolysis was stopped by the addition of PMSF (20 mg/ml in isopropanol) to a final concentration of 1.5 mg/ml. The samples were supplemented with an equal amount of SDS-PAGE sample buffer and analyzed by SDS-PAGE using 16% polyacrylamide gels. Translocation products were quantified on a computer-assisted Geiger densitometer equipped with QuantProbe software (AMBIS Systems, Inc., San Diego, CA).

Metabolic Labeling

Relevant yeast strains were grown at 25°C in minimal medium to midlogarithmic phase. 1.25 OD_{600} cells were harvested, washed twice with salt/dextrose medium (SD) plus complete, and resuspended in 1.3 ml SD plus complete. The cells were incubated at 25°C for 15 min before [^{35}S]translabel was added (0.5 mCi) followed by incubation for 5 min at 25°C. 1.3 ml 2× chase solution was added (final concentration 0.03% methionine, 0.03% cysteine, and 0.03 M ammonium sulfate). Aliquots were removed at different time intervals and quenched on ice with 10 mM NaN_3. The samples were pelleted, washed in PBS, and lysed with glass beads. The cell extracts were analyzed by SDS-PAGE and fluorography.

Immunoprecipitations

For native immunoprecipitation of Sac1p, microsomes were solubilized in 3% Triton X-100, 150 mM Na_2SO_4, 10 mM Tris-HCl, pH 7.4. The extract was incubated with Sac1p mAbs (Whitters et al., 1993) that were covalently coupled to protein G–Sepharose. After three washing steps with extraction buffer and one with PBS, 1% Triton X-100, the beads were eluted with 100 mM glycine, 1% Triton X-100, pH 2. The eluate was immediately adjusted to pH 7.4 with Tris base. For all other immunoprecipitations, yeast cells were lysed with glass beads. Cell extracts were diluted in PBS, 1% Triton X-100, 0.1% SDS, and 10 mM NaN_3. 500-μl samples were treated with 50 μl of a 10% suspension of fixed *Staphylococcus au-*

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; DIDS, 4,4′diisothiocyano-2,2′-stibene disulfonic acid; pp-αF, prepro-α-factor; ppCPY, prepro–carboxypeptidase Y.

reus for 30 min. After centrifugation the appropriate antibodies were added and incubated overnight at 4°C. 25 μl of a 20% protein A–Sepharose suspension was added and the mixture was incubated for 2 h at 4°C. The protein A–Sepharose beads were pelleted and washed three times with PBS, 1% Triton X-100, 0.1% SDS, 10 mM NaN_3, pH 7.4, and once with 10 mM NaCl, 10 mM Tris-HCl, pH 6.8. The bound material was analyzed by SDS-PAGE.

Results

ATP Uptake Activity from Yeast Microsomes Can Be Reconstituted into Proteoliposomes as a Nucleotide Antiport

Yeast ER possesses an ATP uptake activity that is essential for protein translocation in vitro (Mayinger and Meyer, 1993). To understand how ATP is transported into the ER lumen and identify and characterize the transport protein, it was necessary to develop a reconstituted system for the precise analysis of this process. Incorporation of detergent extracts of microsomal membranes into proteoliposomes enabled the accurate measurement of ATP uptake kinetics needed for this kind of analysis. Microsomes were extracted with the nonionic detergents Triton X-100 or $C_{12}E_8$ and the reconstitution was carried out essentially as described for mitochondrial carrier proteins (Krämer and Klingenberg, 1979; Krämer and Heberger, 1986). The extracts were mixed with phosphatidylcholine liposomes, made by sonication and additional detergent. Highest activities were obtained with phospholipid mixtures containing 10% cholesterol. The detergent was removed by repeated passage of the mixture over small columns filled with Bio-Beads adsorbent. The resulting proteoliposomes were separated from nonincorporated material by gel filtration.

Measurements of the time course of nucleotide transport into proteoliposomes revealed that the uptake of radiolabeled ATP was strictly dependent on the presence of suitable counter substrates in the vesicle lumen (Table I). As described in Materials and Methods, nonspecific uptake was determined either by treatment of liposomes with DIDS, an effective inhibitor of ATP transport, or by conducting the transport assay at 0°C. In subsequent studies the liposomes were loaded with 10 mM ATP, and then by adding external radiolabeled ATP, the electroneutral isotope equilibration was followed. Under these conditions (with saturating amounts of internal substrate) the nucleotide exchange is a pseudo first order reaction that can be interpreted as a simple 1:1 nucleotide antiport (Fig. 1). This type of antiport is similar to the ADP/ATP exchange in mitochondria (Klingenberg, 1993). An apparent Km of 11 μM was calculated (Fig. 1) by determining the concentration dependence of the ATP exchange, which is consistent with values found for ATP uptake into intact yeast microsomes (Mayinger and Meyer, 1993). Since AMP was not accepted as a transportable substrate (Table I), it is likely that, in vivo, the uptake of ATP into yeast ER is coupled stoichiometrically with the export of ADP.

