Formation of vesicular intermediates in protein transport between the endoplasmic reticulum and the Golgi apparatus involves a mechanism that sorts and packages two classes of molecules into transport vesicles: targeting molecules, which are required for targeting and consumption of vesicular intermediates, and cargo proteins. In order to examine the importance of cargo in this packaging reaction, we developed an in vitro assay that quantifies vesicle formation based on segregation of targeting molecules. Here we document that endoplasmic reticulum devoid of cargo proteins is competent in the formation and release of targeting molecule-containing vesicles in a fashion indistinguishable from its normal counterpart. This observation implies that packaging of cargo proteins may be uncoupled from the recruitment of targeting molecules during vesicle budding from the endoplasmic reticulum. Using the same assay, we demonstrate that the packaging of targeting molecules into vesicles is not dependent on the luminal chaperone, BiP (Kar2p).

The cytosolic events that lead to the formation and release of vesicles from endoplasmic reticulum (ER)1 were reconstituted with the use of an enriched ER fraction and three purified cytosolic fractions (Sec13/31p, Sec23/24p, and Sar1p) that comprise a novel coat protein complex, COPII (Salama et al., 1993; Barlowe et al., 1994). A system comprising these factors and GTP support the inclusion of cargo proteins such as glycosylated pro-α-factor (gpαf) into 60-nm vesicles that bud from the ER (Barlowe et al., 1994). In addition to cargo proteins, ER-derived vesicles contain synaptobrevin-like proteins such as Bet1p, Bos1p, and Sec22p (Barlowe et al., 1994; Lian and Ferro-Novick, 1993; Rexach et al., 1994), the targeting molecules that function as specificity factors by serving as the docking "v-SNARE" in the consumption of vesicles by the Golgi apparatus (Rothman, 1994). The absence of ER resident proteins in these transport vesicles suggests that cargo proteins are faithfully sorted from other ER luminal proteins (Rexach et al., 1994). Thus, the COPII proteins plus GTP are sufficient to provide the cytosolic information necessary to reproduce an authentic vesicle budding event.

In contrast to advances in the biochemical dissection of cytosolic requirements for vesicle transport, the molecular events within the ER lumen that are responsible for segregating and packaging targeting and cargo molecules into the vesicles are not well understood. Only two proteins known to play a role in post-translational aspects of secretion are lumenerally oriented. Sec12p is a type II membrane glycoprotein with a C-terminal domain of approximately 100 residues in the ER lumen. The cytosolic domain catalyzes nucleotide dissociation on Sar1p which serves to initiate the budding process (Barlowe and Schekman, 1993). In contrast, the luminal domain is dispensable; truncated Sec12p that retains only the N-terminal and membrane anchor domains is sufficient to sustain normal yeast cell growth. BiP (Kar2p), in addition to its role in polypeptide translocation (Vogel et al., 1990; Sanders et al., 1992), is required in the quality control decision to segregate incompletely folded polypeptides away from cargo that is ready for transport (Gething and Sambrook, 1992). However, given the defect in α-factor precursor translocation in kar2 mutant strains, it has not been possible to test a role for Kar2p in the packaging of folded proteins into transport vesicles.

In order to determine if the sorting and packaging of cargo proteins is an essential prerequisite to vesicle budding, we developed an assay that measures the distribution of targeting molecules rather than that of gpαf. By monitoring the release of Bet1p- and Sec22p-containing vesicles from ER membranes isolated from cells treated with cycloheximide or carrying a temperature-sensitive allele of kar2, we demonstrated that neither the packaging of cargo proteins nor Kar2p function are required to form COPII vesicles.

**Materials and Methods**

Preparation of ER Membranes, Crude Cytosol, and COPII—Strain RS607 (lei2-3, 112 ura3-52 pep4:URA3 MATα), which harbors the plasmid pDA 6300 (for overexpressing gpαf in order to enhance its detection in isolated vesicles; see Fig. 2), and RS598 (kar2-159 ura3-52 ade2-101 pep4:URA3 MATα) were used to prepare ER membranes, as described (Barlowe et al., 1994) except that RS598 was grown at 17°C. To prepare cargo-depleted ER membranes or CHX-treated cytosol, we cultured RS607 pDA6300 in YP medium (1% Bacto-yeast extract, 2% Bacto-peptone (Difco)) and 2% dextrose to OD600 = 3 and cycloheximide (Sigma) was added to a final concentration of 50 μg/ml. Cells were incubated for 1 h at 30°C to deplete secretory proteins before ER membranes were isolated or crude cytosol was prepared by lysis of cells in a bead beaker (Rexach et al., 1994). For crude cytosol prepared from cells grown under normal conditions, we cultured RS607 in YP medium and 2% dextrose to OD600 = 4 before cells were subjected to liquid nitrogen lysis (Barlowe et al., 1994). COPII proteins were purified as described (Barlowe et al., 1994).

