Dynamics of the COPII coat with GTP and stable analogues

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We have developed an assay to monitor the assembly of the COPII coat onto liposomes in real time. We show that with Sar1pGTP bound to liposomes, a single round of assembly and disassembly of the COPII coat lasts a few seconds. The two large COPII complexes Sec23/24p and Sec13/31p bind almost instantaneously (in less than 1 s) to Sar1pGTP-doped liposomes. This binding is followed by a fast (less than 10 s) disassembly due to a 10-fold acceleration of the GTPase-activating protein activity of Sec23/24p by the Sec13/31p complex. Experiments with the phosphate analogue BeF_x suggest that Sec23/24p provides residues directly involved in GTP hydrolysis on Sar1p.

The COPII coat promotes the formation of transport vesicles from the endoplasmic reticulum[1,2]. This coat forms by the sequential recruitment of three cytosolic proteins: the small G protein Sar1p, and two large complexes, Sec23/24p and Sec13/31p (refs 3, 4). The coat has two main functions, the physical deformation of the endoplasmic reticulum (ER) membrane into vesicles and the selection of cargo molecules[1,2].

Because the GTP-bound form of Sar1p has the unique ability to interact directly with both lipid membranes and the Sec23/24p complex, the binary switch of Sar1p between GDP-bound and GTP-bound forms governs the assembly and disassembly of the COPII coat. Thus, assembly is driven by the exchange of GDP for GTP on Sar1p, which is catalysed by the ER-resident protein Sec12p (ref. 5), whereas disassembly requires GTP hydrolysis on Sar1p, which is stimulated by one subunit of the COPII coat, Sec23p (ref. 6). This built-in GTPase-activating protein (GAP) makes the COPII coat intrinsically unstable. Nonhydrolysable analogues of GTP have therefore been used to isolate and characterize COPII-coated vesicles[3,4].

The formation of COPII-like vesicles has been observed *in vitro* by using liposomes incubated with the three purified COPII components and the nonhydrolysable analogue of GTP, GMP-PNP[4]. In this minimal system, the sequence of assembly of the COPII coat and the morphology of coated structures mimic closely what is observed with biological membranes[3,4]. Thus, Sar1p binds to liposomes on incubation with GMP-PNP, allowing the subsequent recruitment of Sec23/24p, which itself is required for the recruitment of Sec13/31p (ref. 4). Electron microscopy revealed that the assembly of the COPII coat onto liposomes produced three profiles: partly coated liposomes, coated buds emerging from liposomes, and small (~70 nm) coated vesicles[4]. These structures might represent consecutive snapshots in the pathway of vesicle formation.

To study the dynamics of assembly and disassembly of the COPII coat, we have established a real-time assay based on light scattering. The light scattering signal, which depends on the mass increase associated with coat polymerization on the liposome surface, provided a level of quantitative precision and time resolution that was not possible by more conventional means of analysis[4]. With this technique we describe novel intermediates in coat assembly and an influence of coat polymerization on GTP hydrolysis.

Results

A real-time assay for the coating of liposomes by COPII proteins. The intensity and pattern of the light scattered by an object depends on its size, shape and mass[7]. The coating of liposomes by proteins and eventually their fragmentation into small vesicles should therefore lead to changes in light scattering. In Fig. 1a–c the scattering of light ($\lambda = 350$ nm) of a suspension of liposomes was continuously monitored at 90° in a fluorimeter on the addition of the three COPII components and the nonhydrolysable GTP analogue GMP-PNP. Previous work established a liposome composition (defined as "major–minor mix"; see Methods) optimal for the formation of the COPII coat with GMP-PNP[4]. Lipids with unsaturated acyl chains facilitated the translocation of Sar1p to liposomes upon the exchange of GDP GMP-PNP[4]. Subsequently, the presence of anionic phospholipids promoted the recruitment of Sec23/24p and Sec13/31p (ref. 4). In Fig. 1a the three COPII components were added to liposomes before the addition of GMP-PNP. Whereas none of the three COPII proteins promoted any significant light scattering change, the subsequent addition of GMP-PNP triggered a very large increase in light scattering (+150%) within a few minutes. No signal was observed in incubations conducted in the absence of liposomes. The experiment shown in Fig. 1b demonstrates that the signal observed in Fig. 1a can be dissected into three elementary signals, each of which corresponds to the binding of one COPII component. In this experiment, Sar1pGDP and GMP-PNP were added first to liposomes. Upon the addition of GMP-PNP, a small and slow increase in light scattering was observed. After this first stage, Sec23/24p and Sec13/31p were added successively. For each addition, an instantaneous increase in light scattering was observed. Because no signal was observed when Sec23/24p and Sec13/31p were added directly to liposomes (Fig. 1a), the Sec23/24p and Sec13/31p signals observed in Fig. 1b must be dependent on Sar1p and GMP-PNP. Inverting the order of addition of Sec13/31p and Sec23/24p revealed that the Sec13/31p signal was also dependent on Sec23/24p (Fig. 1c). Indeed, a mixture of Sec13/31p with liposomes preincubated with Sar1pGDP and GMP-PNP did not induce any change in light scattering. However, the subsequent addition of Sec23/24p induced a large jump, the amplitude of which corresponded to the sum of the Sec23/24p and Sec13/31p binding signals (compare Fig.1 b and Fig.1 c).