Table I. Substrate Specificity of ATP Uptake Reconstituted from Triton X-100 Extracts

External substrate	Internal substrate	Total uptake (percent of control)
50 μM ATP	10 mM ATP	100
50 μM ATP	10 mM ADP	112
50 μM ATP	10 mM AMP	7
50 μM ATP	None	4

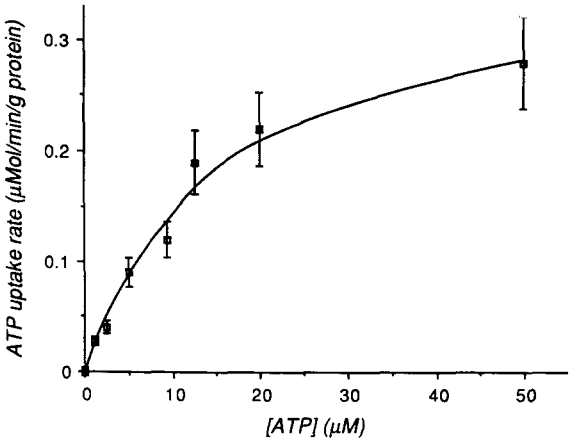

Figure 1. Kinetic analysis of microsomal ATP transport reconstituted into proteoliposomes. Yeast microsomes were extracted with Triton X-100 and Na_2SO_4. The extracts were reconstituted into phosphatidylcholine/cholesterol vesicles (Krämer and Heberger, 1986) resulting in proteoliposomes of ~1 mg protein/ml. The vesicle lumen contained 10 mM ATP. Equilibration kinetics were measured at 25°C by adding external [^{14}C]ATP to 50-μl aliquots of proteoliposomes. Only single time points, obtained within the first 20 s were measured to obtain concentration dependence of uptake. In control experiments it was shown that the time course of isotope equilibration follows pseudo first order kinetics and is close to linear for the first 30 s. For determining specific uptake, values obtained for DIDS-treated (0.5 mM) vesicles or by conducting the transport assay at 0°C were subtracted. Data are from three sets of uptake measurements. Km, 11.2 μM.

Sac1p Is a Constituent of a Highly Purified Fraction Active in ATP Transport

The proteoliposome system was then used as a means to purify components mediating nucleotide exchange. The different fractions obtained during the purification procedure were reconstituted into proteoliposomes followed by analysis of ATP uptake. To rule out that ATP binding rather than ATP uptake was being measured, the minor fraction of ATP bound to proteoliposomes at 0°C was subtracted as a background value. The same level of background ATP binding was obtained when the uptake assay was performed in the presence of 0.5% Triton X-100 or when the proteoliposomes were pretreated with 0.5 mM DIDS, a specific inhibitor of ATP transport into yeast (Mayinger and Meyer, 1993) and rat liver microsomes (Clairmont et al., 1992). The purification was accomplished by first adsorbing Triton X-100 extracts of microsomes onto hydroxyapatite, a method successfully used to purify a number of solute transporters of the inner mitochondrial membrane (Palmieri et al., 1993). Activity was only found in the unadsorbed fraction (Fig. 2, lanes *2* and *4*). Phase partitioning using Triton X-114 (Bordier, 1981) indicated that this fraction consists primarily of hydrophobic proteins (not shown). After the eightfold enrichment in specific activity achieved by chromatography on hydroxyapatite, further purification was carried out by affinity chromatogra-

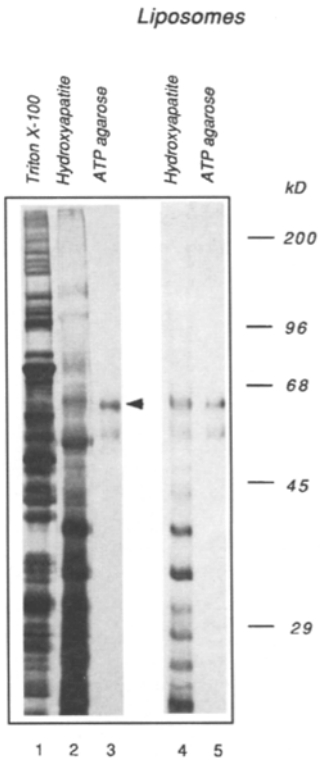

Figure 2. Purification of ATP transport activity from yeast microsomes. Yeast microsomes were extracted with Triton X-100 and Na_2SO_4 (lane *1*). The extracts were mixed with a suspension of hydroxyapatite, incubated on ice, and centrifuged. The supernatant (lane *2*) was applied to an ATP agarose affinity column, washed with 500 mM NaCl and 1% Triton X-100, and then eluted with 50 mM NaCl, 1% Triton X-100, and 3 mM ATP (lane *3*). Aliquots of the hydroxyapatite and ATP agarose fractions (lanes *2* and *3*) were reconstituted into proteoliposomes (lanes *4* and *5*). The different fractions were analyzed by SDS-PAGE and silver stain.

phy on ATP agarose. SDS-PAGE analysis of the ATP eluate showed two bands at ~68 and 50 kD (Fig. 2, lanes *3* and *5*). As in reconstituted detergent extracts from whole microsomes (Table I), ATP transport into proteoliposomes prepared from this fraction was strictly dependent on a suitable counter substrate (ADP or ATP). Although this two-step purification resulted in a 23-fold enrichment of specific activity (Table II), neither anion exchange chromatography nor gel filtration could resolve the polypeptides enriched after ATP agarose. Both polypeptides contained in this fraction therefore underwent microsequence analysis.

Since the NH_2-termini of both proteins were blocked, in-gel cleavage with cyanogen bromide was used to obtain internal sequences (see Materials and Methods). Sequencing of a major cleavage product of ~40 kD derived from the 68-kD band gave the partial amino acid sequence (M)GFIKLSLNREXIIANTVE that corresponds to the sequence of Sac1p, an integral membrane protein of unknown function in yeast ER and Golgi membranes (Novick et al., 1989; Cleves et al., 1989; Whitters et al., 1993). Immunoblot analysis of purified fractions with mAbs against Sac1p (Whitters et al., 1993) confirmed that the 68-kD band (Fig. 2, lanes *3* and *5*) is the product of the *SAC1* gene (not shown). Cleavage products for the 50-kD species could not be generated and this band remains to be identified. However, as the 50-kD protein was not consistently present in fractions active in ATP transport (data not shown), we considered it an unlikely candidate for the ATP transporter.

