Kinase Signaling Initiates Coat Complex II (COPII) Recruitment and Export from the Mammalian Endoplasmic Reticulum*

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The events regulating coat complex II (COPII) vesicle formation involved in the export of cargo from the endoplasmic reticulum (ER) are unknown. COPII recruitment to membranes is initiated by the activation of the small GTPase Sar1. We have utilized purified COPII components in both membrane recruitment and cargo export assays to analyze the possible role of kinase regulation in ER export. We now demonstrate that Sar1 recruitment to membranes requires ATP. We find that the serine/threonine kinase inhibitor H89 abolishes membrane recruitment of Sar1, thereby preventing COPII polymerization by interfering with the recruitment of the cytosolic Sec23/24 COPII coat complex. Inhibition of COPII recruitment prevents export of cargo from the ER. These results demonstrate that ER export and initiation of COPII vesicle formation in mammalian cells is under kinase regulation.

Components comprising the cytosolic coat complex II (COPII) are now recognized to be involved in cargo selection and export from the endoplasmic reticulum (ER) (1, 2). Export from the ER is initiated by the activation of the small GTPase Sar1 through exchange of GDP for GTP by the membrane-associated Sec12 guanine nucleotide exchange factor (GEF). This activation step leads to the recruitment of the COPII subunits Sec23/24 from the cytosol to the membrane to form a tertiary complex that interacts with cargo and cargo receptors, initiating selection prior to export (3, 4). Subsequent recruitment of the Sec13/31 complex allows the selected cargo to be exported from the ER by budding vesicles. Following COPII-mediated sorting from the ER, cargo-containing COPII vesicles are believed to fuse to form pre-Golgi intermediates containing tubular elements (5). Pre-Golgi intermediates are the first step in the exocytic pathway involved in the retrieval of recycling components to the ER using the coat complex I (COPI) components (6), thus separating forward moving cargo from the membrane-bound components of the COPII budding and fusion machinery (2, 7–9). The integration of these two sorting steps enables the forward moving cargo to be selectively delivered to the Golgi complex for transport to the cell surface (7).

The mechanisms by which different sorting steps in the early secretory pathway are regulated remain unknown. We (10, 11) and others (12) have previously implicated kinases and phosphatases in regulating transport between the ER and Golgi compartments. More recently, a qualitative morphological study using indirect immunofluorescence suggested that H89, an isouquinolinesulfonamide that is frequently used as a selective serine/threonine and protein kinase A inhibitor, was involved in a late step of COPII vesicle coat assembly following Sar1 activation (13). We have now obtained quantitatively the step in ER export directly regulated by H89 sensitive kinase(s) utilizing purified COPII components to follow the ordered recruitment of COPII to membranes, COPII-mediated vesicle formation, and cargo export. We show that Sar1 recruitment to ER membranes is sensitive to kinase activation, demonstrating that the first step in COPII vesicle formation, Sar1 activation, is under kinase regulation.

EXPERIMENTAL PROCEDURES

Materials—Recombinant wild type Sar1a was expressed and purified as described previously (14). Sec23/24 and Sec13/31 COPII coat subunits were purified from rat liver cytosol as described previously (3). Antibodies to Sec23, Sar1a, and vesicular stomatitis virus glycoprotein (VSV-G) were described previously (3). GTPyS was purchased from Roche Molecular Biochemicals. H89, PKI, and PKD inhibitor peptides were purchased from Calbiochem.

Coat Recruitment Assays—Sec23 recruitment (two-stage recruitment assay with salt wash) was performed as described previously (7). For Sar1 binding, microsome membranes, prepared as described previously (8) (20–40 mg), were incubated in the presence of wild type Sar1 (0.1 mg) and Sec23/24 (0.7 mg) or rat liver cytosol (200 mg) in a final volume of 60 ml in a reaction mix containing 36 mM Hepes pH 7.2, 70 mM K2OAc, 2.5 mM MgOAc, 250 mM sorbitol, 1.8 mM CaCl2, 1.5 mM MgCl2, 5 mM EGTA, and 100 mM GDP in the presence or absence of an ATP-regenerating system as described previously (11). Following incubation, the reactions were layered on a 15% sucrose cushion (75 mM Hepes pH 7.2, 70 mM K2OAc, 2.5 mM MgOAc, 250 mM sorbitol, 1.8 mM CaCl2, 5 mM EGTA, and 100 mM GDP) and analyzed by SDS-polyacrylamide gel electrophoresis and quantitative immunoblotting (8). All experiments presented were performed at least twice with identical results.

In Vitro Vesicle Formation Assay—In vitro vesicle formation assay was performed with purified COPII components utilizing wild type Sar1 (2 mg), Sec23/24 (1 mg), and Sec13/31 (12 mg) as described previously (3). All experiments presented were performed at least twice with identical results.

RESULTS AND DISCUSSION

The formation of COPII vesicles requires the sequential recruitment of the Sar1 GTPase and the Sec23/24 coat complex followed by the Sec13/31 complex (16). We have previously demonstrated biochemically that the recruitment of the Sec23/24 coat complex to ER membranes requires Sar1 activation and ATP (3, 7, 17). Consistent with these results, and as
expected, ATP is also required for the recruitment of Sec13/31 (13). One possible explanation of the need for ATP was that ER membranes need to be primed with ATP prior to Sar1 activation and COPII recruitment. Alternatively, Sar1 activation may be initiated prior to the ATP-dependent step, which is then required for Sec23/24 recruitment and coat assembly. To test these two possibilities, ER membranes were incubated in a "stage 1" reaction in the presence of crude cytosol with or without ATP and/or the nonhydrolyzable form of GTP, GTPγS, for 10 min at 32 °C as described previously (7) (Fig. 1A). Following incubation, the membranes were either transferred to ice or collected by a brief centrifugation and resuspended in the presence or absence of ATPγS or GTPγS, as indicated, for an additional 10 min at 32 °C (Stage 2). At the end of the stage 2 incubation, membranes were collected and analyzed for Sec23/24 binding by immunoblotting as described under "Experimental Procedures." B, membranes were incubated either with rat liver cytosol (cyt) or purified Sar1 and Sec23/24 COPII coat components, in the presence of GDP, ATPγS, or GTPγS as indicated. Binding of Sar1 and Sec23/24 was determined as described under "Experimental Procedures."