Because no nucleotide exchange factor for Sar1p was included in our assay, the activation of Sar1p upon exchange of GDP for GMP-PNP should be relatively slow. Slow exchange could account for the gradual increase in signal observed upon GMP-PNP addition, on Sar1pGDP (Fig. 1b, c), or on a complete COPII mixture (Fig. 1a). To assess directly the time course of Sar1p activation, we used tryptophan fluorescence. Sar1p, ARF and the α-subunit of heterotrimeric G proteins share a tryptophan residue in a switch

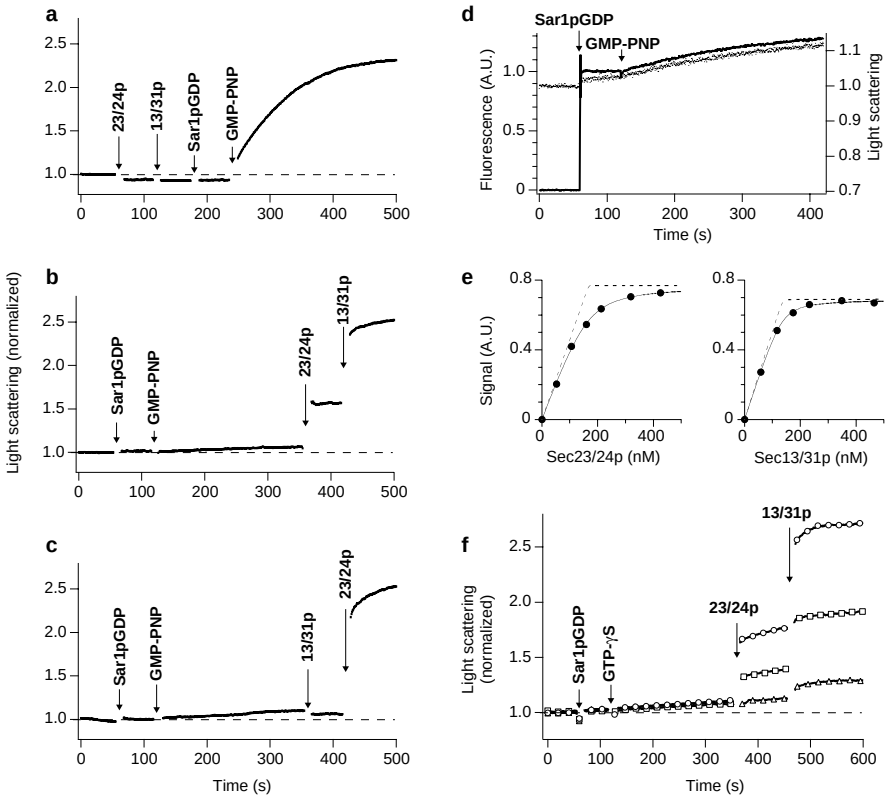

Figure 1 **Sequential assembly of the COPII coat onto liposomes as detected by light scattering. a–c**, Order-of-addition experiments. The light scattering of a suspension of major–minor mix liposomes (100 μg ml^{-1}) was continuously monitored upon the addition of 950 nM Sar1pGDP, 160 nM Sec23/24, 260 nM Sec13/31p and 100 μM GMP-PNP. Note the different order of additions between the three experiments. Mixing was performed manually for each injection, giving a dead time of ~10 s for each addition. **d**, Correlation between the binding of Sar1p to liposomes as monitored by light scattering (dotted trace) and the conformational change upon GDP/GMP-PNP exchange as monitored by tryptophan fluorescence (thick trace). **e**, Titration of the binding signals of Sec23/24p and Sec13/31p onto major–minor mix liposomes preloaded with Sar1pGMP-PNP. The same protocol as in **b** was used except that increasing amounts of Sec23/24p or Sec13/31p were added to the sample. For the titration of the Sec13/31p binding signal, the concentration of Sec23/24p was 160 nM. **f**, Effect of lipid composition. This is the same experiment as in **b** but with GTP-γS instead of GMP-PNP and with PC/PE/PI (triangles), major mix (squares) or major–minor mix (circles) liposomes. A.U., arbitrary units.

