Genetic and molecular interactions of the Erv41p-Erv46p complex involved in transport between the endoplasmic reticulum and Golgi complex

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Summary

Erv41p and Erv46p are integral membrane proteins conserved across species. They were originally identified as abundant constituents of COPII-coated vesicles, and form a complex which cycles between the endoplasmic reticulum and Golgi complex. Yeast strains lacking these proteins are viable but display subtle secretory phenotypes. In order to obtain information about possible biological roles of this protein complex in endoplasmic reticulum to Golgi transport, we employed the Synthetic Genetic Array approach to screen for synthetic genetic interactions with the *erv46* null mutation. We identified synthetic interactions with *vma12*, *vma21*, *vma22* and *vps1* deletion mutations. The *vma21Δ* mutation exacerbates transport defects caused by the *erv46Δ* mutation. Unexpectedly, yeast strains lacking Vma21p fail to sort the endoplasmic reticulum to Golgi v-SNARE, Bos1p, efficiently into COPII vesicles, yet these vesicles are fully fusion competent. In addition, we set out to identify, by a biochemical approach, proteins interacting with the Erv41p-Erv46p complex. We report a strong interaction between the Erv41p-Erv46p complex and endoplasmic reticulum glucosidase II. Strains lacking a cycling Erv41p-Erv46p complex display a mild glycoprotein processing defect.

Key words: Vesicle trafficking, Endoplasmic reticulum, Golgi, COPII, Erv proteins

Introduction

Secretory proteins are synthesized at the rough endoplasmic reticulum (ER). After translocation into the ER lumen, they undergo folding and post-translational modifications before being transported downstream to the Golgi complex and beyond. Membrane-bound vesicles or transport intermediates play a crucial role in such transport steps. They are formed by the polymerization of distinct coat protein complexes on donor membranes and consumed by homotypic or heterotypic fusion events (Bonifacino and Glick, 2004). At ER exit sites, the COPII coat is thought to act in vesicle formation coupled with secretory cargo selection (Lee et al., 2004). Although progress has been made over the past few years, the mechanisms underlying regulated vesicle formation and, in particular, cargo selection remain poorly understood.

To discover proteins involved in transport between the ER and Golgi complex, we have previously used a reconstituted COPII vesicle formation assay combined with proteomics techniques (Otte et al., 2001). This study identified a number of previously uncharacterized gene products, which we termed Erv (for ER vesicle proteins), followed by a number indicating their molecular mass. Two of them, Erv41p and Erv46p, form a complex that is efficiently sorted into COPII vesicles and cycles between the ER and Golgi complex. Both are integral membrane proteins with two membrane spanning segments each, short N- and C-terminal tails exposed to the cytosol, and large central luminal domains. Hydrophobic signals present on both C-terminal tails of the Erv41p-Erv46p complex control sorting into COPII vesicles for anterograde transport, and retrieval from the Golgi is mediated by a COPI binding KKxx motif (Cosson and Letourneur, 1994) on Erv46p. Expression and localization of both proteins are interdependent such that Erv41p is not detected in an *erv46* null mutant strain, whereas Erv46p accumulates in the ER in an *erv41Δ* strain. A knockout of either gene therefore removes function of the whole complex (Otte and Barlowe, 2002). Strains lacking Erv41p, Erv46p or both are viable but display subtle secretory phenotypes such as a 25% reduction in ER to Golgi transport caused by a defect at the late stages of an in vitro transport assay. These phenotypes of the *erv41* and *erv46* deletions are not additive. Further, genetic studies using a candidate gene approach revealed interactions with *erv14Δ* and *ypt1-3*, two other mutations that impede transport in the early secretory pathway (Otte et al., 2001).

Yeast Erv41p and Erv46p have one orthologue each in all eukaryotes studied and have 30% and 41% amino acid sequence identity, respectively, with the corresponding human proteins. In particular, the membrane topology of both proteins and the KKxx retrieval signal on Erv46p are conserved across species. Mammalian Erv46 (mErv46) is ubiquitously expressed in various tissues and cell lines, and localizes predominantly to the ER-Golgi intermediate compartment

(ERGIC) and *cis*-Golgi, whereas smaller amounts are also detected in the ER. The mammalian orthologue is thus localised to equivalent compartments to the yeast protein. An RNA interference experiment excluded a structural role for mErv46 in maintenance of the Golgi complex (Orci et al., 2003). Recently, a proteomics approach using enriched ERGIC membranes demonstrated that human Erv46 (hErv46) in addition interacts with ERGIC-32, a novel 32 kDa protein with sequence similarity to hErv41 and hErv46 (Breuza et al., 2004). The high degree of conservation and equivalent localization patterns suggest similar roles for the yeast and mammalian proteins in the secretory pathway.

Although the precise biological role of the Erv41p-Erv46p complex is unknown, we envisaged a number of possible functions. First, these proteins may act in the sorting of as yet unidentified secretory cargo molecules during vesicle formation, similar to the function of the p24 proteins (Muniz et al., 2000), Erv29p (Belden and Barlowe, 2001; Otte and Barlowe, 2004) and ERGIC-53 (Appenzeller et al., 1999). Second, the Erv41p-Erv46p complex could operate to retrieve or localize other components of the transport and protein processing machinery, as in the case of Rer1p (Sato et al., 1995). Third, the complex might play a role in lipid transport, and finally, it could be involved in protein folding and glycoprotein processing in the ER and early Golgi complex. In order to gain more insight into these possible biological functions, we embarked on an analysis of interactions of the Erv41p-Erv46p complex with other components of the early secretory pathway, using both genetic and biochemical approaches. Here, we report genetic interactions between the *erv46* deletion and *vma12*, *vma21* and *vma22* deletions as well as a strong molecular interaction between Erv41p-Erv46p and ER glucosidase II (Rot2p, Gls2p) (Trombetta et al., 1996). The *VMA12*, *VMA21* and *VMA22* genes encode ER-localized chaperones required for the assembly and sorting of the vacuolar ATPase (V-ATPase) (Hill and Stevens, 1994; Malkus et al., 2004). In vitro vesicle formation and transport assays reveal functional interactions between the Erv41p-Erv46p complex and Vma21p, as well as an unexpected role for yeast Vma21p in sorting of the ER to Golgi SNARE, Bos1p, into COPII vesicles.

Results

Synthetic genetic interactions with the *erv46Δ* mutation

We employed the advantages of yeast genetics to study possible functions of the Erv41p-Erv46p complex. To identify additional gene products that might act in the same or parallel pathways as the Erv41p-Erv46p complex, we set out to identify second-site mutations that would be lethal or sick in combination with the non-lethal *erv46Δ* mutation. Rather than follow a classical synthetic lethal screen protocol, we made use of the synthetic genetic array (SGA) method (Tong et al., 2001; Tong et al., 2004). In this automated high-throughput technique, a SGA starting strain carrying a defined query mutation is crossed to an array of ~4700 viable gene deletion mutants. Following sporulation, double-mutant haploid cells are selected and their growth is assessed. One advantage of this approach is that it can readily be performed under different conditions for any given query strain. An *erv46Δ* query strain (SOY01417) was constructed and tested, but an initial screen at 30°C did not produce any synthetic lethal or sick interactions. As *erv41Δ* and *erv46Δ* strains are cold sensitive (Otte et al., 2001), we reasoned that incubating the double mutant haploids at a lower temperature might uncover interactions that had previously been missed. Indeed, at 22°C, 39 synthetic sick or lethal interactions were identified in the automated screen. The high-throughput protocol generates false-positive hits at a certain frequency (Tong et al., 2004), and we therefore validated these observations by manual tetrad analysis. We were able to confirm interactions with three genes: *vma21Δ*, *vma22Δ* and *vps1Δ*. *VMA21* and *VMA22* as well as the related *VMA12* gene were originally identified as being required for biogenesis of the vacuolar V-ATPase (Hill and Stevens, 1994). The corresponding proteins are localised to the ER, where according to current models they act as chaperones in the assembly of the transmembrane V_0 sector of the V-ATPase (Graham et al., 1998). Vma21p escorts the assembled V_0 structure into budding COPII vesicles (Malkus et al., 2004). Vps1p is a GTPase of the dynamin family required for vacuolar protein sorting, for retention of some proteins including Kex2p in the Golgi (Rothman et al., 1990; Nothwehr et al., 1995) and for normal actin cytoskeleton organization (Yu and Cai, 2004).

