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Authors
Mayinger, P
Bankaitis, VA
Meyer, DI

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

Peter Mayinger,* Vytas A. Bankaitis,† and David I. Meyer*

*Department of Biological Chemistry, University of California, Los Angeles School of Medicine, Los Angeles, California 90024; and †Department of Cell Biology, The University of Alabama at Birmingham, Birmingham, Alabama 35294

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.

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ecretion 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-
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 postranslational 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 Na2SO4, 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 10 mg/ml. The extract was mixed with a suspension of hydroxyapatite (100 mg/ml 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 centrifuge 10 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 paraffin 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 with three 20-min washes in 1 ml water followed by phosphilization. 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 polyvinylidene 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 translation reaction was 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 QuanProbe software (AMBUS Systems, Inc., San Diego, CA).

Metabolic Labeling

Relevant yeast strains were grown at 25°C in minimal medium to midlogarithmic phase. 1.25 OD600 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 [35S]translabeled was added (0.5 MCl) followed by incubation for 5 min at 25°C. 1.3 ml 2X chase solution was added (final concentration 0.03% mercaptoethanol, 0.03% cysteine, and 0.03 M ammonium sulfate). Aliquots were removed at different time intervals and quenched on ice with 10 mM Na2N3. 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 Na2SO4, 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 NaCl. 500-μl samples were treated with 50 μl of a 10% suspension of fixed Saphyilococcus au-
from Triton X-IO0 Extracts

ATP Uptake Activity from Yeast Microsomes

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_{2}SO_{4}. The extracts were reconstituted into phosphatidylincholine/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 [^{3}H]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 chromatography.
Saclp (Whitters et al., 1993) confirmed that the 68-kD band (Fig. 2, lanes 3 and 5) is the product of the vick et al., 1989; Cleves et al., 1989; Whitters et al., 1993). Since the NH2-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 Saclp, an integral membrane protein of un-known function in yeast ER and Golgi membranes (Whitters et al., 1993). As Saclp 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 2. Purification of ATP transport activity from yeast microsomes. Yeast microsomes were extracted with Triton X-100 and Na2SO4 (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.

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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

**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 [14C]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 [14C]ATP into microsomes from wild-type and from sac1Δ strains was determined. Where indicated 0.1 mM carboxy-tractylloside (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 [14C]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.
prepared from the overproducer strain contained 10-fold more Saclp 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 Saclp level. Little, if any ATP exchange could be measured in the saclΔ 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 Saclp 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.

ATP Transport Activity Can Be Immunopurified Using mAbs Against Saclp

To obtain more direct evidence for the role of Saclp in ATP transport, Saclp was immunopurified before reconstitution into proteoliposomes. For this purpose, we coupled mAbs raised against Saclp (Whitters et al., 1993) to protein G–Sepharose beads. These beads were used to purify Saclp 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 Saclp-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 saclA microsomes (not shown). This fraction was reconstituted into proteoliposomes and assayed for ATP transport as described above. Although Saclp 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 Saclp, but only a fraction of the purified protein is functioning after reconstitution. Immunopurification of extracts from saclΔ 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 Saclp. This putative transporter is the subject of further investigation.

Saclp-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 saclΔ microsomes would impair protein translocation. Accordingly the translocation of pp-αF and prepro-carboxypeptidase Y (ppCPY) into wild-type and saclΔ 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 saclΔ 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 Saclp-dependent translocation, the posttranslational translocation of pp-αF was examined in wild-type and saclΔ 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 saclΔ membranes to the incubation. At the indicated times, aliquots were removed and translocation was stopped by cooling the samples on ice. Again, saclΔ microsomes showed impaired translocation and it appeared

\[ \text{Figure 5. ATP transport activity can be immunoprecipitated with Saclp-specific mAbs. (A) Yeast microsomes were extracted with Triton X-100 and Na}_2\text{SO}_4. The extracts were incubated with Saclp mAbs covalently bound to protein G–Sepharose. After washing, the bound material was eluted at low pH and analyzed by SDS-PAGE. (B) The material eluted from the protein G–Sepharose was adjusted to pH 7.4, reconstituted into proteoliposomes, and assayed for ATP transport as in Fig. 4.} \]
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 secretary 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 pl 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 cleared 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 pl (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 pl 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
into yeast microsomes is coupled to export of ADP in vivo. 

Proteoliposomes enriched in ATP transport were generated from detergent extracts provided several lines of evidence that Saclp 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 Saclp present. Proteoliposomes prepared from a Saclp-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 Saclp (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 Saclp 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-Saclp 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 Saclp leads to an up-regulation of another transporter, thereby maintaining a sufficient level of luminal ATP for survival.