region (Trp 84 in Sar1p), which can serve as an intrinsic fluorescent sensor of the conformation, the GTP-bound state displaying a higher fluorescence than the GDP-bound state[8,9]. Fig. 1d compares the time course of Sar1p activation as monitored by tryptophan fluorescence (bold trace) with the time course of the binding of Sar1p to liposomes as monitored by light-scattering (dotted trace). We observed a correlation between the two time courses, each displaying a half-time of ~2 min. We conclude that the activation of Sar1p upon spontaneous GDP/GMP-PNP exchange, which correlates with its translocation to liposomes, is much slower than the subsequent recruitment of the Sec23/24p and Sec13/31p complexes and is therefore the rate-limiting step for the formation of the COPII coat.

Titration reactions were used to estimate the stoichiometry of the COPII subunits assembled onto liposomes. On major–minor mix liposomes incubated with 1 μM Sar1pGDP and 100 μM GMP-PNP, titration of the Sec23/24p binding signal revealed a maximum binding at ~160 nM Sec23/24p (Fig. 1e, left panel). At this concentration of Sec23/24p, titration of the Sec13/31p signal showed saturation at ~145 nM (Fig. 1e, right panel), indicating a 1:1 stoichiometry between the two complexes. To estimate the stoichiometry of the heterodimers to Sar1pGMP-PNP, we determined the fraction of Sar1p bound to GMP-PNP by comparing the amplitude of the GMP-PNP-induced fluorescence change on Sar1p at the concentration of lipids used here to its extrapolated value at a saturating concentration of lipid. This fraction was estimated to be 30% (data not shown), yielding a 2:1 stoichiometry between Sar1pGMP-PNP and the other COPII subunits. These results are in good agreement with the stoichiometry observed for isolated COPII vesicles formed in the presence of GMP-PNP[3,4].

We showed previously that Sar1pGMP-PNP binds to neutral liposomes, but that the subsequent binding of Sec23/24p and Sec13/31p requires the presence of anionic lipids[4]. The light-scattering signals associated with the binding of the three proteins conformed to these observations (Fig. 1f). Thus, the small and slow light-scattering change triggered by the addition of the nonhydrolysable GTP analogue (here GTP-γS) after the addition of Sar1pGDP did not depend on the presence of anionic lipids in our lipid mixture. In contrast, the binding signals of Sec23/24p and Sec13/31p gradually diminished as the contribution of anionic lipids to the liposomes formulation was decreased (Fig. 1f).

In conclusion, the experiments shown in Fig. 1 suggest that light scattering provides a real-time, quantitative assay to monitor the coating of liposomes by the three components of the COPII coat.

Assembly and disassembly of the COPII coat with Sar1pGTP. The time resolution of the light-scattering assay allowed us to investigate the dynamics of the COPII coat in the presence of GTP, in which coat assembly is followed by disassembly promoted by the