In order to examine the synthetic effects of *erv46* with these genes more closely, we constructed the double deletion strains at the permissible temperature of 30°C. Dilution series of isogenic wild-type, single and double deletion strains were spotted onto plates and incubated at 30°C or 16°C (Fig. 1). All strains grew at 30°C, whereas the *vma12Δ*, *vma21Δ* and *vma22Δ* strains and to a lesser extent the *vps1Δ* strain showed reduced growth at 16°C. Strikingly, if the *vma12*, *vma21* and *vma22* deletions were combined with the *erv46Δ* mutation, strains were inviable at low temperature. Even after 1 week at 16°C, no growth was observed for these double mutants. The *vps1 erv46* double deletion showed a slightly increased cold sensitivity as well. As a negative control, no interaction was observed if the *erv46Δ* mutation was combined with the *vps27* deletion. For the remainder of this report, we will focus on the interaction with *vma21Δ*. The other interactions will be characterized elsewhere.

Effects of the *erv46Δ* and *vma21Δ* mutations on ER to Golgi transport

To test whether the observed synthetic growth defects were concomitant with synthetic effects on ER to Golgi transport functions, we made use of a sensitive in vitro transport system (Barlowe, 1997). This assay can detect even weak transport phenotypes which are not easily observed using pulse-chase experiments (Conchon et al., 1999; Otte et al., 2001; Liu and Barlowe, 2002; Miller et al., 2002; Ballew et al., 2005). ^{35}S-labelled α factor pheromone precursor (ppαf) was translocated into washed semi-intact cells, and transport of the core glycosylated form of α factor (gpαf) was reconstituted by the addition of purified COPII subunits and the tethering and fusion factors, Uso1p and LMA1, in the presence of ATP. Protease resistant, outer-chain modified gpαf which had reached the Golgi was then precipitated with saturating amounts of an α1,6-mannose-specific antibody (Fig. 2A) and quantified. As reported earlier, transport was reduced by about 25% with the *erv46Δ* membranes (Otte et al., 2001). The *vma21* deletion showed a similarly modest but significant transport defect, while a combination of both mutations caused

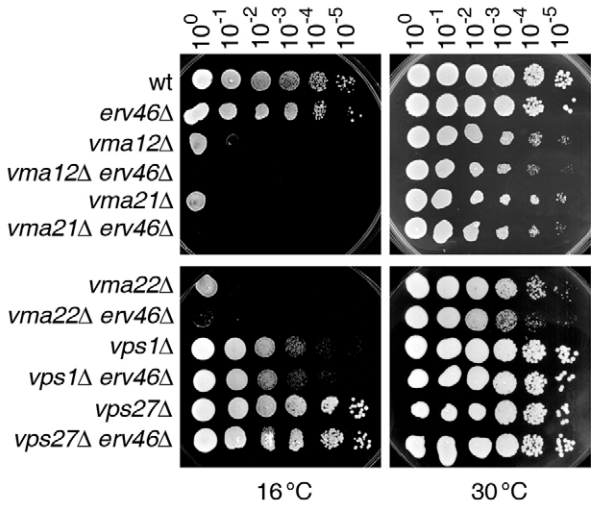

Fig. 1. Synthetic cold-sensitive effects with the *erv46* deletion. Wild-type (wt, BY4742), *erv46Δ* (SOY01409), *vma12Δ* (SOY01628), *vma12Δ erv46Δ* (SOY01654), *vma21Δ* (SOY01629), *vma21Δ erv46Δ* (SOY01655), *vma22Δ* (SOY01630), *vma22Δ erv46Δ* (SOY01656), *vps1Δ* (SOY01631), *vps1Δ erv46Δ* (SOY01657), *vps27Δ* (SOY01632) and *vps27Δ erv46Δ* (SOY01658) cells were grown to saturation in YPD medium and adjusted on an OD_{600} of 3.0. 5 μl of a 10-fold dilution series were spotted onto YPD plates. One set of plates was incubated at 30°C for 2 days and the other at 16°C for 1 week in order to visualize the synthetic growth phenotype of the double mutants. After this long incubation, the slight cold sensitivity of the *erv46Δ* strain is not visible.

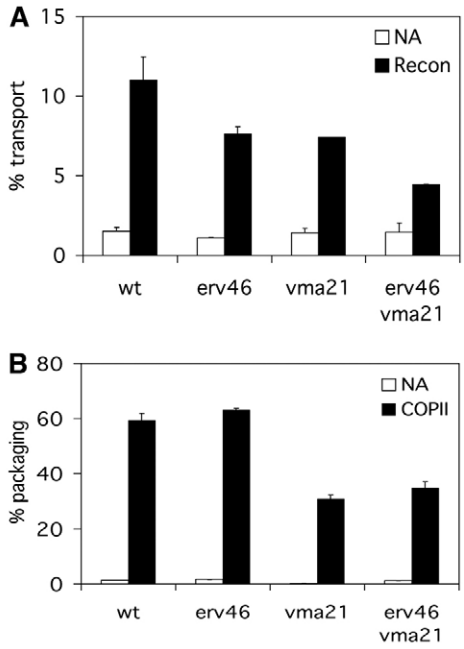

Fig. 2. Influence of *erv46* and *vma21* deletion mutations on α factor precursor transport and packaging into COPII vesicles. (A) Washed semi-intact wild-type (BY4742), *erv46Δ* (SOY01409), *vma21Δ* (SOY01629) or *erv46Δ vma21Δ* (SOY01655) cells containing ^{35}S-labelled gpαf were incubated without (NA) or with purified COPII subunits, Uso1p and LMA1 (Recon) in the presence of an ATP regeneration system. After 90 minutes at 23°C, outer-chain modified ^{35}S-gpαf was immunoprecipitated and amounts were plotted relative to ^{35}S-gpαf precipitated on concanavalin A beads. (B) Washed microsomes prepared from the same strains as in A containing ^{35}S-gpαf were incubated without (NA) or with purified COPII subunits (COPII) in the presence of an ATP regeneration system. After 20 minutes at 23°C, the relative amounts of gpαf in the diffusible vesicle fraction were measured. All assays were performed in duplicate and error bars represent the range.

a synthetic reduction in gpαf transport. To determine whether the *vma21* and *erv46* deletions influenced the same stage of the transport reaction, we performed an in vitro budding assay with microsomes containing ^{35}S-gpαf, COPII subunits and energy (Barlowe et al., 1994) but no tethering or fusion factors (Fig. 2B). After the reaction, ^{35}S-gpαf was precipitated from the vesicle fraction on concanavalin A Sepharose, allowing us to calculate the percentage of input ^{35}S-gpαf that was sorted into vesicles. As reported (Otte et al., 2001), the *erv46* deletion did not affect this stage, but less ^{35}S-gpαf was present in the vesicle fraction in the case of the *vma21* deletion strain. As expected, this sorting phenotype was not exacerbated in the *erv46 vma21* double deletion strain, indicating that both gene products act at different stages.