Absence of Sac1p Results in a Defect in Microsomal ATP Transport

The *SAC1* gene was originally identified in genetic screens for allele-specific suppressers of actin mutations (Novick et al., 1989) and bypass suppressers of mutations in *SEC14*, the structural gene for the yeast phosphatidylinositol transfer protein (Cleves et al., 1989). Recent results show that Sac1p is an integral membrane protein of 70 kD localized to ER and Golgi membranes (Whitters et al., 1993). As Sac1p was present as one of two polypeptides in a highly purified fraction containing ATP transport activity, it is likely that this protein represents the transporter itself. To pursue this hypothesis, the ATP uptake abilities of microsomes prepared from wild-type yeast versus membranes from a yeast strain where the *SAC1* gene had been disrupted (*sac1Δ*) (Whitters et al., 1993) were compared (Fig. 3). ATP uptake was measured as described earlier (Mayinger and Meyer, 1993). As seen in Fig. 3 *A*, microsomal membranes from *sac1Δ* strains showed significantly reduced ATP transport. A consistent background activity was observed, however, allowing ATP to enter the lumen of these microsomes at a rate equal to 15% of wild type. To determine the nature of the residual activity, we examined the effects of several inhibitors on microsomal ATP exchange (Fig. 3 *B*). The background ATP transport in *sac1Δ* microsomes was insensitive to carboxyatractyloside, a specific inhibitor of mitochondrial nucleotide transport but completely suppressed by DIDS, a strong inhibitor of several anion transporters including ATP transport into yeast ER (Mayinger and Meyer, 1993) and into rat liver ER and Golgi (Capasso et al., 1989; Clairmont et al., 1992). This suggests that an additional transporter is present in the microsomal membrane that mediates ATP transport at very low efficiency in the *sac1Δ* strain, which may account for the viability of the strain.

ATP Transport Reconstituted into Proteoliposomes Is Dependent on the Amount of Sac1p Present

It remains a possibility that the absence of a functioning Sac1p may only indirectly influence ATP transport. It has been shown that the absence of a functional Sac1p effects a bypass of the cellular requirement for the yeast phosphatidylinositol/phosphatidylcholine transfer protein Sec14p (Bankaitis et al., 1990; McGee et al., 1994). One could postulate that Sac1p is involved in the regulation of the phospholipid composition of membranes and is thereby only

Table II. Purification of ATP Transport Activity from Yeast Microsomes

Fraction	Total activity	Protein	Specific activity	Yield	Enrichment
	pMol/min	*mg*	*pMol/mg/min*	*%*	
Triton X-100 extract	406	2.26	180	100	1
Hydroxyapatite	189	0.138	1,369	47	8
ATP agarose	51	0.012	4,290	13	24

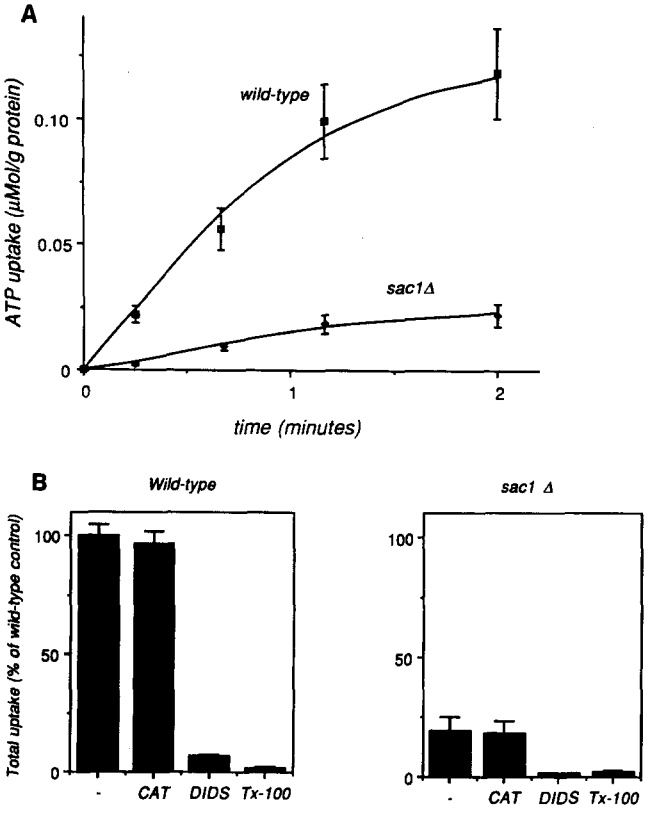

Figure 3. Microsomes from yeast with a deleted *SAC1* gene display a defect in ATP uptake. (*A*) Microsomes from wild-type and from *sac1Δ* strains were analyzed for ATP transport. Transport was started by addition of 25 μM [^{14}C]ATP to 50-μl aliquots of microsomes (10 mg protein/ml) and stopped at different time points by quick filtration over a strong anion exchange resin (Dowex X-1) to remove free substrate. For determining specific uptake, the amount of ATP associated with microsomes at 0°C or with DIDS-treated (0.5 mM) microsomes was subtracted. (*B*) Total uptake within 5 min of [^{14}C]ATP into microsomes from wild-type and from *sac1Δ* strains was determined. Where indicated 0.1 mM carboxy-atractyloside (*CAT*), 0.5 mM DIDS, or 0.1% Triton X-100 was added to the microsomes before uptake was measured.

