The Emp24 Complex Recruits a Specific Cargo Molecule into Endoplasmic Reticulum-derived Vesicles

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Abstract. Members of the yeast p24 family, including Emp24p and Erv25p, form a heteromeric complex required for the efficient transport of selected proteins from the endoplasmic reticulum (ER) to the Golgi apparatus. The specific functions and sites of action of this complex are unknown. We show that Emp24p is directly required for efficient packaging of a luminal cargo protein, Gas1p, into ER-derived vesicles. Emp24p and Erv25p can be directly cross-linked to Gas1p in ER-derived vesicles. Gap1p, which was not affected by emp24 mutation, was not cross-linked. These results suggest that the Emp24 complex acts as a cargo receptor in vesicle biogenesis from the ER.

Key words: COPII-coated vesicles • ER • Erv25p • Saccharomyces cerevisiae • protein sorting

Introduction

In eukaryotic cells, protein transport between the organelles of the secretory pathway is mediated by vesicles that bud from a donor compartment and fuse with an appropriate acceptor compartment (Palade, 1975). The starting point of the exocytic route is the ER. There, correctly folded and assembled cargo molecules can enter COPII-coated vesicles for transport to the cis-Golgi compartment (Schekman and Orci, 1996). Many cytosolic and transmembrane proteins have been identified that mediate this vesicular transport step (Rothman and Wieland, 1996; Schekman and Orci, 1996).

p24 proteins are present in heteromeric complexes that have been proposed to cycle between ER and Golgi compartments because they are found in both COPII and COPI vesicles in addition to ER and Golgi membranes (Schimmöller et al., 1995; Stamnes et al., 1995; Belden and Barlowe, 1996; Søhn et al., 1996; Rojo et al., 1997; Dominguez et al., 1998; Füllekrug et al., 1999; Marzioch et al., 1999). In mammals, functional data suggest a role for these proteins in the formation of ER exit sites (Lavoie et al., 1999). In yeast, eight genes encode p24 family members: EMP24, ERV25 and ERP1-ERP6 (Marzioch et al., 1999). Mutants in several of these genes show a protein transport defect for invertase, a soluble secreted protein. Several other cargo molecules, including α factor, a secreted pheromone, do not require p24 proteins for efficient transport. Mutations of several yeast p24 genes result in secretion of the ER luminal protein, Kar2p (Elrod-Erickson and Kaiser, 1996; Marzioch et al., 1999).

The Emp24 complex in yeast comprises Emp24p, Erv25p, and most likely Erp1p and Erp2p. Deletion of EMP24 causes a strong reduction in the levels of the other three proteins of the complex (Belden and Barlowe, 1996; Marzioch et al., 1999). In this study we provide evidence that the Emp24 complex is directly required for efficient packaging of Gas1p into ER-derived vesicles. Two subunits of this complex can be directly cross-linked to the cargo protein in purified ER-derived vesicles, consistent with the hypothesis that the Emp24 complex plays a role as a cargo receptor in ER to Golgi transport.

Materials and Methods

Strains

A nmyc epitope was introduced at the NH$_2$ terminus of mature Emp24p. The myc-tagged emp24-E178A mutant was constructed by substituting an appropriate fragment of EMP24 with the sequenced mutant version obtained by PCR techniques. R H 4443 (MATa, ura3, leu2, his4, bar1, emp24::KanMx) was obtained by replacing the entire EMP24 coding sequence of R H 1959 (MATa, ura3, leu2, his4, bar1) with a KanMX cassette. EMP24 alleles were cloned into a YCplac111 (CE/N A R S) plasmid. The S. cerevisiae strain R H 696-2B (MATa, sec18-20 gap1-L::L E U2 ura3 ade2 leu2 lys2,1201 pP269) was obtained by crossing PLY129 (pP269) (MATa gap1-L::L E U2 ura3 ade2 leu2 lys2,1201 pP269) (Kuehn et al., 1996), with R H 478 (MATa sec18-20 leu2 his4). Cytosols were prepared from R H 732 (MATa his4 leu2 ura3 lys2,1201 pep4::URA3 bar1) and R H 2043 (MATa sec18-20 his4 leu2 ura3 pep4::URA3 bar1).
Emp24p Is Required for Efficient Recruitment of Gas1p into ER-derived Vesicles