During the course of this study, it was discovered that Vma21p is not required for normal COPII vesicle biogenesis, but escorts the assembled V_0 integral membrane sector of the vacuolar ATPase (V-ATPase) into vesicles (Malkus et al., 2004). We, therefore, asked whether the Vma21p-deficient membranes would fail to sort any other proteins efficiently into COPII vesicles. As Erv46p function is required at the fusion stage (Otte et al., 2001), we were especially interested in the sorting of fusion factors such as the ER-to-Golgi SNAREs Bet1p, Bos1p, Sec22p and Sed5p. Vesicle fractions from the in vitro budding assay performed with wild-type, *erv46,* or *vma21* deletion membranes were probed for the presence of these ER vesicle proteins by western blot (Fig. 3). With wild-type and *erv46* deletion membranes, we observed packaging efficiencies which are in line with previous reports (Cao and Barlowe, 2000; Otte et al., 2001), whereas Sec12p, an ER resident protein, was not found in the vesicle fraction, as expected. With membranes lacking Vma21p, packaging of most proteins was only marginally reduced, but, surprisingly, almost no Bos1p and a modestly reduced amount of Erv29p were detected in the vesicles. Taken together, these results suggest that Vma21p is not required for vesicle formation, but for the efficient sorting of a subset of proteins into vesicles, including the V_0 sector of the V-ATPase, the v-SNARE Bos1p and, possibly and to a lesser extent, Erv29p. As Erv29p in turn sorts gpαf into vesicles (Belden and Barlowe, 2001; Otte and Barlowe, 2004), this may explain the reduced amount of gpαf seen in the *vma21Δ* vesicle fraction in Fig. 2B and the additive effects of the *vma21* and *erv46* deletions on overall gpαf transport in Fig. 2A. Alternatively, Vma21p might modulate Erv29p function, for example by regulating Erv29p-cargo interactions. The subcellular distribution of Vph1p, a subunit of the V-ATPase, was indistinguishable in wild-type and *erv41 erv46* deletion strains, indicating that the Erv41p-Erv46p is not required for the localization and function of Vma12p, Vma21p or Vma22p (not shown).

Although all four ER to Golgi SNAREs are distributed and

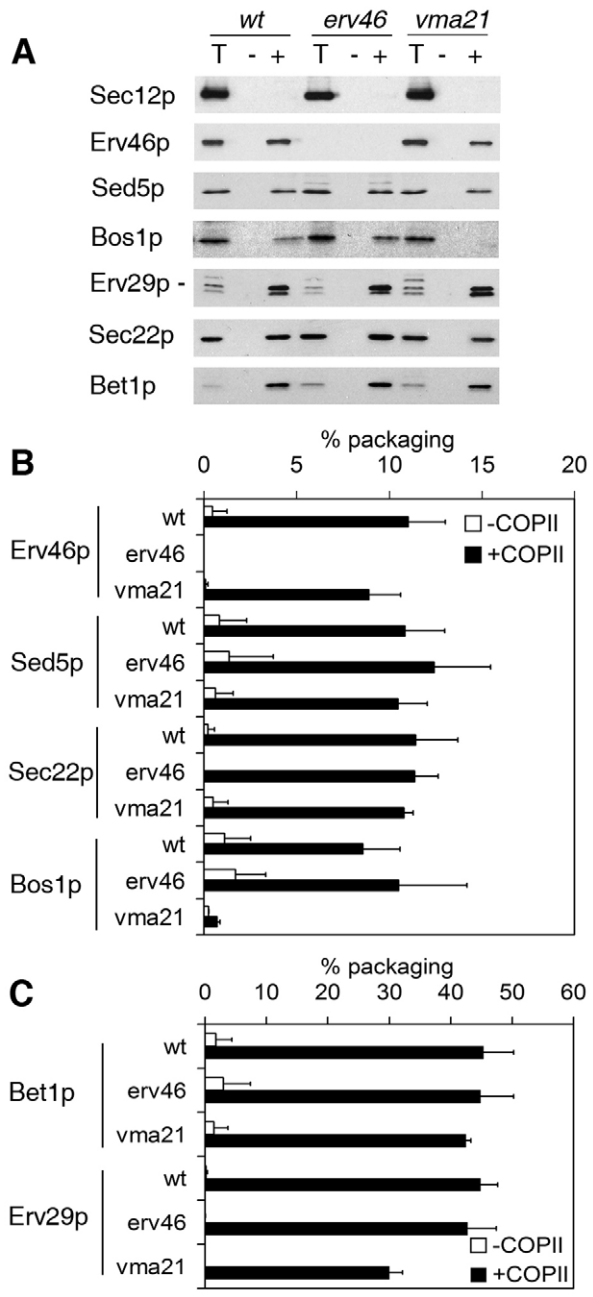

Fig. 3. The v-SNARE, Bos1p, is less efficiently sorted into COPII vesicles in a *vma21* deletion strain than in the wild type. (A) Microsomes from wild-type (BY4742), e*rv46Δ* (SOY01409) or *vma21Δ* (SOY01629) strains were incubated without (–) or with (+) purified COPII subunits in presence of GTP and an ATP regeneration system. Membranes from 10% of the total reaction (T) and vesicle fractions were collected by centrifugation and detected by western blotting with antibodies against various vesicle proteins as well as the ER resident, Sec12p, as a negative control. A line marks the Erv29p band among bands cross-reacting with the polyclonal antiserum. (B,C) Densitometric analysis of three independent experiments performed as in A. Mean packaging efficiencies as a percentage of input were plotted, and error bars represent the standard deviation.

cycle similarly between these compartments, only Bet1p and Bos1p are functionally required on the vesicle membrane during membrane fusion at the Golgi (Cao and Barlowe, 2000). Since the *vma21Δ* vesicles contained little Bos1p, we therefore asked whether they would fuse inefficiently with wild-type membranes. At the same time, we sought to determine whether Erv46p function was required on the vesicle or target membrane during fusion. To this end, we performed two-stage reactions (Cao and Barlowe, 2000), in which vesicles were first generated from wild-type, *erv46Δ* or *vma21Δ* microsomes, and in the second stage, the isolated vesicles were incubated with wild-type or *erv46Δ* acceptor membranes in the presence of tethering and fusion factors and energy (Fig. 4A). Vesicles lacking Erv46p fused normally with wild-type acceptor membranes, whereas fusion of all three vesicle preparations was reproducibly and significantly reduced by about 20-25% of the wild-type signal above background if the acceptor membranes did not contain Erv46p. This reduction corresponds to the previously observed approximately 25% fusion defect in an *erv46* strain (Otte et al., 2001), and it indicates that during fusion, the Erv41p-Erv46p complex is functionally required only on the acceptor membrane but not on vesicles. Surprisingly, *vma21Δ* vesicles displayed normal fusion competence with both wild-type and *erv46Δ* membranes.

Since Bos1p was less efficiently packaged in the cold-sensitive *vma21Δ* strain than in the wild type, we tested whether overexpression of Bos1p would rescue the cold sensitivity of *vma21Δ* and *vma21Δ erv46Δ* double deletion strains. Cells were transformed with a multicopy plasmid containing the *BOS1* sequence, or, for comparison, with a multicopy plasmid carrying *BET1*. Overexpression of either gene did not suppress the cold-sensitive growth phenotypes (Fig. 4B). These results indicate that the reduced Bos1p packaging in *vma21Δ* strains probably does not cause the cold-sensitive phenotype.

Erv41p and Erv46p are present in large heterogeneous complexes

As a second strategy to identify factors interacting with the Erv41p-Erv46p complex, we chose a biochemical approach using preparative co-immunoprecipitation. Expression of Erv41p and Erv46p is interdependent, and both proteins can be co-immunoprecipitated. Early observations suggested that Erv41p and Erv46p are physically associated in a heteromeric complex probably containing single Erv46p and Erv41p subunits (Otte et al., 2001). To estimate the size of such a complex, we resolved a detergent solubilized extract of a post-nuclear fraction on a sucrose velocity gradient (Fig. 5). Erv25p, which is present in a 100 kDa complex with Emp24p, Erp1p, and Erp2p (Marzioch et al., 1999), and ferritin, which migrates as a 440 kDa species (Conibear and Stevens, 2000), were used as size markers. Erv41p and Erv46p cofractionate on the gradient, with the main peak in fractions three and four, indicating a size of the complex of more than 100 kDa. In addition, a second smaller peak was observed deeper in the gradient in fractions eight and nine. These results suggest that Erv41p and Erv46p are present in large, heterogeneous complexes with molecular masses of approximately 200-400 kDa. We, therefore, set out to identify other proteins present in these complexes.