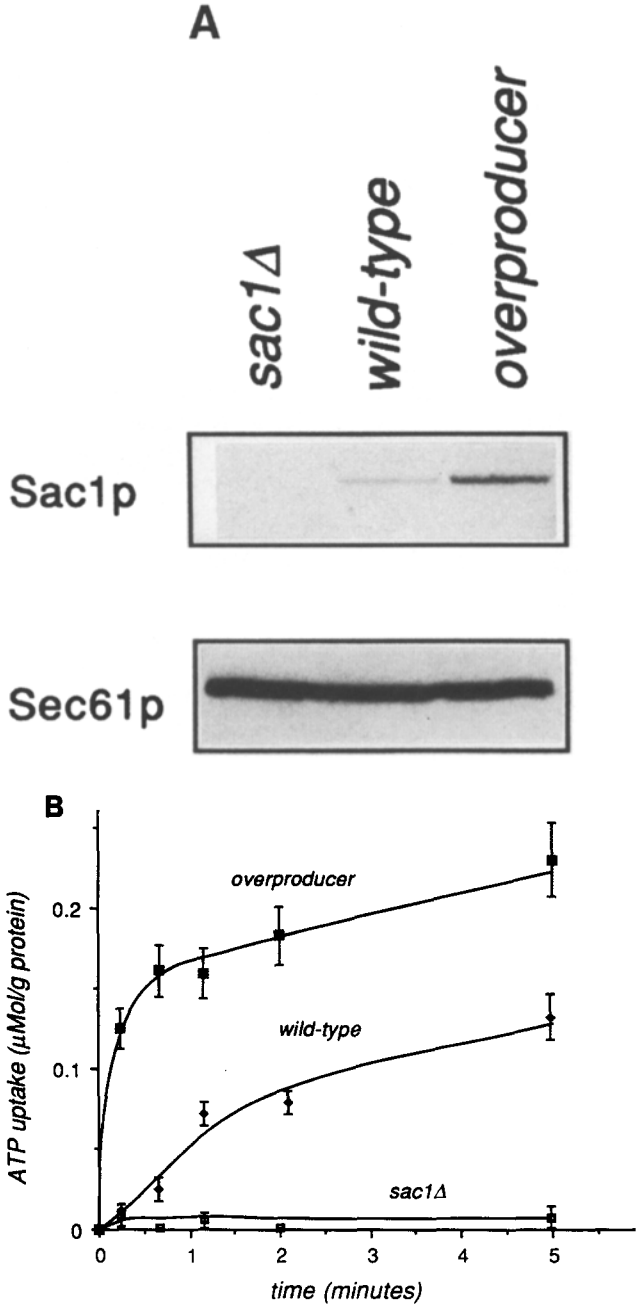

Figure 4. ATP transport into reconstituted proteoliposomes is dependent on the relative amounts of Sac1p present. Microsomes from wild-type, from overproducer, and from *sac1Δ* strains were solubilized and reconstituted into proteoliposomes as in Fig. 1. (*A*) Aliquots of the proteoliposomes were analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Sec61p and anti-Sac1p antibodies. (*B*) ATP uptake was assayed at 25°C by addition of 25 μM [^{14}C]ATP to 50-μl aliquots of proteoliposomes (1 mg protein/ml), preloaded with 10 mM ATP. The transport reaction was stopped at different time points by quick filtration over a strong anion exchange resin (Dowex X-1) to remove free substrate. Nonspecific uptake was determined either by treatment of liposomes with 0.5 mM DIDS, an effective inhibitor of ATP transport, or by conducting the transport assay at 0°C.

indirectly influencing the activity of ATP transport. To exclude an altered lipid composition in the *sac1Δ* strain as being responsible for decreased ATP transport, all detergent extracts used in the following studies were reconstituted into proteoliposomes using the same phosphatidylcholine/cholesterol mixture as in Fig. 1.

Three different yeast strains were used for reconstitution. If ATP transport is indeed an intrinsic property of Sac1p, the reconstituted activity should depend on the level of Sac1p present in the reconstituted vesicles. Therefore, microsomes from a *sac1Δ* strain, a wild-type strain, and a strain containing multiple copies of a plasmid encoding the *SAC1* gene (overproducer strain), were extracted with Triton X-100 and reconstituted in proteoliposomes. Aliquots of these liposome preparations were analyzed by immunoblotting to quantify the relative amounts of Sac1p. Sac1p was not detected in extracts of *sac1Δ* membranes that were used for reconstitution, while proteoliposomes

prepared from the overproducer strain contained 10-fold more Sac1p compared with wild type (Fig. 4 *A*). As can be seen in Fig. 4 *B*, the initial rate of ATP transport is ~11-fold higher in the overproducer liposomes compared with the wild type. This is consistent with the increased transport activity being a direct consequence of the elevated Sac1p level. Little, if any ATP exchange could be measured in the *sac1Δ* proteoliposomes (Fig. 4 *B*). The steady state isotope incorporation, which is a measure of the total vesicle volume active in the ATP exchange reaction, reached about twice the wild type level when Sac1p overproducer proteoliposomes were used. This observation supports the idea that more than one active transporter is present per liposome and incorporation of additional carriers no longer increases the total uptake value. It also implies that less than one functional carrier per proteoliposome is present under wild type conditions.