In vitro VSV budding assay—Vesicle budding measuring secretion of exogenously introduced 35S-labeled gpαf was performed as described (Barlowe et al., 1994). To detect budding by assay of Bet1p and Sec22p, we used ER fractions mixed with the indicated reagents and incubated at 20°C for 30 min, but without the translocation of radioactive α-factor precursor. ER-derived vesicles were separated from the fast sedimenting ER by centrifugation and were analyzed for the presence...
RESULTS

COPII- and GTP-dependent Budding of v-SNARE-containing Vesicles from ER Membranes—Vesicle budding was monitored in the absence of exogenously introduced radioactive cargo proteins by quantifying the segregation of Bet1p from the ER. In a typical reaction, 30% of Bet1p budded in vesicles from the ER in a COPII protein- and GTP-dependent manner (Fig. 1, columns 1, 5, and 6, black bars). Omission of any one of the purified COPII proteins prevented Bet1p release (Fig. 1, columns 2–4, black bars). In order to isolate the Bet1p-containing compartment for biochemical analysis, the reaction product was subjected to isopycnic gradient centrifugation in Nycodenz step gradients (Barlowe et al., 1994). Bet1p was detected at the 25–15% (w/v) Nycodenz interface, suggesting this targeting molecule was associated with a membrane compartment with a buoyant density similar to that of ER-derived vesicles (Barlowe et al., 1994; Rexach et al., 1994) (Fig. 2a, lane 2). The endogenous soluble cargo protein gppf was detected in the same fraction, and the appearance of both proteins was dependent on the presence of the three purified fractions and GTP (Fig. 2a, compare lanes 1 and 2). In addition, this fraction exhibited a polypeptide pattern similar to that of ER-derived vesicles previously described (Barlowe et al., 1994; Rexach et al., 1994) (Fig. 2b, lane 2). The absence of ER resident proteins Kar2p (BIP) and Sec61p in either the crude reaction product (Fig. 1, hatched and white bars) or in gradient-purified vesicles (Fig. 2a, lane 2) indicated that this budding reaction enriched for targeting proteins, and ruled out the possibility that the Bet1p-containing compartment was produced by nonspecific fragmentation of the ER membrane. These results demonstrate that the import of exogenous α-factor precursor is not required for Bet1p packaging. Similar results were obtained in evaluating the packaging of Sec22p, another v-SNARE molecule (Fig. 3).

Cargo-depleted ER Membranes Are Capable of Vesicle Budding—Vesicle formation by the ER involves the selection of cargo proteins and concentration of these molecules at discrete sites within ER before the assembly of vesicles (Mizuno and Singer, 1993; Balch et al., 1994). A similar process seems to function in concentrating and packaging targeting molecules into ER-derived vesicles. To examine whether packaging of endogenous cargo proteins is essential for vesicle budding, we investigated the capacity of ER devoid of cargo proteins to participate in this reaction. The ER luminal was depleted of cargo proteins by inhibiting protein synthesis in vivo with cycloheximide (CHX) during a period long enough to purge even the most slowly secreted proteins in yeast (Esmon et al., 1981; Novick et al., 1981; Novick and Schekman, 1983). In the absence of protein synthesis, endogenous gppf was depleted from the ER, whereas the level of ER resident proteins and targeting molecules remained unchanged (Fig. 2a, compare lanes M+ and M−). The packaging of targeting molecules was assessed in these cargo-free ER membranes using the modified budding assay. Bet1p and Sec22p were released from the CHX-treated ER fraction in a reaction dependent on COPII proteins and GTP (Fig. 3, column 5, and Fig. 4, column 2). As before, the Bet1p-containing vesicles sedimented at the 25–15% (w/v) Nycodenz interface upon isolation by ultracentrifugation (Fig. 2a, compare lanes 2 and 3). The polypeptide pattern of the membrane species derived from CHX-treated ER was virtually identical to that of vesicles from mock-treated ER fractions (Fig. 2b, compare lanes 2 and 3). We found no detectable budding of Sec61p and Kar2p from these ER fractions, demonstrating that formation of Bet1p-containing vesicles was not triggered by erroneous packaging of ER resident proteins or ER fragmentation into vesicles (Fig. 2a, lane 3, and Fig. 4, hatched and white bars).
Crude Cytosol Promotes Vesicle Budding from Cargo-depleted ER Membranes—Although the above results show that COPII proteins promote the formation of authentic vesicles by cargo-depleted ER, it remained a possibility that cells possess a cytosolic regulator that modulates or arrests traffic when cargo production falls. Such a protein could act on the ER membrane or could modify the activity of a COPII subunit, for example, by reversible covalent modification. This regulator may not be represented in COPII proteins isolated from exponentially growing cells. We therefore tested the ability of crude cytosol prepared from either normal or CHX-treated cells to stimulate vesicle budding from cargo-depleted ER. As shown in Fig. 5, crude cytosol and GTP promoted the formation of Bet1p-containing vesicles to the same extent as COPII. Thus, we find no evidence for a factor extrinsic to COPII that may sense the availability of cargo for transport of targeting proteins from the ER.