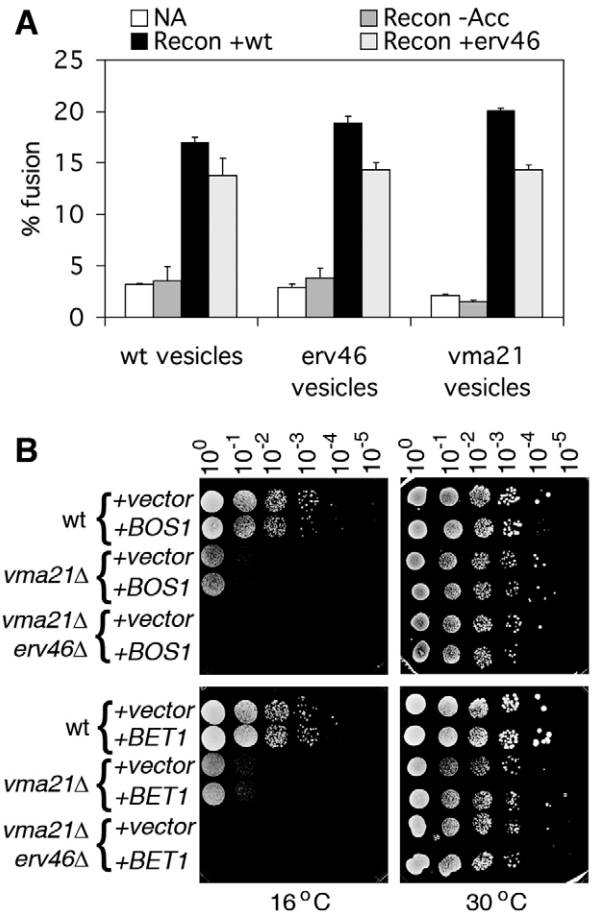

Fig. 4. Vesicles from a *vma21* deletion strain are fusion competent, and Erv46p is required only on the acceptor membrane. (A) In the first stage of a transport assay, COPII vesicles were generated from wild-type (BY4742), e*rv46Δ* (SOY01409) or *vma21Δ* (SOY01629) microsomes containing gpαf. In the second stage, these vesicles were incubated with washed semi-intact wild-type (BY4742, Recon +wt) or e*rv46Δ* (SOY01409, Recon +erv46) cells in presence of Uso1p, LMA1 and the ATP regeneration system. NA, mock reaction with wild-type semi-intact cells but no Uso1p or LMA1; Recon –Acc, control containing Uso1p and LMA1 but no semi-intact cells. Percentage fusion was quantified after precipitation of the outer-chain modified forms of gpαf. All assays were done in duplicate and error bars represent the range. (B) Overexpression of Bos1p does not suppress the cold-sensitive phenotypes of *vma21Δ* or *vma21Δ* e*rv46Δ* strains. Wild-type, single or double deletion strains (SOY163-SOY174) carrying empty vectors or multicopy *BOS1* or *BET1* overexpression plasmids as indicated were grown in appropriate selective media, spotted on plates and incubated as described in Fig. 1.

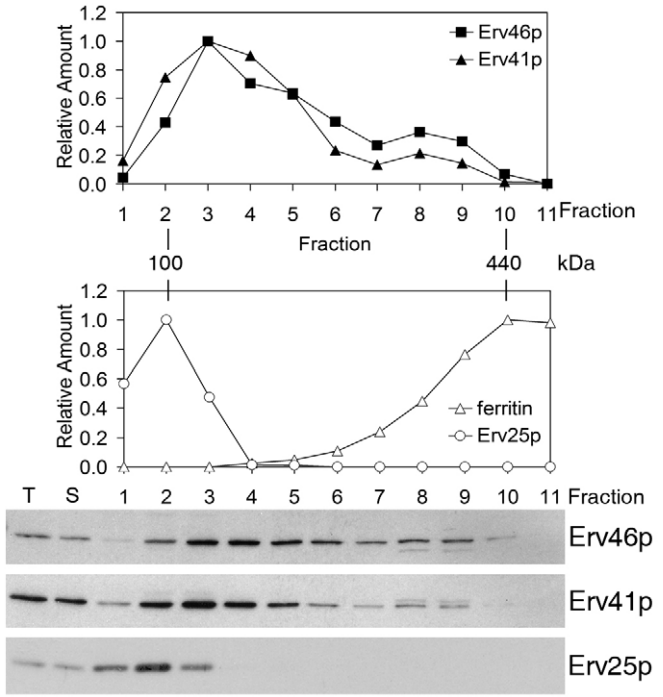

Fig. 5. Erv41p and Erv46p co-sediment on a sucrose velocity gradient. A post-nuclear supernatant (T) of FY834 cells was solubilized in 1% Triton X-100 (S) and separated on a 5-19% sucrose gradient. Fractions were collected from the top and blotted for Erv41p and Erv46p, as well as for Erv25p, which is present in a 100 kDa complex that serves as a molecular mass marker. Relative amounts of these proteins were determined by densitometry of immunoblots and plotted. The position of the 440 kDa marker ferritin was determined by spectrophotometry.

Erv41p and Erv46p interact with glucosidase II

To identify other proteins binding to Erv41p and Erv46p, we chose a preparative scale co-immunoprecipitation approach with gently solubilized microsomal membranes isolated from strains expressing N-terminally 3HA-tagged Erv46p or Erv41p (Otte et al., 2001). As shown in Fig. 6A, a band of an apparent molecular mass of 41 kDa co-precipitated with 3HA-Erv46p, and a slightly larger band was precipitated from the 3HA-Erv41p-containing membranes. These represent Erv41p and 3HA-Erv41p, respectively, whereas a band of 46 kDa co-precipitating with 3HA-Erv41p represents Erv46p, as confirmed by immunoblot (not shown). 3HA-tagged Erv46p is masked on the gel by the heavy chain of the antibody. This indicates that the Erv41p-Erv46p complex was intact under our solubilization conditions. In both samples, a band migrating near the 116 kDa molecular mass marker was also detected. These interactions were specific, as none of the bands were present in a mock reaction using wild-type microsomes or in controls without antibody. These bands were excised from a gel and both were identified as glucosidase II (Rot2p, Gls2p, YBR229C) by tryptic digest and mass spectrometry. Rot2p is an enzyme localised to the ER lumen, where it trims the two remaining glucose residues from N-linked core oligosaccharides after glucosidase I (Cwh41p, Gls1p) has removed the most distal glucose (Trombetta et al., 1996). We tried unsuccessfully to obtain protein identifications for the doublet of bands of approximately 75 kDa apparent molecular mass. These may contain other interacting proteins or glucosidase II breakdown products. A very weak band around 100 kDa molecular mass may represent glycosylated Gtb1p, the recently identified β subunit of yeast glucosidase II (Wilkinson et al., 2006).