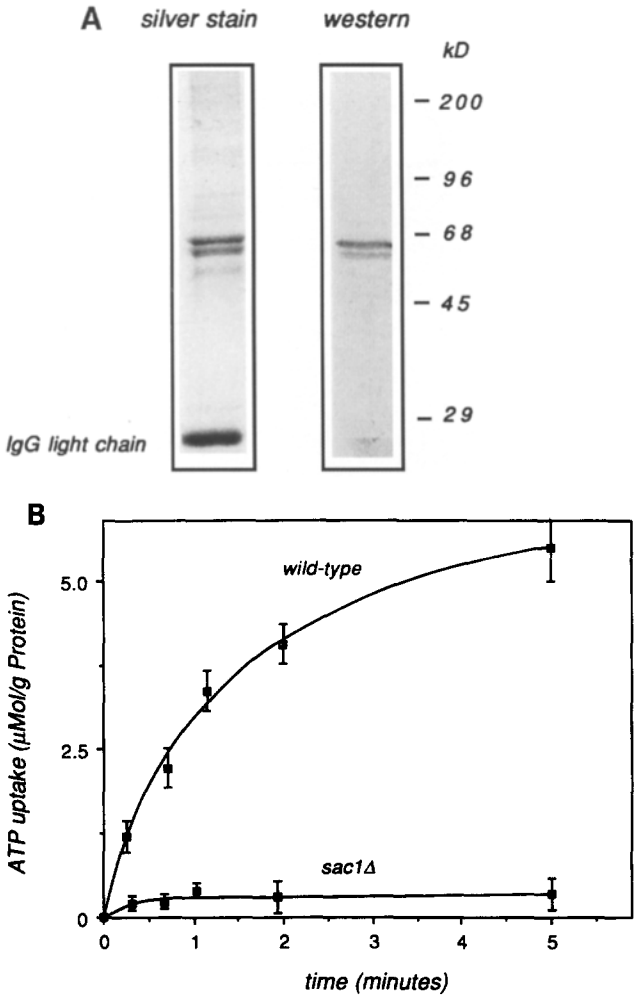

Figure 5. ATP transport activity can be immunoprecipitated with Sac1p-specific mAbs. (*A*) Yeast microsomes were extracted with Triton X-100 and Na_2SO_4. The extracts were incubated with Sac1p mAbs covalently bound to protein G–Sepharose. After washing, the bound material was eluted at low pH and analyzed by SDS-PAGE. (*B*) The material eluted from the protein G–Sepharose was adjusted to pH 7.4, reconstituted into proteoliposomes, and assayed for ATP transport as in Fig. 4.

ATP Transport Activity Can Be Immunopurified Using mAbs Against Sac1p

To obtain more direct evidence for the role of Sac1p in ATP transport, Sac1p was immunopurified before reconstitution into proteoliposomes. For this purpose, we coupled mAbs raised against Sac1p (Whitters et al., 1993) to protein G–Sepharose beads. These beads were used to purify Sac1p from detergent extracts. The material specifically bound to the conjugated antibodies was eluted by treatment with a low pH buffer and immediately neutralized. The immunopurified fraction consisted of two major species, and as both were recognized by Sac1p-specific antibodies on an immunoblot (Fig. 5 *A*), the band of lower apparent mol wt is most likely a breakdown product. The additional minor contaminating band (Fig. 5 *A*) was also present in control fractions obtained from *sac1Δ* microsomes (not shown). This fraction was reconstituted into proteoliposomes and assayed for ATP transport as described above. Although Sac1p was almost quantitatively removed from the detergent extracts by the antibody treatment and quantitatively incorporated into the liposomes (not shown), only ~10% of the total activity could be recovered, resulting in a 15-fold enrichment in specific activity (Fig. 5 *B*). These results suggest that the ATP transport activity is indeed associated with Sac1p, but only a fraction of the purified protein is functioning after reconstitution. Immunopurification of extracts from *sac1Δ* microsomes also yielded a fraction possessing some ATP uptake activity, representing ~5% of wild type (Fig. 5 *B*). This result is consistent with the presence of an additional ATP transporter that is antigenically related to Sac1p. This putative transporter is the subject of further investigation.

Sac1p-mediated ATP Transport Is Crucial for Preprotein Translocation In Vitro

A minimal level of ATP transport is necessary for maintaining the ability of yeast microsomes to translocate preproteins (Mayinger and Meyer, 1993). Based on these findings, one could expect that the significant reduction of ATP uptake seen in *sac1Δ* microsomes would impair protein translocation. Accordingly the translocation of pp-αF and prepro–carboxypeptidase Y (ppCPY) into wild-type and *sac1Δ* microsomes was analyzed. For better visualization of translocated species, a pp-αF construct was used in which the three glycosylation sites had been removed (Mayinger and Meyer, 1993). In the standard translocation assay, cotranslational translocation of both precursors into *sac1Δ* microsomes was clearly reduced (Fig. 6 *A*). Translocation of pp-αF was reduced by >80% while the translocated form of CPY species was not detected at all. To explore Sac1p-dependent translocation, the posttranslational translocation of pp-αF was examined in *sac1Δ* and wild-type membranes. This assay allowed a detailed examination of the time course of the translocation process. Translation was blocked by the addition of cycloheximide before the translocation reaction was started by the addition of wild-type or *sac1Δ* membranes to the incubation. At the indicated times, aliquots were removed and translocation was stopped by cooling the samples on ice. Again, *sac1Δ* microsomes showed impaired translocation and it appeared