Cargo-depleted ER Membranes Are Active in Packaging gpaf—We next considered the possibility that cargo proteins may be distinct from targeting molecules in requiring ongoing protein synthesis for packaging. Membranes from CHX-treated cells were incubated in a standard budding reaction with 35S-labeled α-factor precursor as an exogenous cargo protein. As shown in Fig. 6, cargo-depleted ER membranes produced 35S-gpaf-containing vesicles in the presence of COPII or crude cytosols prepared from normal or CHX-treated cells. This result suggests that CHX-treated ER retains an intact cargo protein sorting and packaging machinery whose operation is not required for the formation of transport vesicles. In summary, the CHX-treated ER fraction, which was virtually identical to its mock-treated counterpart except for the lack of cargo proteins, was capable of producing a membrane compartment with physical and biochemical properties identical to those of ER-derived transport vesicles.

ER Membranes Defective in Kar2p Function Are Active in Vesicle Budding—Stable ER lumenal chaperones, such as Kar2p, that are not depleted during inhibition of protein synthesis may contribute to the packaging of cargo and targeting molecules. Because Kar2p is required to translocate α-factor precursor into the ER in vitro (Sanders et al., 1992), we quantified the formation of Bet1p-containing vesicles using kar2–159 membranes isolated from cells grown under normal conditions (cytosol) or in the presence of CHX (CHX cytosol). Dose-response assays showed that half-maximal budding was achieved with 7 μg of cytosol from untreated cells and 8 μg of cytosol from cycloheximide-treated cells.
Uncoupled Packaging of Targeting and Cargo Molecules in ER

The role of secretory cargo in membrane traffic between the ER and Golgi apparatus has been difficult to resolve. In the extreme case of vesicular stomatitis virus infection, which shuts down host protein synthesis (Zilberstein et al., 1981), the capsid G protein is transported through the secretory pathway as the sole cargo molecule. In yeast, glycoproteins accumulated in the ER in a sec mutant strain are secreted when cells are returned to a permissive temperature in the presence or absence of an inhibition of protein synthesis (cycloheximide) (Novick and Schekman., 1983). In this case the cargo accumulated in the ER may include or represent structural proteins necessary for the traffic event. In support of this view, small vesicles detected as an intermediate in the transport of proteins from the ER in yeast depend upon protein synthesis to accumulate to morphologically observable levels (Kaiser and Schekman, 1990). However, even in this case, a basal level of vesicle traffic may persist in cycloheximide-treated cells. Unfortunately, in vivo experiments have not addressed the cycling of ER to Golgi vesicle targeting (SNARE) proteins in cells treated with cycloheximide.

We investigated this problem using a cell-free vesicle budding reaction where the cargo content of the donor ER membrane may be manipulated and the role of cargo in the formation of an anterograde transport vesicle can be distinguished from other steps in the ER to Golgi limb of the secretory pathway. The yeast SNARE proteins Bet1p and Sec22p continue to be packaged into transport vesicles irrespective of the presence of cargo molecules. SNARE protein packaging requires the full set of Sec proteins that comprise the COPII coat shown previously to envelope a model cargo protein, gspf. No requirement for cargo was detected when this assay was conducted with ER membranes from cycloheximide-treated cells incubated with COPII proteins or cytosol from exponentially growing or cycloheximide-treated cells. Nevertheless, we cannot rule out the possibility that cells possess a feedback mechanism that we have not reproduced in vitro. No requirement for the luminal chaperone Kar2p was detected when the packaging of SNARE molecules was assessed using kar2 mutant membranes incubated with COPII proteins at permissive or restrictive temperatures. Furthermore, we observed no influence of cargo or Kar2p on the segregation of SNARE and resident ER membrane proteins that accompanies the formation of transport vesicles.

We conclude that the availability and packaging of cargo proteins is not essential to the formation of ER-derived vesicles. A similar phenomenon was first discovered in endocytosis of low density lipoprotein receptors in human fibroblasts (Anderson et al., 1978) and more recently in the recycling of pheromone receptors in yeast (Davis et al., 1993), in which the receptors are retrieved from the cell surface irrespective of their liganded state. By analogy to the role of clathrin and its adaptors in receptor-mediated endocytosis, we propose that SNARE and certain membrane cargo (or cargo adaptor) molecules possess structural or sequence information that allows their recognition and capture by COPII. These membrane proteins may share a common domain of interaction on a COPII subunit and thus be packaged together by a stochastic process. Ordinarily, the abundance of both types of membrane protein would ensure their coincident packaging into a single vesicle species. Alternatively, SNARE and membrane cargo (or cargo adaptor) molecules may interact with different COPII subunits, or different portions of the same subunit, thus ensuring a coordinate packaging event. In either case, our model predicts that in the absence of one type of membrane protein packaging of the other would continue.

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FIG. 7. Budding of v-SNARE-containing vesicles is independent of Kar2p activity. ER membranes prepared from RSY598 (kar2-159) were tested for the packaging of Sec61p (hatched bars) and Bet1p (black bars) in the presence of COPII or 100 μg of crude cytosol at 20 °C (lanes 1–4) or 30 °C (lanes 5–8).