To confirm the interaction between Erv41p-Erv46p and Rot2p, an epitope-tagged version of Rot2p was constructed. A 3HA tag was appended to the C terminus of Rot2p and stable

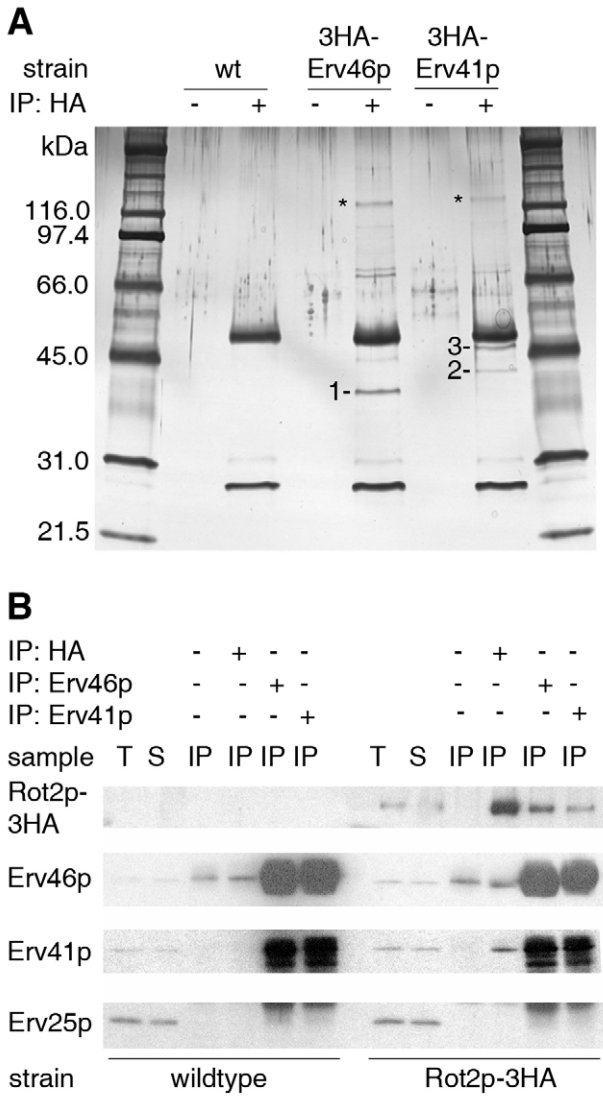

Fig. 6. Glucosidase II is co-immunoprecipitated with the Erv41p-Erv46p complex. (A) Microsomes were prepared from wild-type (FY834) and strains expressing 3HA-Erv46p (CBY770) or 3HA-Erv41p (CBY783) and solubilized with 1% digitonin. Soluble extracts were incubated in the absence (–) or presence (+) of a monoclonal antibody against the HA epitope. Precipitates were resolved on a silver stained gel. Numbers indicate bands representing Erv41p (1), 3HA-Erv41p (2) and Erv46p (3). The band indicated by and asterisk (*) was excised from a Coomassie Blue-stained gel and identified by mass spectrometry as glucosidase II. (B) Microsomes were isolated from wild-type (FY834) and Rot2p-3HA-expressing strains (SOY01117) and solubilized as above. Antibodies against the HA epitope, Erv46p or Erv41 were added as indicated at the top. Proteins eluted from the precipitate (IP), totals (T) and solubilized extracts (S) were resolved on a 12.5% polyacrylamide gel and immunoblotted with 3HA-, Erv46p-, Erv41p- and Erv25p-specific antibodies. T and S each represent 2.6% of input.

expression of the tagged protein was confirmed by western blot. The tagged protein was functional as it displayed normal glucose trimming activity (not shown). Microsomes were prepared from wild-type and Rot2p-3HA-expressing strains, solubilized, and subjected to immunoprecipitation (Fig. 6B). The HA antibody immunoprecipitated 17% of total Rot2p-3HA, 8% of Rot2p-3HA co-immunoprecipitated with Erv46p and 4% co-immunoprecipitated with Erv41p. Conversely, 9% of total Erv41p was co-immunoprecipitated with Rot2p-3HA. In this lane, Erv46p could not be quantified because it is again masked by the heavy chain band. These interactions are specific as an unrelated protein, Erv25p, was not present in the precipitates. To confirm that the co-immunoprecipitation results reflect a biologically significant interaction rather than an artefact generated during solubilization, we repeated this experiment with membranes isolated individually from an *erv41Δ erv46Δ* strain expressing Rot2p-3HA and from the isogenic wild-type strain. If these solubilized extracts were mixed immediately prior to immunoprecipitation, very little co-immunoprecipitation was observed (not shown). Taken together, these results indicate that the Erv41p-Erv46p complex interacts directly, or indirectly via another protein, with ER-localised glucosidase II.

Mammalian glucosidase II consists of a catalytic α subunit and a 58 kDa β subunit that localizes the enzyme to the ER (Trombetta et al., 1996; Pelletier et al., 2000). Although a β subunit has recently been identified in yeast as well, it is not required for localization of Rot2p (Wilkinson et al., 2006). Rot2p itself does not contain any known ER retention or retrieval signal. Therefore, we asked whether normal expression or ER localization of Rot2p required the Erv41p-Erv46p complex. First, the Rot2p-3HA allele was crossed into an *erv41 erv46* double deletion strain. Rot2p-3HA was stably expressed in both wild-type and *erv41Δ erv46Δ* backgrounds. Subcellular localization of Rot2p-3HA to the ER was indistinguishable in both strains as determined by immunoblots of sucrose gradient fractions (not shown), indicating that the Erv41p-Erv46p complex does not act as a retrieval system for steady-state localization of Rot2p-3HA to the ER. Next, we tested whether Rot2p might cycle between ER and Golgi while bound to the Erv41p-Erv46p complex. To explore this possibility, we used microsomes containing Rot2p-3HA in the in vitro COPII vesicle formation assay with purified COPII subunits and an ATP regeneration system (Barlowe et al., 1994). As shown in Fig. 7A, Erv46p and Erv25p were efficiently packaged into vesicles, whereas Kar2p, as a negative control, was not detected in the vesicle fraction. Surprisingly, Rot2p-3HA was not sorted into vesicles either. Taken together, these results demonstrate that only the ER-localized fraction of the Erv41p-Erv46p complex interacts with Rot2p in the ER, and that this interaction does not persist upon export of the Erv41p-Erv46p complex from the ER.

erv41 and *erv46* deletions cause a glycoprotein processing defect

We then asked whether the Erv41p-Erv46p complex is functionally required for Rot2p glucose trimming activity in the ER. Strains lacking glucosidase I and glucosidase II activity display reduced electrophoretic mobility of glycoproteins compared to the fully trimmed species (Trombetta et al., 1996). In the following experiments, we monitored the glucose trimming of in vitro translocated and core glycosylated α factor pheromone precursor (gpαf) as a model substrate (Fig. 7B). As expected, strains lacking Rot2p or Cwh41p contain incompletely trimmed gpαf forms of reduced electrophoretic mobility. Disruption of the Erv41p-Erv46p complex in an *erv41Δ* strain did not cause a *rot2Δ*-like phenotype, but