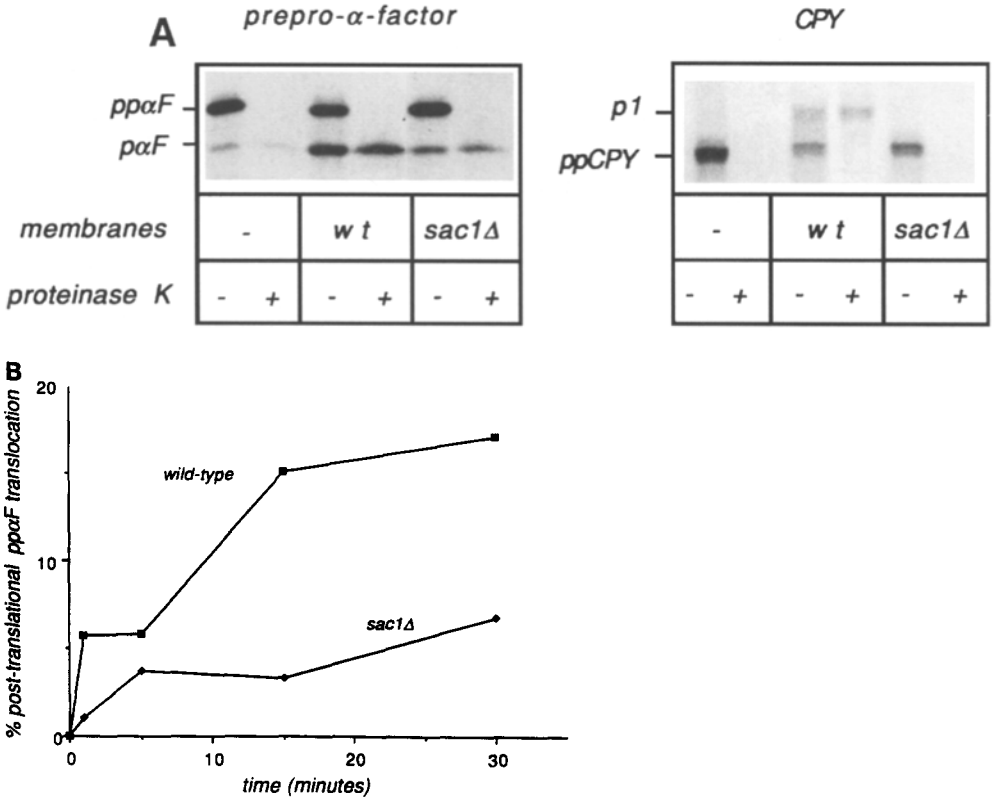

Figure 6. Translocation of prepro-α-factor into microsomes from *sac1Δ* strains is impaired. (*A*) Cotranslational translocation of CPY and a pp-αF without glycosylation sites was assayed using membranes from wild-type and *sac1Δ* strains. Protection of pro-α-factor and p1-CPY from proteinase K digestion was used to detect translocated species. (*B*) Posttranslational translocation. Translation of pp-αF was stopped by the addition of cycloheximide before membranes were added. At different time points aliquots were removed and the reaction was quenched on ice. The amount of translocated pro-α-factor was assayed by protection from proteinase K digestion and quantified by Geiger densitometry.

that the reduced ATP transport seen in the absence of Sac1p correlates with a slowdown in the posttranslational translocation of pp-αF (Fig. 6 *B*). These results are consistent with previous data showing that an inhibition of ATP uptake with specific inhibitors reduced all modes of protein transport into yeast ER (Mayinger and Meyer, 1993).

Strains Lacking Sac1p Display a Defect in Intracellular Protein Transport In Vivo

If Sac1p indeed regulates ATP levels in the ER lumen, one could predict that a reduced luminal concentration of ATP would be reflected in other intracellular transport steps known to be ATP sensitive. For example, it has been suggested that the ADP/ATP ratio may be a crucial factor for the chaperone-mediated folding of proteins. Gething and Sambrook (1992) have reported that the association between members of the hsp70 protein family such as BiP and secretory proteins is dependent on ADP and ATP. In more recent studies it was shown that binding to hsp70 is potentiated when ADP is bound to these chaperones and release of the bound polypeptide is mediated by replacement of ADP with ATP (McKay et al., 1994; Hightower et al., 1994). Thus, decreased ATP levels in the ER lumen should lead to a decrease in the ATP-mediated release from BiP and a subsequent inhibition or delay in the exit of secretory proteins from the ER. A *sac1Δ* strain should therefore exhibit a defect in secretory protein transport from ER to Golgi. Kar2p has been shown to be critical for CPY folding inside the ER lumen (Simons et al., 1995). Accordingly, the intracellular transport of CPY was analyzed by pulse labeling and immunoprecipitation. CPY enters the secretory pathway via translocation into the ER (Stevens et al., 1982) and is transported to the vacuole, undergoing multiple modifications upon transit through the ER and the Golgi complex. Signal sequence cleavage and core glycosylation result in the p1 form (ER), transit through the Golgi complex leads to further oligosaccharide modification to the p2 form, and the protein is finally sorted to the vacuole where it is cleaved to the mature form by the *PEP4* gene product (Hemmings et al., 1981). In wild-type cells (Fig. 7) the prepro form cannot be detected and the conversion from the p1 (ER) to the p2 (Golgi) form is comparatively fast. In *sac1Δ* yeast, however, the ER to Golgi transport step was significantly slower (Fig. 7). The half-life of the p1 form was approximately three times longer in *sac1Δ* cells than in wild-type cells. Sorting from the Golgi to the vacuole seemed to be less affected in *sac1Δ* cells. This result is consistent with the hypothesis that *sac1Δ* strains possess lowered luminal ATP levels.