To investigate whether Emp24p plays a role in cargo exit from the ER, we quantified the packaging of different secretory proteins into vesicles that were generated from wild-type and emp24 mutant ER membranes in vitro. Budding of Gas1p was much less efficient (>70% less) from emp24Δ membranes than from wild-type membranes, whereas the budding efficiencies of gpαF and an amino acid permease (Gαp1p) were similar from the two membrane sources (Fig. 1). gpαF is an established control for selectivity, because its transport from the ER to the Golgi is not affected in emp24Δ cells (Schimmöller et al., 1995). Gαp1p was also investigated because it has been shown to be efficiently incorporated into COPII-coated vesicles in vitro (Kuehn et al., 1996). Therefore, Emp24p is required for efficient packaging of at least one secretory protein into COPII-coated vesicles. The magnitude of the defect in Gas1p budding from the ER is consistent with the ER to Golgi transport defect seen in vivo (Schimmöller et al., 1995) and could, therefore, be the entire explanation for the transport delay.

It has been proposed that Emp24p may be a negative regulator of vesicle formation (Elrod-Erickson and Kaiser, 1996) and that its absence would result in increased K ar2p exit from the ER. To address this point, we compared the incorporation of K ar2p into vesicles using wild-type and emp24Δ membranes. K ar2p was poorly packaged into ER-derived vesicles using either membrane source (Fig. 1). Therefore, it does not seem that Emp24p is required for excluding K ar2p from ER-derived vesicles. This suggests, but does not prove, that the reason for K ar2p secretion in the emp24 mutant is a defect in retrograde transport.
Emp24p Is Directly Involved in the Selective Packaging of Cargo Molecules

Since Emp24p could play a role in retrograde transport it is possible that the inefficient budding of Gas1p from emp24Δ membranes is an indirect consequence of a retrograde transport defect. To address this point, we used antibodies against the cytosolic tail of Emp24p to inhibit the function of Emp24p in wild-type membranes. Preincubation of membranes with antibodies on ice reduced the budding efficiency of Gas1p greater than threefold (Fig. 2A). This inhibition was specific for Gas1p, because the packaging of Gap1p and gpaF was not affected. As a control for the specificity of our anti-tail antibodies, we used an emp24D mutant with an E178A substitution in the cytosolic tail. This point mutation does not affect the function of Emp24p (Fig. 2B), but the anti-tail antibodies no longer recognize the mutant protein (Fig. 2B). Preincubation of membranes derived from emp24Δ cells with anti-tail antibodies had no effect on budding efficiencies of Gas1p, gpaF, or Gap1p (Fig. 2A). Therefore, we conclude that Emp24p is required directly for the efficient incorporation of Gas1p into ER-derived vesicles.

One requirement for Gas1p exit from the ER is the attachment of the GPI anchor (Doering and Schekman, 1996). To test whether the GPI anchor is efficiently attached to Gas1p in the emp24Δ mutant, we performed a pulse-chase analysis followed by separation of the protein extracts into detergent and aqueous phases using Triton X-114. GPI-anchored Gas1p partitions efficiently into the detergent phase, whereas the unanchored protein partitions approximately equally between detergent and aqueous phases (Nuoffer et al., 1993). After the 4-min pulse-labeling, ~90% of the Gas1p was already found in the detergent phase in both wild-type and emp24Δ mutant strains (Fig. 2C), demonstrating that the GPI anchor is efficiently attached to Gas1p in the emp24Δ mutant. Therefore, a defect in GPI anchoring cannot explain the transport defect of Gas1p in the emp24Δ mutant.