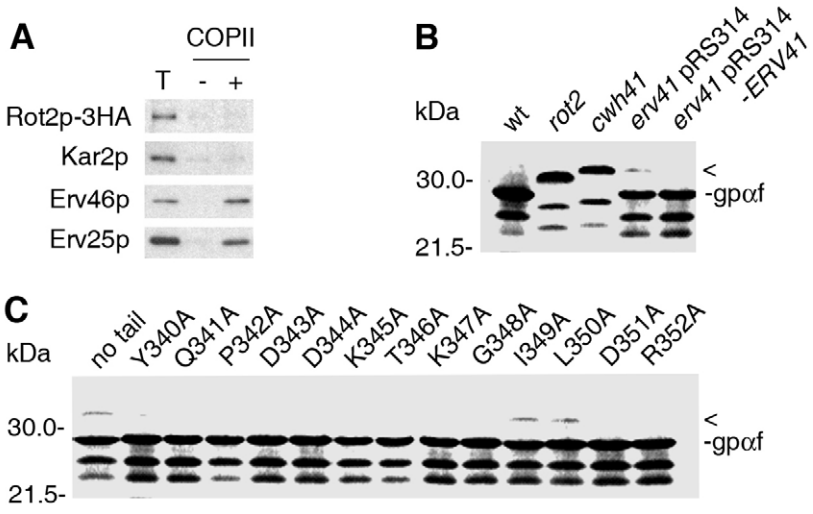

Fig. 7. Glucosidase II is not packaged into COPII vesicles in vitro and strains lacking a cycling Erv41p-Erv46p complex have a glycoprotein processing defect. (A) Microsomes from a strain expressing Rot2p-3HA (SOY01117) were incubated without (–) or with (+) purified COPII subunits in the presence of GTP and an ATP regeneration system. Membranes from 10% of the total reaction (T) and vesicle fractions were collected by centrifugation, resolved on a polyacrylamide gel and immunoblotted. (B) ^{35}S-labelled α factor pheromone precursor was translocated into semi-intact cells. Membranes were solubilized and glycoproteins precipitated on concanavalin A Sepharose beads. Bound radiolabelled protein was eluted from the beads, resolved on polyacrylamide gels and detected by phosphorimaging. Fully core glycosylated α factor pheromone precursor is indicated (gpαf). The lower bands represent partially glycosylated species. Arrowheads mark the band representing incompletely trimmed gpαf. Semi-intact cells were prepared from wild-type (wt, FY834), *rot2Δ* (CBY1087), *cwh41Δ* (CBY1086), an *erv41Δ* strain containing empty vector pRS314 (CBY1036), or an *erv41Δ* strain containing the *ERV41* gene in vector pRS314 (CBY1037). (C) Same procedure as in B, but semi-intact cells were prepared from an *erv41Δ* strain containing a plasmid which encodes a truncated version of *ERV41* lacking C-terminal residues 340-352 (CBY1038), or from *erv41Δ* strains transformed with pRS314 plasmids containing alanine scan *ERV41* mutants (CBY1074-1080, CBY1089-1096, CBY1158 and CBY1159, respectively).

surprisingly, a weak *cwh41Δ*-like glucose trimming defect. This defect was rescued by expression of wild-type Erv41p from a single copy vector. A similar effect was observed in an *erv46Δ* strain, and the trimming phenotype was not further exacerbated in an *erv41 erv46* double deletion strain. We confirmed, by endoglycosidase H treatment, that the bands of low electrophoretic mobility represented glycosylated forms and are not caused by other covalent modifications (not shown).

As the Erv41p-Erv46p complex cycles between the ER and Golgi complex but Rot2p does not, we tested whether cycling of the Erv41p-Erv46p complex is required to overcome the observed glucose-trimming defect, or whether a static complex arrested in the ER would be sufficient. Export of the Erv41p-Erv46p complex from the ER is controlled by two hydrophobic signals present on both subunits in trans, and mutations in either signal cause it to accumulate in the ER. When the Erv41p tail was deleted, the complex could not leave the ER, and a glucose trimming defect similar to that in the *erv41* deletion strain was observed (Fig. 7C). We had performed an alanine scan on this tail sequence to define its hydrophobic ER export signal (Otte and Barlowe, 2002), and we used these mutants to test for complementation of the glucose-trimming phenotype. The same residues that form the ER export signal on Erv41p, isoleucine in position 349 and leucine in position 350, are also required for complete glucose trimming. We conclude that the Erv41p-Erv46p complex is required for efficient glycoprotein trimming in the ER, and that it must be cycling between the ER and Golgi to perform this function.

Discussion

Using genetic and biochemical approaches, we have identified new interactors of the Erv41p-Erv46p complex. A synthetic genetic array screen identified genetic interactions of *erv46Δ* with *vma12Δ*, *vma21Δ* and *vma22Δ*, genes required for V-ATPase biogenesis, and also *vps1Δ*. Reconstituted transport assays revealed that Vma21p is required for normal packaging of the SNARE Bos1p and possibly the ER to Golgi transport receptor, Erv29p. At the fusion stage, Erv46p activity was only required on the acceptor compartment but was dispensable on the vesicle membrane. Surprisingly, vesicles lacking Vma21p and containing only trace amounts of Bos1p were able to fuse normally. Biochemically, we detected a molecular interaction between Erv41p and Erv46p and ER-localised glucosidase II, which does not persist during packaging of the Erv41p-Erv46p complex into COPII vesicles. Loss of the Erv41p-Erv46p complex or inhibition of its cycling leads to a mild glycoprotein processing defect. Although both approaches detected very different interactions, all proteins identified as Erv41p-Erv46p interactors are localized to the early secretory pathway and are involved in protein maturation and processing in the ER and/or sorting into COPII vesicles for transport to the Golgi. The Erv41p-Erv46p complex is therefore expected to play a biological role in the same processes.

We had reported earlier that loss of the Erv41p-Erv46p complex affects a late stage during ER to Golgi transport. In vitro, we had observed an approximately 25% reduction in the amount of COPII vesicles fusing with the acceptor compartment (Otte et al., 2001). We therefore set out to assign the requirement for the Erv41p-Erv46p complex to either the vesicle or target membrane. Our results clearly show that these proteins are functionally only required on the target membrane but dispensable on the vesicle during fusion. This is consistent with the fact that about 70% of the yeast Erv41p-Erv46p complex cofractionates with Golgi membranes. Likewise, 70% of mammalian Erv46 localises to early Golgi cisternae and another 20% to the ERGIC (Orci et al., 2003). Fig. 3 also demonstrates that the Erv41p-Erv46p complex is not required for sorting of fusion factors such as SNAREs into vesicles. Moreover, subcellular distribution of the ER-to-Golgi SNAREs was normal in an *erv41 erv46* deletion strain (not shown). The Erv41p-Erv46p complex may, however, play an accessory role

during fusion or act in localizing other accessory fusion factors. In addition, the present fusion assay does not allow us to distinguish between fusion defects and effects on glycoprotein processing such as the addition of α1,6-mannose residues in the *cis*-Golgi. Therefore, a role of the Erv41p-Erv46p complex in early Golgi glycoprotein processing cannot be ruled out. Future studies using more direct fusion assays, such as methods measuring SNARE pairing (Flanagan and Barlowe, 2006), should help distinguish between these possibilities.

Our genetic analysis uncovered interactions between *erv46* and *vma12*, *vma21*, *vma22* as well as *vps1* deletions. Of these, *vma12Δ*, *vma21Δ* and *vma22Δ* are the most interesting since they are all localized to the ER at steady state, where they act as chaperones during assembly of the transmembrane V_0 sector of the vacuolar V-ATPase (Hill and Stevens, 1994). Vma12p is an integral membrane protein (Jackson and Stevens, 1997) which anchors the peripheral membrane protein Vma22p to the luminal side of the ER membrane (Hill and Stevens, 1995). Vma21p is a small, 8.5 kDa integral membrane protein that is sorted into COPII vesicles, cycles between the ER and Golgi and is thought to function in sorting the assembled V_0 sector into vesicles (Malkus et al., 2004). As the overall transport defects caused by the *vma21* and *erv46* deletions were additive and the *erv46Δ* mutation affects the fusion stage, we asked whether Vma21p might have a broader role in sorting other proteins into COPII vesicles, for example, factors required for membrane fusion. Most vesicle proteins tested were sorted efficiently in the *vma21Δ* strain, in agreement with normal sorting of Chs3p as reported earlier (Malkus et al., 2004). Vma21p is therefore dispensable for vesicle biogenesis. Surprisingly, we found that in contrast to the other three ER-to-Golgi SNARE proteins, Bos1p was inefficiently sorted into COPII vesicles in this strain. We also detected a slight reduction of Erv29p and gpαf packaging with *vma21Δ* membranes, which had not been found in an experiment using antibodies to partially inhibit sorting of Vma21p and Vph1p (Malkus et al., 2004). We propose that yeast Vma21p may have a wider, so far unappreciated, function in the packaging of a larger subset of proteins, besides the V_0 sector, into COPII vesicles. These sorting mechanisms may be limited to lower eukaryotes, as database searches reveal Vma21p homologues in other yeasts, but no obvious homologues in higher eukaryotes. Since Bos1p overexpression did not rescue the cold sensitivity of the *vma21Δ* strain, this phenotype might be caused by a defect in the sorting of other, as yet unidentified cargo molecules. Evidence is mounting that many secretory cargo molecules and vesicle proteins are sorted specifically into COPII-coated vesicles, and it will be important to more systematically determine the cargo spectrum of the handful of described ER to Golgi cargo receptors by a combination of genetic, biochemical and proteomics approaches.