As in wild-type yeast, a nontranslocated prepro form of CPY was not observed in *sac1Δ* cells. Thus, in contrast to our in vitro experiments (Fig. 6 *A*), a translocation defect for CPY was not visible in vivo. The significance of this finding is discussed below.

Discussion

The results presented provide strong evidence linking ATP uptake into yeast microsomes to preprotein translocation and to transport from ER to Golgi. The presence of microsomal ATP-uptake systems in mammalian cells (Capasso et al., 1989; Clairmont et al., 1992) as well as in yeast

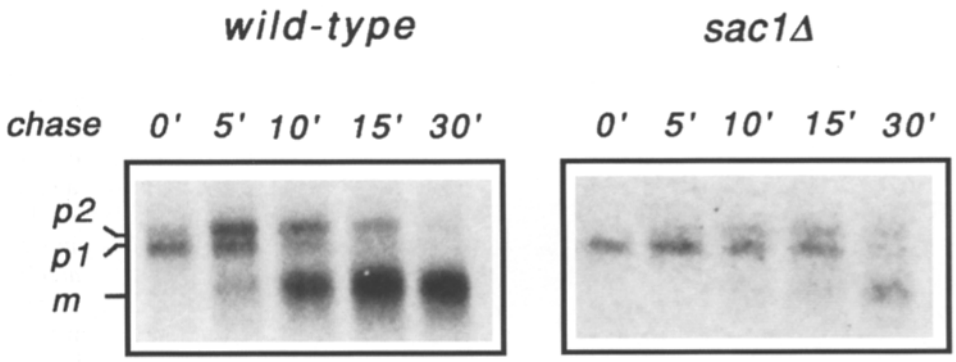

Figure 7. Transport of CPY from ER to Golgi is slowed down in *sac1Δ* strains. Cells from wild-type and *sac1Δ* strains were grown to mid-logarithmic phase. The cells were pulsed with [^{35}S]methionine and [^{35}S]cysteine for 5 min at 25°C. The reaction was chased with nonlabeled amino acids for the times indicated. The cells were lysed and the extracts immunoprecipitated with anti-CPY polyclonal antibodies. The precipitated material was analyzed by SDS-PAGE and fluorography. *p1*, ER form; *p2*, Golgi form; *m*, mature CPY.

(Mayinger and Meyer, 1993) has been described. Here, we analyzed microsomal ATP uptake in yeast by reconstitution of this activity into proteoliposomes. We found that uptake of ATP into these vesicles was strictly dependent on a transportable counter substrate. Accordingly, the ATP uptake into yeast microsomes was defined as nucleotide antiport. This mechanism is also seen in mitochondrial nucleotide exchange (Klingenberg, 1993) and many other solute transporters (Palmieri et al., 1993). Since AMP is not accepted as a substrate, we propose that ATP uptake into yeast microsomes is coupled to export of ADP in vivo.

The reconstituted system proved to be a powerful tool to purify ATP transport activity. We found that Sac1p, the product of a previously described gene of unknown function (Novick et al., 1989; Cleves et al., 1989; Whitters et al., 1993), is the main constituent of a highly purified fraction enriched in ATP transport. A detailed analysis of ATP transport into microsomes and into proteoliposomes generated from detergent extracts provided several lines of evidence that Sac1p possesses ATP transport activity. First, deletion of the *SAC1* gene resulted in a drastic reduction of ATP uptake activity into microsomes. Second, when reconstituted into proteoliposomes, ATP transport rates were proportional to the amount of Sac1p present. Proteoliposomes prepared from a Sac1p-overproducing yeast strain showed elevated transport kinetics, whereas practically no activity could be reconstituted from *sac1Δ* yeast microsomes. Third, mAbs raised against the central portion of Sac1p (Whitters et al., 1993) could be used to immunopurify ATP transport with high specific activity from detergent extracts.

We cannot unequivocally rule out the possibility that Sac1p represents a regulatory factor interacting with the actual ATP transporter, since minor contaminating bands were consistently present in our purified fractions. The fact that the *sac1Δ* strain is still viable, but cold sensitive for growth (Whitters et al., 1993), may be explained by the fact that microsomes from *sac1Δ* yeast are still capable of ATP transport albeit with very low efficiency. This is also supported by the fact that some ATP transport activity could be immunopurified from *sac1Δ* microsomes using the anti-Sac1p mAb. Further characterization of this activity will be pursued. It is important to note that as our initial kinetic analysis of ATP transport into yeast microsomes did not indicate the presence of additional ATP-uptake systems (Mayinger and Meyer, 1993), it is possible that, in a *sac1Δ* strain, the absence of a functional Sac1p leads to an up-regulation of another transporter, thereby maintaining a sufficient level of luminal ATP for survival.