Emp24p and Gas1p Can be Found in the Same Protein Complex in ER-derived Vesicles

Since Emp24p is efficiently incorporated into ER-derived vesicles (Schimmöller et al., 1995) and is directly required for Gas1p recruitment, we investigated if Gas1p could be found in a protein complex with Emp24p in ER-derived vesicles. ER-derived vesicles were generated in vitro and uncoated vesicles were exposed to the cleavable membrane-permeable cross-linker DSP. After lysis and denaturation, the samples were immunoprecipitated using antibodies against the c-myc epitope or against the cytosolic tail of Emp24p. Mutant (emp24Δ) cells were transformed with empty vector (emp24Δ), the wild-type allele (EMP24), myc-tagged Emp24p (mEMP24), or the myc-tagged emp24Δ (mE178A) allele. After pulse-chase labeling of wild-type (EMP24) and mutant (emp24Δ) strains, cells were lysed and extracted with Triton X-114 followed by detergent phase separation. A aqueous (A) and detergent (D) phases were subjected to immunoprecipitation and SDS-PAGE. Gas1p (both forms combined) was quantified using a PhosphorImager. Immature Gas1p (105) was converted to the mature form (125) with delayed kinetics in the emp24Δ mutant, but was efficiently extracted into the detergent phase.
was not cross-linked to Emp24p. These data clearly demonstrate that Emp24p and Gas1p can be found in the same protein complex in ER-derived vesicles and suggest that this complex is specific.

An alternative explanation for the absence of a role of Emp24p in Gap1p budding and of Gap1p from the cross-linked Emp24 complex would be if Emp24p and Gap1p were found in different ER-derived vesicles. To test this we produced vesicles using membranes and cytosol from a sec18 mutant. SEC18 encodes yeast N-ethylmaleimide-sensitive fusion protein, and its inactivation under the conditions of this experiment blocks fusion of ER-derived vesicles with the Golgi apparatus (Muñiz, M., and H. Riezman, manuscript in preparation). These conditions ensure that we are analyzing primary ER-derived vesicles. The vesicles were immunoprecipitated using antibody against the cytosolic tail of Emp24p. 83% of the Gas1p and 96% of the Gap1p copurified with the immunoprecipitated vesicles (Fig. 4). Virtually no cargo proteins were isolated when Emp24p tail antibody was omitted. Therefore, Emp24p is found in the same vesicles as Gap1p and Gas1p. The lack of effect of the emp24 mutation on Gap1p budding and Gap1p absence from the cross-linked Emp24 complex cannot be due to their presence in distinct vesicles.

**Emp24p and Erv25p Can be Directly Cross-Linked to Gas1p in ER-derived Vesicles**

The cross-linking results presented above suggest that Emp24p may be part of a cargo receptor that improves the efficiency of cargo entry into ER-derived vesicles. However, they do not show that Emp24p binds the cargo molecule directly. To address this issue we used a noncleavable cross-linker, DSG, and performed similar cross-linking studies on ER-derived vesicles. Emp24p was cross-linked to Gas1p as seen by a smear starting at around 25,000 higher apparent molecular mass than the ER form of Gas1p (Fig. 5 A). Additional bands were seen at higher molecular weights. The band at 130 kD must represent a directly cross-linked product between Emp24p (24 kD) and Gas1p (105 kD). All of the precipitated material was specific because it was not seen when the vesicles were generated from emp24-E178A mutant membranes.

Next, we immunoprecipitated the cross-linked material with antibodies against Erv25p, followed by antibodies against Gas1p. A strong band appeared at the position expected for a directly cross-linked product between Erv25p (24 kD) and Gas1p (105 kD). All of the precipitated material was specific because it was not seen when the vesicles were generated from emp24-E178A mutant membranes.