Detailed structure-function studies using site-directed mutagenesis combined with structural and biochemical analyses have revealed a wealth of information regarding cargo selection by the COPII coat, in particular by the Sec24p subunit. Three independent cargo binding sites have been identified on Sec24p. The 'A-site' interacts with a YNNSNPF motif on the SNARE Sed5p (Miller et al., 2005), whereas the 'B-site' recognizes another motif, LxxLE, on Sed5p, as well as a related motif on Bet1p (Mossessova et al., 2003). The 'C-site' binds an unknown motif on Sec22p (Miller et al., 2003). By contrast, the sorting of Bos1p is less well understood. Packaging of Bos1p is largely independent of Sed5p sorting, and mutations in the A-site only slightly reduced Bos1p packaging (Miller et al., 2005). B-site mutations have a stronger effect but Bos1p is still packaged at about 15% of normal levels, whereas Bet1p is almost undetectable. The ER export signal on Bos1p is not known (Miller et al., 2003). We speculate that in yeast, Vma21p might act as a receptor in recruiting Bos1p to the COPII coat, or it may stabilize a direct Bos1p-Sec24p interaction. We have so far not been able to show a physical interaction between Vma21p and Bos1p by co-immunoprecipitation. However, such an interaction may be weak and transient and thus difficult to detect. In similar cases, crosslinking (Muniz et al., 2000; Belden and Barlowe, 2001) or accumulating a cycling receptor in the ER (Powers and Barlowe, 2002) have been helpful.

Previous reports indicate that Bos1p, Bet1p, Sed5p and Sec22p mediate fusion of ER-derived vesicles with an early Golgi compartment (Kaiser and Schekman, 1990; Newman et al., 1990; Hardwick and Pelham, 1992; McNew et al., 2000). Bos1p and Bet1p have been shown to be functionally required on the vesicle membrane, whereas Sed5p was necessary only on the acceptor membrane using an in vitro assay and temperature sensitive alleles (Cao and Barlowe, 2000). Therefore, it is surprising that *vma21Δ* vesicles, containing only reduced amounts of Bos1p, were able to fuse normally. It appears that either only a small percentage of the Bos1p pool contained on wild-type vesicles is actually required for optimal fusion, or that another factor may compensate for Bos1p function on these vesicles. In this context, it has been reported that function of the non-essential SNARE Sec22p can be substituted by Ykt6p, a related SNARE, which is up-regulated in a *sec22* deletion strain (Liu and Barlowe, 2002). A similar substitution might occur in the *vma21* deletion mutant but not in a *bos1* temperature sensitive mutant shifted to non-permissive temperature.

In many ways, Erv41p and Erv46p are reminiscent of the p24 proteins, which are also non-essential, display subtle secretory phenotypes, are present in heteromeric complexes and cycle between the ER and Golgi complex. Mammalian p24 proteins have been reported to be present in heterogeneous complexes depending on their localization (Jenne et al., 2002). Erv41p and Erv46p both have large luminal domains and two transmembrane segments each, which would be available for protein-protein interactions in the ER lumen or membrane. Our sizing experiment showed that they are indeed present in large complexes. Their size heterogeneity may reflect differential interactions, which may include binding of an ER-localized pool of Erv41p and Erv46p to ER resident proteins such as Rot2p, which we identified here. Interestingly, in a high-throughput tandem affinity purification study (Gavin et al., 2002), an interaction between Erv46p and another ER resident protein, Ero1p, has been reported. Ero1p acts during formation of disulphide bonds in the ER by transferring oxidizing equivalents to protein disulphide isomerase (Frand et al., 2000). We have not been able to reproduce this interaction by co-immunoprecipitation of a Myc epitope-tagged version of Ero1p. However, like Rot2p-3HA, this construct was not packaged into COPII vesicles, nor did its localization depend on the presence of the Erv41p-Erv46p complex (not shown).

Mammalian glucosidase II comprises a catalytic α subunit

Table 1. Strains used in this study

Strain	Genotype	Reference
BY4742	*MATα his3 leu2 lys2 ura3*	Winzeler et al., 1999
FY834	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*	Winston et al., 199
CBY738	*MAT**a** his3 leu2 met15 ura3 erv46::kanR*	Winzeler et al., 1999
CBY770	FY834 with *ERV46::HIS3MX6-PGAL1-3HA*	Otte et al., 2001
CBY783	FY834 with *ERV41::TRP1-PGAL1-3HA*	Otte et al., 2001
CBY795	FY834 with *erv41::HIS3 erv46::kanR*	Otte et al., 2001
CBY797	FY834 with *erv41::HIS3*	Otte et al., 2001
CBY799	FY834 with *erv46::kanR*	Otte et al., 2001
CBY1036	CBY797 containing pRS314	Otte et al., 2002
CBY1037	CBY797 containing pRS314-*ERV41*	Otte et al., 2002
CBY1038	CBY797 containing pRS314-*ERV41-ΔC*	Otte et al., 2002
CBY1074	CBY797 containing pRS314-*ERV41-Y340A*	Otte et al., 2002
CBY1075	CBY797 containing pRS314-*ERV41-P342A*	Otte et al., 2002
CBY1076	CBY797 containing pRS314-*ERV41-D343A*	Otte et al., 2002
CBY1077	CBY797 containing pRS314-*ERV41-T346A*	Otte et al., 2002
CBY1078	CBY797 containing pRS314-*ERV41-K347A*	Otte et al., 2002
CBY1080	CBY797 containing pRS314-*ERV41-G348A*	Otte et al., 2002
CBY1086	BY4742 with *cwh41::kanR*	Winzeler et al., 1999
CBY1087	BY4742 with *rot2::kanR*	Winzeler et al., 1999
CBY1089	CBY797 containing pRS314-*ERV41-D344A*	Otte et al., 2002
CBY1090	CBY797 containing pRS314-*ERV41-L350A*	Otte et al., 2002
CBY1091	CBY797 containing pRS314-*ERV41-D351A*	Otte et al., 2002
CBY1092	CBY797 containing pRS314-*ERV41-R352A*	Otte et al., 2002
CBY1096	CBY797 containing pRS314-*ERV41-Q341A*	Otte et al., 2002
SOY01117	FY834 with *ROT2::TRP1-ROT2-3HA*	This study
CBY1158	CBY797 containing pRS314-*ERV41-K345A*	Otte et al., 2002
CBY1159	CBY797 containing pRS314-*ERV41-I349A*	Otte et al., 2002
SOY01409	*MAT**a** his3 leu2 met15 ura3 erv46::natR*	This study
SOY01417	*MATα can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 erv46::natR ura3 leu2 his3 lys2 met15*	This study
SOY01628	BY4742 with *vma12::kanR*	Winzeler et al., 1999
SOY01629	BY4742 with *vma21::kanR*	Winzeler et al., 1999
SOY01630	BY4742 with *vma22::kanR*	Winzeler et al., 1999
SOY01631	BY4742 with *vps1::kanR*	Winzeler et al., 1999
SOY01632	BY4742 with *vps27::kanR*	Winzeler et al., 1999
SOY01654	BY4742 with *erv46::natR vma12::kanR*	This study
SOY01655	BY4742 with *erv46::natR vma21::kanR*	This study
SOY01656	BY4742 with *erv46::natR vma22::kanR*	This study
SOY01657	BY4742 with *erv46::natR vps1::kanR*	This study
SOY01658	BY4742 with *erv46::natR vps27::kanR*	This study
SOY163	BY4742 containing pRS426	This study
SOY164	BY4742 containing pRS426-*BOS1*	This study
SOY165	SOY01629 containing pRS426	This study
SOY166	SOY01629 containing pRS426-*BOS1*	This study
SOY167	SOY01655 containing pRS426	This study
SOY168	SOY01655 containing pRS426-*BOS1*	This study
SOY169	BY4742 containing pRS425	This study
SOY170	BY4742 containing pRS425-*BET1*	This study
SOY171	SOY01629 containing pRS425	This study
SOY172	SOY01629 containing pRS425-*BET1*	This study
SOY173	SOY01655 containing pRS425	This study
SOY174	SOY01655 containing pRS425-*BET1*	This study