Saclp 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 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 Saclp 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 Saclp 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-oF 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-oF 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 Saclp 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 \textit{sec} \textit{A} 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 yeast homologue of mammalian BiP, represents the ATP-dependent shuttling of polypeptides to the Golgi (Rose et al., 1989; Normington et al., 1996). 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, 1994; Hightower et al., 1994). The drastic reduction of ATP uptake into \textit{sec} \textit{A} 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 \textit{sec} \textit{A} yeast. The most likely explanation for this finding is that the translocation rate was decreased in \textit{sec} \textit{A} 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 \textit{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, Sactlp-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 Sactlp's role in microsomal ATP transport and secretion are the known pleiotropic phenotypes associated with a loss of Sactlp function (Cleves et al., 1989; Novick et al., 1989; Whitters et al., 1993). It has been found that mutations in \textit{SAC1} display multiple interactions with \textit{sec} mutations. The \textit{sactl-6} \textsuperscript{ts} allele, which results in loss of Sactlp function, aggravates \textit{sec6} mutations connected with ER to Golgi transport (Cleves et al., 1989), \textit{sec13-l} \textsuperscript{ts} and \textit{sec20-l} \textsuperscript{ts} display synthetic lethality with \textit{sactl-6} \textsuperscript{ts}, whereas \textit{sec17-l} \textsuperscript{ts}, \textit{sec18-l} \textsuperscript{ts}, \textit{sec21-l} \textsuperscript{ts}, and \textit{sec23-l} \textsuperscript{ts} are significantly more temperature sensitive in a \textit{sactl-6} \textsuperscript{ts} background (Cleves et al., 1989). Loss of Sactlp function also bypasses the requirement for phosphorylaminositol 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 \textit{SEC9} (Cleves et al., 1989; Whitters et al., 1993). Importantly, \textit{SACI} was originally found through its ability to suppress certain mutations in \textit{ACT7} (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 \textit{actl-l}. The result would be the suppression of the \textit{actl-l} phenotype. As it is an open question whether the sole in vivo function of Sactlp is in ATP transport, further studies are required to understand completely how Sactlp functions in this and other vital cellular processes.

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References

Aquila, H., T. A. Link, and M. Klingenberg. 1985. The uncoupling protein from brown fat mitochondria is related to the mitochondrial ADP/ATP carrier. Analysis of sequence homologies and of folding of the protein in the membrane. \textit{EMBO J.} (Eur. Mol. Biol. Organ.) 4:2369-2376.

Bankaitis, V. A., J. R. Aitken, A. E. Cleves, and W. Dowhan. 1990. An essential role for a phospholipid transfer protein in yeast Golgi function. \textit{Nature (Lond.)}, 345:561-562.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. \textit{J. Biol. Chem.} 256:1604-1607.

Broshsky, J. L., and R. Schekman. 1993. A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. \textit{J. Cell Biol.} 123:1355-1363.

Capasso, J. M., T. W. Keenan, C. Abejon, and C. B. Hirschberg. 1989. Mechanism of phosphorylation in the lumen of the Golgi apparatus. Translocation of adenosine 5'-triphosphate into Golgi vesicles from rat liver and mammary gland. \textit{J. Biol. Chem.} 264:5233-5240.

Clairmont, C. A., A. Da Maio, and C. B. Hirschberg. 1992. Translocation of ATP into the lumen of rough endoplasmic reticulum-derived vesicles and its binding to luminal proteins including BiP (GRP 78) and GRP 94. \textit{J. Biol. Chem.} 267:3983-3990.

Cleves, A. E., P. J. Novick, and V. A. Bankaitis. 1989. Mutations in the \textit{SACI} gene suppress defects in yeast Golgi and yeast actin function. \textit{J. Cell Biol.} 109:2895-2899.

Cleves, A. E., P. J. Novick, and V. A. Bankaitis. 1989. Mutations in the \textit{SACI} gene suppress defects in yeast Golgi and yeast actin function. \textit{J. Cell Biol.} 109:2895-2899. 80:

Cleves, A. E., P. J. Novick, and V. A. Bankaitis. 1989. Mutations in the \textit{SACI} gene suppress defects in yeast Golgi and yeast actin function. \textit{Cell.} 67:131-144.

Hann, W. P. D. Garcia, and P. Walter. 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of prepro-alpha-factor. \textit{Cell.} 45:397-406.

Hemnings, B. A., G. S. Zabbenko, A. Hassilik, and E. W. Jones. 1981. Mutant

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defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 78:435-439.

Hightower, L. E., S. E. Sadis, and I. M. Takenaka. 1994. Interactions of vertebrate hsc70 and hsp70 with unfolded proteins and peptides. *In The Biology of Heat Shock Proteins and Molecular Chaperones.* R. I. Morimoto, A. Tissieres, and C. Georgopoulos, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 179-207.

Klingenberg, M. 1993. Mitochondrial carrier family: ADP/ATP carrier as a carrier paradigm. *Soc. Gen. Physiol. Ser.* 48:201-212.