Finally, we performed a titration of DSG on the isolated vesicles. When cross-linker was omitted, no Erv25p-Gas1p product could be detected (Fig. 5 B). A low concentration of cross-linker the directly cross-linked product was by far the main product seen. At higher concentrations of cross-linker more cross-linked products were seen and the bands at higher molecular weight became more apparent. The cross-linking studies shown above demonstrate that Gas1p can be specifically and directly cross-linked to the
A major finding of our study, that Emp24p and Erv25p can be directly cross-linked to Gas1p, supports this hypothesis. This model raises two issues. Why does mutation of EMP24 or other genes encoding members of the p24 family affect the transport of so few proteins? And why is transport of these proteins not completely defective in emp24 mutant cells?

One possibility to explain the small number of proteins affected is that there could be a large and heterogeneous family of cargo receptors with overlapping function. Evidence exists for another type of cargo receptor acting in ER to Golgi transport, namely ERGIC 53, a lectin that also apparently facilitates transport of some glycoproteins (A ppenzeller et al., 1999). It is interesting to note that mutation of either receptor only affects a small number of cargo molecules in a highly specific manner. There is a large number of proteins that cycle between ER and Golgi compartments. Many of these could have cargo receptor functions in direct or indirect ways.

Another way to explain the small number of proteins affected in a cargo receptor mutant would be that only a small subset of proteins requires a receptor at this step. It seems clear that some very abundant proteins in specialized secretory cells are not concentrated in C OPII-coated vesicles (M artinez-M enarquez et al., 1999). Therefore, not all secretory proteins require a receptor for efficient ER exit. So why would some proteins require receptors for this transport step? Some proteins may bind with low affinity to other proteins that are retained in the E R lumen. These cargo molecules would require a higher affinity interaction to liberate them from the E R. Alternatively, some proteins may require high rates of secretion, for example, a protein with an activity that would be detrimental in the E R. Gas1p probably has a function in cross-linking β 1,3 glucans with β 1,6 glucans and chitin in the cell wall (Popolo and Vai, 1999). Such an activity may have negative consequences in the E R.

Previously, a bulk flow mechanism (Wieland et al., 1987) where proteins leave the E R simply by being available for entry into the forming vesicle, similar to the process of fluid-phase pinocytosis, has been proposed. This could explain why mutation of genes encoding members of the p24 family affects transport of some cargo proteins strongly, but not completely. Without a receptor, the cargo protein would still be able to enter E R-derived vesicles by bulk flow. When the function of other cargo receptors, such as the mannos e-6-phosphate receptor (K ornfeld, 1992), or yeast V ps10p (M arcusson et al., 1994) is blocked, the lysosomal or vacuolar proteins that are affected still exit the Golgi. However, they are secreted rather than targeted to the lysosome or vacuole. Since, as far as we know, there is only one destination for E R-derived vesicles, namely the Golgi apparatus, protein mis-sorting would not occur in the absence of receptors in the E R. This may also help to explain why only a small subset of proteins actually needs a receptor. As long as they can exit the E R they will go to the Golgi compartment.

A role in specific cargo recruitment is not likely to be the only role of the Emp24 complex. Secretion of Kar2p caused by mutations in subunits of this complex implies a role in the retrograde transport from the Golgi compartment to the E R (E lrod-E rickson and Ka iser, 1996; M arzi och et al., 1999). Consistent with this, p24 tails from animal

**Discussion**

A major finding of this study is that Emp24p is directly required for efficient incorporation of Gas1p into E R-derived vesicles in vitro. Two independent assays were used to show this. One used mutant membranes devoid of Emp24p. The other used antibodies to inhibit Emp24p function directly in wild-type membranes. Both assays showed that Gas1p budding depended strongly on Emp24p, whereas budding of other proteins did not. This striking specificity suggests that this complex has a highly specific role in packaging of Gas1p into E R-derived vesicles.