FIG. 1. ATP is required for Sar1 recruitment to ER membranes. A, membranes were incubated in the presence or absence of an ATP regenerating system (ATPγS) and/or 100 μM GTPγS, as indicated, for 10 min at 32 °C (Stage 1) as described under "Experimental Procedures." Following incubation, membranes were either transferred to ice or collected by a brief centrifugation and resuspended in the presence or absence of ATPγS or GTPγS, as indicated, for an additional 10 min at 32 °C (Stage 2). At the end of the stage 2 incubation, membranes were collected and analyzed for Sec23/24 binding by immunoblotting as described under "Experimental Procedures."
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36 nM). Concentrations of up to 250 nM PKI failed to affect Sar1 and Sec23/24 recruitment (Fig. 2C) or COPII vesicle formation (Fig. 3B, see below). These results are consistent with the inability of other PKA inhibitors to block ER-to-Golgi transport (12, 13). H89 was also utilized previously as an inhibitor of serine/threonine kinases of the PKC family. However, related inhibitors such as H7 or chelerythrine do not affect ER-to-Golgi transport (13). Moreover, recent morphological observations raised the possibility that the primary step affected by the H89-sensitive kinase is the Sec12 GEF. Consistent with this observation, in the presence of either PKD peptide substrate, using a specific PKD peptide substrate (PKD) (250 nM), the PKD-inhibiting peptide (50 μM), or H89 (160 μM). The recruitment of Sar1 and Sec23/24 was determined as described under “Experimental Procedures.”

COP II components, in the absence of cytosol, support efficient export. However, further experiments are necessary to address the potential role of lipid kinases, as a PI4-kinase of unknown function has been demonstrated to be enriched on ER membranes (20).

Our results demonstrate that an H89-sensitive kinase initiates Sar1 recruitment, the first step in COPII vesicle formation. Our conclusions differ significantly from a recent report in which H89 was proposed to affect a late, but not early, step in COPII vesicle formation (13); that report based its conclusion on experimental results in which H89 was observed to inhibit GTPγS stabilization of the Sec13/31 complex to membranes. It was assumed that the inclusion of GTPγS in the assay was sufficient for passive Sar1 activation. In contrast, we now demonstrate that the recruitment and activation of Sar1 by GTPγS is dependent on ATP and inhibited by H89. These results are consistent with the requirement for physiological temperatures for recruitment of COPII to membranes in vitro and with the relatively low affinity of Sar1 for guanine triphosphate nucleotides in the absence of a Sar1 specific exchange activity (3, 7, 8, 17). Thus, Sar1 binding of GTPγS is not passive and requires the Sec12 GEF. Consistent with this observation, in the present experiments we have observed that in the absence of Sar1 activation by Sec12, the recruitment of the Sec23/24 complex, and as a consequence, the subsequent recruitment of the Sec13/31 complex, cannot occur. Indeed, the inability morphologically to detect ER export sites using indirect immunofluorescence based on the detection of recruited Sec13/31 complex (13), is entirely consistent with our results that demonstrate that the primary step affected by the H89-sensitive kinase is the first step, Sar1 recruitment and activation.

Control of small GTPase activation by kinases is not unpre-
edented. Activation of Ras through the recruitment of the Grb2-SOS GEF complex is initiated by protein tyrosine kinases (21). By analogy to Ras, recruitment and activation of Sar1 through Sec12, a transmembrane GEF, may be regulated by a membrane-associated kinase. This conclusion is consistent with our observations that recruitment of Sar1 is the limiting component in our budding reaction (3). In preliminary experiments we have failed to detect direct phosphorylation of either Sar1 or Sec12 during the formation of COP II vesicles. However, further studies are required to establish or preclude a role for Sar1 or Sec12 phosphorylation during these potentially transient events involved in coat assembly. Alternatively, our previous studies have suggested a role for cargo in modulating the COP II export machinery (17). Although it remains to be seen whether cargo availability per se can regulate kinase and Sar1 activation, ER chaperones, which interact with cargo during the folding cascade, may mediate these events, thereby linking cargo availability to ER export. This interpretation is consistent with the role of the ER resident chaperone Ig heavy chain-binding protein (BIP) in activation of ER stress receptor kinases in response to misfolded cargo in the ER (22), an event that leads to a global up-regulation of the exocytic pathway (23). Additional targets for kinase activity may therefore include proteins of the ER folding and selection machinery that can interact with cargo prior to its association with the recruited COP II coat (24–26).

Placing ER budding under kinase control would allow the export machinery to respond to extracellular signaling pathways, thus integrating the secretory pathway with cellular physiology. We have previously demonstrated that activation of IgE receptors in mast cell lines enhances ER export (27) and that ER export is regulated by a calphostin C-sensitive, diacylglycerol-binding protein (28). Moreover, it has been shown that modulation of kinase activity plays a major role during mitosis, leading to cessation of ER export and dispersal of ER export sites (29). We propose that the ability of the ER export machinery to integrate with kinase signaling cascades may represent an important first step in the general function of cargo selection by COP II machinery in the exocytic pathway in mammalian cells (26).

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