and a 58 kDa β subunit, which is required for ER localisation (Trombetta et al., 1996; Pelletier et al., 2000). Recently, a β subunit, Gtb1p, has been described for yeast glucosidase II, but Gtb1p was not necessary for localization of Rot2p to the ER (Wilkinson et al., 2006). In contrast to the mammalian β subunit, Gtb1p does not terminate in a known ER retrieval signal. Our results exclude a function of the Erv41p-Erv46p complex in retrieval of this enzyme to the ER, and how Rot2p remains anchored in the ER remains unclear. In mammalian cells, glucose trimming by glucosidase II is part of the calnexin-calreticulin cycle of the ER quality control mechanism (Helenius and Aebi, 2004), although it is not certain whether the same process operates in yeast since no corresponding glucosyltransferase has been identified. The mild glycoprotein trimming defect observed in the *erv41Δ* and *erv46Δ* mutants might indicate that these proteins could act as cofactors for efficient glycoprotein processing or transport. Surprisingly, this defect resembles a partial loss of glucosidase I, rather than glucosidase II, activity. We currently cannot explain this discrepancy. There is no evidence so far that the Erv41p-Erv46p complex interacts with glucosidase I, but in addition to the interaction with glucosidase II, the Erv41p-Erv46p complex might modulate glucosidase I activity. It could also act as a cofactor for this enzyme, or function in localizing the enzyme or accessory factor(s) to the ER. At present, we do not understand the significance of the interactions of subpopulations of Erv41p and Erv46p with ER-resident proteins, but they indicate a poorly characterized, close relationship between protein folding, processing and export from the ER by COPII vesicles.

Materials and Methods

Strain construction

Yeast strains are listed in Table 1. Strains CBY738, SOY01628 to SOY01632 and the isogenic wild-type strain BY4742 were obtained from a systematic deletion project (Winzeler et al., 1999) (Invitrogen). SOY01409 was generated by transforming CBY738 with linearized plasmid p4339 as described previously (Tong et al., 2001). SOY01409 was crossed with Y3598 (Tong et al., 2001) to yield SOY01417. To generate SOY01117, a 3HA epitope tag was appended to the C terminus of Rot2p by targeting a cassette amplified from plasmid pFA6a-3HA-TRP1 for homologous recombination at the *ROT2* locus in FY834 as described previously (Longtine et al., 1998). Transformants were selected for tryptophan prototrophy and screened for expression of tagged Rot2p by western blotting of membrane fractions as reported by Otte et al. (Otte et al., 2001). Double deletion strains SOY01654 to SOY01658 were generated by crossing SOY01409 with strains SOY01628 to SOY01632. Strains SOY163 to SOY174 were obtained by transforming BY4742, SOY01629 and SOY01655 with high-copy-number shuttle vectors pRS426 or pRS425 (Christianson et al., 1992), or with plasmids pRS426-*BOS1* or pRS425-*BET1* (VanRheenen et al., 1998).

Subcellular fractionation

Cells were grown to mid-logarithmic phase in yeast extract/peptone/dextrose (YPD) medium and harvested. Strains CBY770 and CBY783 were grown in YP supplemented with 1.5% galactose and 0.5% glucose to induce expression of 3HA-tagged protein. Microsomes were isolated as described previously (Wuestehube and Schekman, 1992). Semi-intact cells were prepared as reported by Baker et al. (Baker et al., 1988). Detergent velocity gradient centrifugation was done as described by Conibear and Stevens (Conibear and Stevens, 2000) except that gradients consisted of eight steps of 5% to 19% sucrose. As a molecular mass marker ferritin (Sigma) was loaded on a parallel gradient. After centrifugation, 11 fractions were collected from the top. Ferritin was detected by spectrophotometry at 370 nm, whereas Erv46p, Erv41p and Erv25p were visualized by immunoblot.

Antibodies, immunoprecipitation and protein identification

For preparative scale immunoprecipitations, approximately 1.3 mg of membrane protein per reaction was solubilized in 600 μl B88-8 (20 mM Hepes pH 8.0, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate) containing 1% digitonin (Calbiochem) and 1 mM PMSF for 10 minutes at 25°C. Samples were centrifuged for 5 minutes at 14,000 ***g***. Supernatants were pre-absorbed with 75 μl of a 20% protein A Sepharose slurry (GE Biotech) for 10 minutes, centrifuged briefly, and transferred to new tubes. For immunoprecipitations, 25 μl of the 20% protein A Sepharose slurry and a saturating amount of HA.11 antibody (Covance, Research Products, Berkeley, CA) were added and samples rotated for 2 hours at 4°C. The precipitates were washed five times with 1 ml 0.05% digitonin in B88-8, eluted from the beads with 50 μl sample buffer and heating to 95°C, and resolved on a silver-stained 4-20% Tris-glycine gel (Invitrogen). For protein identification, gels were stained using a colloidal Coomassie Blue staining kit (Invitrogen). Bands were excised for analysis by mass spectrometry as described previously (Jensen et al., 1999). Polyclonal antibodies against Erv41p and Erv46p (Otte et al., 2001), Erv25p (Belden and Barlowe, 1996), Kar2p and Sec12p (Powers and Barlowe, 1998), Bos1p (Sogaard et al., 1994), Sed5p (Cao et al., 1998), Bet1p (Rexach et al., 1994), Erv29p (Belden and Barlowe, 2001) and Sec22p (Liu and Barlowe, 2002) have been described. Proteins were detected using the ECL method (GE Biotech, Piscataway, NJ), and documented on a Chemidoc cooled CCD camera system (Bio-Rad) or X-ray films (Pierce). Densitometric analysis of bands free of saturated pixels was done using Quantity One Basic (Bio-Rad).

COPII vesicle formation and transport assays

COPII vesicle budding assays were done as described previously (Barlowe et al., 1994). Transport assays (Barlowe, 1997), and the two-stage budding and fusion assays (Cao and Barlowe, 2000) were performed as reported.

Glucose trimming assay

^{35}S-labelled α factor precursor (ppαf) was prepared by in vitro translation and translocated into semi-intact cells (Baker et al., 1988). After the translocation reaction, membranes were solubilized with 1% SDS and glycoproteins were precipitated on concanavalin A Sepharose beads (GE Biotech). Washed precipitates were dissolved in sample buffer, resolved on polyacrylamide gels and detected using a Storm 860 phosphorimager (Molecular Dynamics).

Synthetic genetic array analysis (SGA)

SGA analysis was performed as described previously (Tong et al., 2001). Automated screens were performed in triplicate. Reproducible synthetic sick or lethal interactions were confirmed by sporulating the diploid strains, followed by manual tetrad analysis.

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