This direct role of Emp24p in selective budding from the E R can be explained in three ways. First, Emp24p could be required for efficient anchor attachment to Gas1p. We have shown that this is not the case. Second, Emp24p could be required for the formation of E R-derived vesicles. It has been suggested recently that α 2p24, a mammalian member of the p24 family, plays a role in the formation of E R cargo exit sites (L avoie et al., 1999). However, in yeast, the absence of Emp24p or the inhibition of its function by the tail antibody does not lead to a general defect in vesicle formation because budding of other cargo proteins was not affected. A lso, mutant membranes efficiently package the vSNA RE Sec22p (B elden and B arlowe, 1996). M oreover, since Emp24p is found in vesicles with Gas1p as well as with Gas1p, it is highly unlikely that Emp24p is required only for formation of vesicles containing Gas1p.

The third explanation for the direct role of Emp24p in selective budding from the E R is to consider Emp24p as part of a cargo receptor that is required for the packaging of a very small subset of cargo proteins. This cargo receptor would recruit specific proteins into the E R-derived vesicles, increasing the efficiency of packaging. A second major finding of our study, that Emp24p and Erv25p can
cells can bind COPI proteins (Fiedler et al., 1996; Soin et al., 1996; Dominguez et al., 1998) and when displayed on liposomes they can stimulate the formation of COPI-coated vesicles (Bremser et al., 1999). Quality control in the ER involves recycling from the Golgi compartment to the ER of misfolded proteins (Hammond and Hellenius, 1994). Interestingly, a mutation in a p24 protein from C. elgans, SE L-9, allows some misfolded proteins to escape the quality control mechanisms in the early secretory pathway (Wen and Greenwald, 1999) which could perhaps be explained by a retrograde defect. Studies that can directly address the retrograde or other putative functions of p24 proteins in vesicular traffic would be desirable.

We thank C. Barlowe, M. J. Kuehn, and R. Schekman for materials and advice; B. A. ndré for antibody; Riezman laboratory members and M. J. Gei for critical reading of the manuscript; and T. A. ust and T. E berle for technical assistance.

This work was supported by Swiss National Foundation grants (to H. Riezman and H.-P. Hauri), Federation of European Biochemical Societies and Human Frontiers Science Program Organization fellowships (to M. Muñiz).

Submitted: 29 November 1999
R evised: 14 January 2000
A ccepted: 19 January 2000

References

A ppenzeller, C., H. A ndersson, F. K appeler, and H. P. Hauri. 1999. The lectin E R G I C-53 is a cargo transport receptor for glycoproteins. Nat. Cell Biol. 1:330–334.

Barlowe, C., L. Or ci, T. Y eung, M. H osobuchi, S. Hamamoto, N. Salama, M. F. R exach, M. R avazolla, M. A mherdt, and R. Schekman. 1994. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell. 77:895–907.

B rremser, M., W. N ickel, M. S chweikert, M. R avazolla, M. A mherdt, C. A. Hughes, T. H. Soliner, J. E. R otman, and F. T. W ieland. 1999. Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. Cell. 96:495–506.

Do ering, T. L., and R. S chekman. 1996. GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum to COPI II vesicles. EM B O (E ur. M ol. B iol. O rgan.) J. 15:182–191.

D ominguez, M., K. D eja egard, J. F üllekrug, S. D hahan, A. F azel, J. P. Paccaud, D. Y. T homas, J. J. B ergeron, and T. N ilsson. 1998. gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COPI I and II coatomer. J. Cell Biol. 140:751–765.

E nd- E rickson, M. J., and C. A. K aiser. 1996. Genes that control the fidelity of endoplasmic COPI to Golgi transport identified as suppressors of vesicle budding mutations. M ol. Cell. Biol. 7:1043–1058.

Fiedler, K., M. Vei t, M. A. Stammes, and J. E. R otman. 1996. Bimodal interaction of coatomer with the p24 family of putative cargo receptors. Science. 273:1396–1399.

F üllekrug, J., T. S ukanuma, B. L. T ang, W. H ong, B. S torrie, and T. N ilsson. 1999. Localization and recycling of gp27 (hp24amann3): complex formation with other p24 family members. M ol. B iol. C ell. 10:1939–1955.