Kramer, R., and C. Heberberger. 1986. Functional reconstitution of carrier proteins by removal of detergent with a hydrophobic ion exchange column. *Biochim. Biophys. Acta.* 863:289-296.

Krämer, R., and M. Klingenberg. 1979. Reconstitution of adenine nucleotide transport from beef heart mitochondria. *Biochemistry.* 19:4209-4215.

Mandon, E. C. M. E. Milla, E. Kempner, and C. B. Hirschberg. 1994. Purification of the Golgi adenine 3'-phosphate 5'-phosphosulfate transporter, a homodimer within the membrane. *Proc. Natl. Acad. Sci. USA.* 91:10707-10711.

Mayinger, P., and D. I. Meyer. 1993. An ATP transporter is required for protein translocation into yeast endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.)* 12:659-666.

McGee, T. P., H. B. Skinner, E. A. Whitters, S. A. Henry, and V. A. Bankaitis. 1994. A phosphatidylinositol transfer protein controls the phosphatidylinositol content of yeast Golgi membranes. *J. Cell Biol.* 124:273-287.

Mckay, D. B., S. M. Wilbanks, K. M. Flaherty, J.-H. Ha, M. C. O'Brien, and L. L. Shirvanee. 1994. Stress-70 proteins and their interaction with nucleotides. *In The Biology of Heat Shock Proteins and Molecular Chaperones.* R. I. Morimoto, A. Tissieres, and C. Georgopoulos, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 153-177.

Nguyen, T. H., T. D. S. Law, and D. B. Williams. 1991. Binding protein BiP is required for translocation of secretory proteins into the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 88:1565-1569.

Normington, K., K. Kohno, Y. Kozotsumi, M.-J. Gething, and J. Sambrook. 1989. *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell.* 57:1223-1236.

Novick, P., B. C. Osmond, and D. Botstein. 1989. Suppressors of yeast actin mutations. *Genetics.* 121:659-674.

Nunnari, J., and P. Walter. 1992. Protein targeting to and translocation across the membrane of the endoplasmic reticulum. *Curr. Opin. Cell Biol.* 4:573-580.

Ogg, S. C., M. A. Poritz, and P. Walter. 1992. Signal recognition particle receptor is important for cell growth and protein secretion in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 3:895-911.

Palmeri, F., C. Indiveri, F. Bissaccia, and R. Krämer. 1993. Functional properties of purified and reconstituted mitochondrial metabolite carriers. *Journal of Bioenergetics and Biomembranes.* 25:525-535.

Panzner, S., L. Dreier, E. Hartmann, S. Koutka, and T. A. Rapoport. 1995. Post-translational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell.* 81:561-570.

Pollard, T. D. 1990. *Actin.* *Curr. Opin. Cell Biol.* 2:33-40.

Rose, M. D., L. M. Mirza, and J. P. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell.* 57:1211-1221.

Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertase in a homologous cell-free system. *Cell.* 44:619-628.

Sadis, S., and L. E. Hightower. 1992. Unfolded proteins stimulate molecular chaperone hsc70 ATPase by accelerating ADP/ATP exchange. *Biochemistry.* 31:9406-9412.

Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell.* 65:353-365.

Sanz, P., and D. I. Meyer. 1988. Signal recognition particle (SRP) stabilizes the translocation-competent conformation of pre-secretory proteins. *EMBO (Eur. Mol. Biol. Organ.)* 7:3555-3557.

Sanz, P., and D. I. Meyer. 1989. Secretion in yeast: preprotein binding to a membrane receptor and ATP-dependent translocation are sequential and separable events in vitro. *J. Cell Biol.* 108:2101-2106.

Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.

Schekman, R. 1994. Translocation gets a push. *Cell.* 78:911-913.

Simon, S. M., C. S. Peskin, and G. F. Oster. 1992. What drives the translocation of proteins? *Proc. Natl. Acad. Sci. USA.* 89:3770-3774.

Simons, J. F., S. Ferro-Novick, M. D. Rose, and A. Helenius. 1995. BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y folding in yeast. *J. Cell Biol.* 130:41-49.

Stevens, T., B. Emon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439-448.

Toyn, J., A. R. Hibbs, P. Sanz, J. S. Crowe, and D. I. Meyer. 1988. In vivo and in vitro analysis of ptfl, a yeast mutant with a membrane associated defect in protein translocation. *EMBO (Eur. Mol. Biol. Organ.)* 7:4347-4353.

Vogel, J. P., L. M. Misra, and M. D. Rose. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* 110:1885-1895.

Waters, M. G., and G. Blobel. 1986. Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. *J. Cell Biol.* 102:1543-1559.

Whitters, E. A., A. E. Cleves, T. P. McGee, H. B. Skinner, and V. A. Bankaitis. 1993. SAC1p is an integral membrane protein that influences the cellular requirement for phospholipid transfer protein function and inositol in yeast. *J. Cell Biol.* 122:79-94.