Sac1p is known to be an integral protein of ER and Golgi membranes. However, its predicted secondary structure based on hydrophobicity analysis does not give a clear homology to other solute transporters. Its primary sequence shows that it is rather hydrophilic with one putative transmembrane domain (Cleves et al., 1989). However, further analysis of the primary structure of Sac1p indicates that it contains additional amphipathic helices which could span the membrane (Mayinger, P., and D. I. Meyer, unpublished observations). An unusually high hydrophilicity was also found in some mitochondrial transporters including the ADP/ATP carrier (Klingenberg, 1993). A more refined analysis was necessary to assign to relatively hydrophilic segments of the primary structure a role as potential amphipathic transmembrane helices (Aquila et al., 1985). Analysis of the transmembrane topology of Sac1p together with a determination of how this polypeptide mediates ATP transport will be pursued. Recently, a Golgi adenosine 3′-phosphate 5′-phosphosulfate transporter was purified from rat liver that functions as a homodimer of 70-kD subunits (Mandon et al., 1994). It is therefore possible that Sac1p represents a member of a microsomal solute transporter family in yeast.

ATP transport into yeast microsomes is essential for protein translocation in vitro (Mayinger and Meyer, 1993). However, ATP transport was not rate limiting for this process and could be reduced significantly before an effect on translocation of proteins was observed. In agreement with these earlier data, cotranslational translocation of pp-αF and CPY into ATP transport-deficient microsomes from *sac1Δ* yeast was substantially reduced when measured in vitro. Analysis of the time course of posttranslational translocation of pp-αF into the mutant membranes suggested that the rate, rather than the overall amount of transmembrane passage of proteins, is reduced. This result suggests that ATP transport into membranes lacking Sac1p was reduced to a level where it became rate limiting for translo-

cation. As a consequence the overall translocation process measured in vitro was significantly slowed.

In vivo analysis of CPY sorting to the vacuole showed that reduced microsomal ATP transport led to a clear decrease in the exit rate of precursors from the ER. We believe that the prolonged retention of a secretory protein in the ER lumen in *sac1Δ* yeast was a direct consequence of the impaired ATP transport into this organelle. It is known that binding and dissociation of unfolded proteins to members of the Hsp70 family is regulated by adenine nucleotides (Hightower et al., 1994; McKay et al., 1994). Kar2p, the yeast homologue of mammalian BiP, represents the Hsp70 species of the ER (Rose et al., 1989; Normington et al., 1989). Recent work shows that polypeptides associate tightly with Hsp70 proteins when ADP is bound to the chaperone. This complex dissociates in the presence of ATP, whereby the release is mediated by ATP binding rather than by ATP hydrolysis (Sadis and Hightower, 1992; Hightower et al., 1994). The drastic reduction of ATP uptake into *sac1Δ* ER membranes should effect a significant decrease of the luminal ATP/ADP ratio. As a consequence, association of Kar2p with translocated proteins is stabilized, which leads to a prolonged retention of those polypeptides inside the ER lumen causing the observed slowdown of CPY transfer to the Golgi. On the other hand, our in vivo analysis of CPY processing did not show a clear translocation defect in *sac1Δ* yeast. The most likely explanation for this finding is that the translocation rate was decreased in *sac1Δ* cells in vivo, but no accumulation of non-translocated precursor was found as preprotein translocation is not the rate-limiting step in the intracellular transport of CPY.

Certainly more work will be required to elucidate in detail all ATP-dependent events in the ER lumen that have importance in protein transport. Our results strongly indicate that ATP is required for different steps in this process and they underscore the importance of Kar2p in secretion. It has been shown genetically that the absence of a functioning *KAR2* gene significantly influences translocation (Vogel et al., 1990; Nguyen et al., 1991). Biochemical studies indicate that Kar2p interacts with translocating chains (Sanders et al., 1992) and Kar2p is also essential for translocation of precursors into reconstituted proteoliposomes (Brodsky et al., 1993; Panzner et al., 1995). Once a secretory protein has entered the ER lumen, Sac1p-mediated ATP transport is essential for a second important role of Kar2p in protein transport through the secretory pathway, i.e., an ATP-dependent folding of translocated proteins, an essential step in their exit from the ER and subsequent trafficking to the Golgi complex (Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Simons et al., 1995).

Another important aspect of the characterization of Sac1p's role in microsomal ATP transport and secretion are the known pleiotropic phenotypes associated with a loss of Sac1p function (Cleves et al., 1989; Novick et al., 1989; Whitters et al., 1993). It has been found that mutations in *SAC1* display multiple interactions with *sec* mutations. The *sac1-6^{cs}* allele, which results in loss of Sac1p function, aggravates *sec^{ts}* mutations connected with ER to Golgi transport (Cleves et al., 1989). *sec13-1^{ts}* and *sec20-1^{ts}* display synthetic lethality with *sac1-6^{cs}*, whereas *sec17-1^{ts}*, *sec18-1^{ts}*, *sec21-1^{ts}*, and *sec23-1^{ts}* are significantly more temperature sensitive in a *sac1-6^{cs}* background (Cleves et al., 1989). Loss of Sac1p function also bypasses the requirement for phosphatidylinositol transfer protein activity (Sec14p), which is essential for cell viability and Golgi secretory function, and is responsible for partial suppression of mutations in other secretory genes like *SEC9* (Cleves et al., 1989; Whitters et al., 1993). Importantly, *SAC1* was originally found through its ability to suppress certain mutations in *ACT1* (Novick et al., 1989). As polymerization and depolymerization of actin filaments are influenced by ADP and ATP levels in the cytosol (Pollard, 1990), a relative rise in cytosolic ATP concentration caused by the defect in microsomal ATP uptake, may be important in stabilizing actin filaments in yeast with mutations like *act1-1*. The result would be the suppression of the *act1-1* phenotype. As it is an open question whether the sole in vivo function of Sac1p is in ATP transport, further studies are required to understand completely how Sac1p functions in this and other vital cellular processes.

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