Hammond, C., and A. Hellenius. 1994. Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. J. Cell Biol. 126:41–52.

Knofeld, S. 1992. Structure and function of the mannose 6-phosphate/insulin-like growth factor II receptors. A m. Rev. B iochem. 61:307–330.

Kuehn, M. J., R. S chekman, and P. D. L jungdahl. 1996. Amino acid permeases require COPII components and the ER resident membrane protein Sh3p for packaging into transport vesicles in vitro. J. Cell Biol. 135:585–595.

Lavoie, C., J. P aement, M. D emongeot, L. R oy, S. Dahan, J. N. Gushue, and J. J. B ergeron. 1999. Roles for alpha2p24 and COPI in endoplasmic reticulum cargo exit site formation. J. Cell Biol. 146:285–299.

Marcusson, E. G., B. F. H ozdovszky, J. L. C ereghino, E. G harkahanian, and S. D. E. M r. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the V PS10 gene. Cell. 77:579–586.

Martínez-Mañá, J. A., H. B. G reuze, J. W. S chimmoller, and H. Riezman. 1999. Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. Cell. 98:81–90.

Marzioch, M., D. C. Henthorn, J. H. H errmann, R. W ilson, D. Y. T homas, J. J. B ergeron, R. C. S oliari, and A. R owley. 1999. Erp1p and Erp2p, partners for Erp24p and Erp25p in a yeast p24 complex. M ol. B iol. C ell. 10:1923–1938.

Nuoffer, C., P. Jeno, A. Conzelmann, and H. Riezman. 1991. Determinants for glycosphospholipid anchoring of the Saccharomyces cerevisiae Gas1 protein to the plasma membrane. M ol. Cell. B iol. 11:27–37.

Nuoffer, C., A. Hovarth, and H. Riezman. 1993. A analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of Saccharomyces cerevisiae Gas1 proteins. B iol. C hem. 268:10558–10563.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. S cience. 189:347–358.

Popolo, L., and M. Vai. 1999. The Gas1 glycoprotein, a putative wall polymer cross-linker. B iochim. B iophys. Acta. 1426:385–400.

Rojo, M., R. Pepperkok, G. E mery, R. K ellem, E. Stang, R. P. Garten, and J. G ruenberg. 1997. Involvement of the transmembrane protein p23 in biosynthetic transport. J. Cell B iol. 130:1119–1135.

Rothman, J. E., and F. T. W ieland. 1996. Protein sorting by transport vesicles. S cience. 272:227–234.

Sche kman, R., and L. O rci. 1996. Coat proteins and vesicle budding. S cience. 271:1526–1533.

Schimmoller, F., B. S inger-K ruger, S. S chroeder, U. K ruger, C. B arlowe, and H. Riezman. 1995. The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. EM B O (E ur. M ol. B iol. O rgan.) J. 14:1329–1339.

Sohn, K., L. O rci, M. R avazolla, M. A mherdt, M. B renemer, F. L ottspeich, K. Fiedler, J. B. H elms, and F. T. W ieland. 1996. A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatomer binding. J. Cell B iol. 135:1239–1248.

Stammes, M. A., M. W. C raignhead, M. H. H oe, N. L ampen, S. G eromanos, P. T empst, and J. E. R otman. 1995. An integral membrane component of coatomer-coated transport vesicles defines a family of proteins involved in budding (published erratum appears in P roc. N at. A cad. S ci. U SA. 92: 10816). P roc. N atl. A cad. S ci. U SA. 92:8011–8015.

Süttérin, C. L. D oering, F. S chimmoller, S. S chroeder, and H. Riezman. 1997. Specific requirements for the ER to Golgi transport of GPI-anchored proteins in yeasts. J. Cell B iol. 110:2703–2714.

Wen, C., and I. G reenwald. 1999. p24 proteins and quality control of LIN-12 and GLP-1 trafficking in Caenorhabditis elegans. J. Cell B iol. 145:1165–1175.

Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. C ell. 50:289–300.