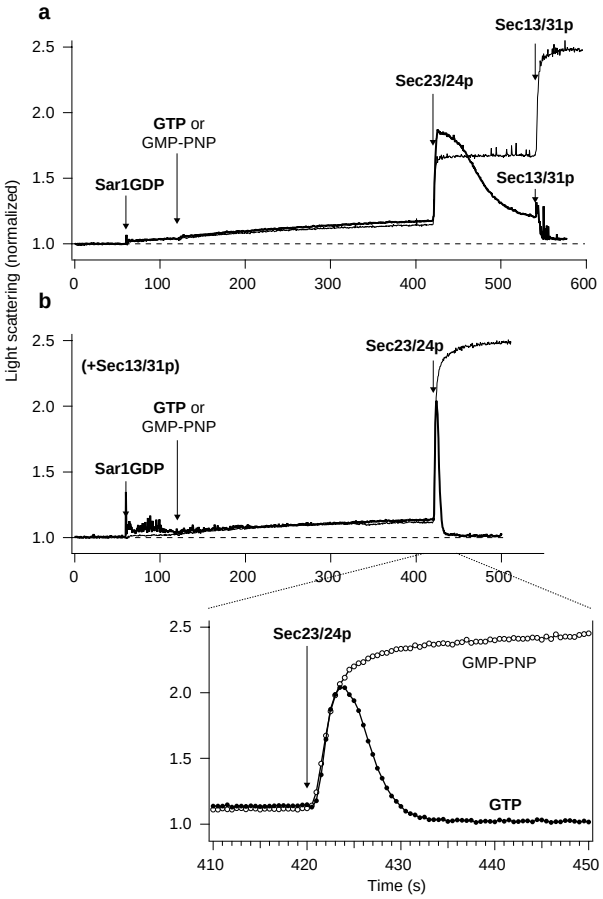

Figure 2 **Transient assembly of the COPII coat on liposomes preloaded with Sar1pGTP.** The cuvette initially contained major–minor mix liposomes (100 μg ml^{-1}) with (**b**) or without (**a**) Sec13/31p (260 nM). In a first stage, Sar1pGDP (950 nM) was activated by the addition of 100 μM GMP-PNP (thin trace) or GTP (bold trace). After 5 min, Sec23/24p (160 nM) was added. In **a**, Sec13/31p (260 nM) was added at the end of the experiment. For better temporal resolution, these experiments were conducted in a large cuvette in which the sample was stirred continuously with a magnetic bar. In **b**, the transient or permanent binding-signal of the complete COPII coat in the presence of Sar1pGTP or Sar1pGMP-PNP is shown on an expanded time scale. The full width at half-maximum amplitude of the transient GTP signal was 5.15 ± 0.60 s (mean ± s.e.m.) in three independent experiments.

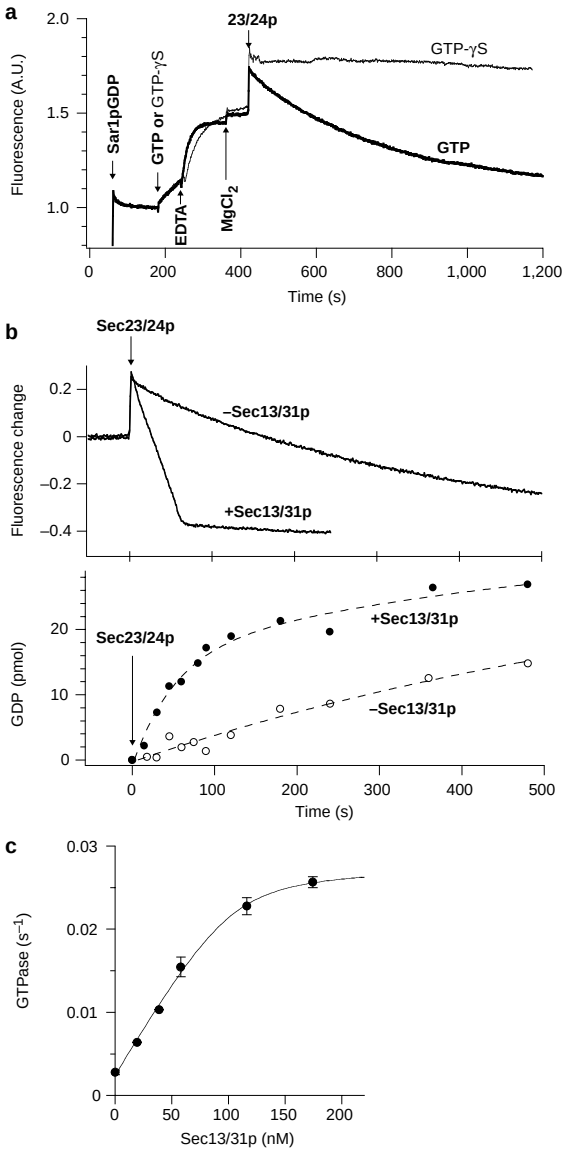

Figure 3 **Sec13/31p stimulates the GAP activity of Sec23/24p. a**, Measurements of the GAP activity of Sec23/24p by tryptophan fluorescence. In a first stage, Sar1pGDP (2 μM) was added to major–minor mix liposomes (300 μg ml^{-1}) and activated by the addition of GTP (bold trace) or GTP-γS (thin trace) (30 μM). To accelerate GDP–GTP exchange, we temporarily decreased the concentration of free Mg^{2+} from 1 mM to 1 μM by the addition of EDTA (2 mM). Sec23/24p (55 nM) was then added to promote GTP hydrolysis. A.U., arbitrary units. **b**, Effect of Sec13/31p on the GAP activity of Sec23/24p. The experiments were performed as in **a** with or without 60 nM Sec13/31p. Sec13/31p did not affect the GTP-loading step, and only the deactivation phase is shown. The upper panel shows the fluorescence recordings. The bottom panel shows the time course of GTP hydrolysis as measured by an assay with radiolabeled GTP. **c**, Dose–response curve for the effect of Sec13/31p on the GAP activity of Sec23/24p. The GAP activity was determined from the initial slope of recordings similar to that shown in **b**. Error bars indicate variations observed in two independent experiments with different preparations of Sar1p and Sec13/31p.

GAP activity of Sec23p (ref. 6). In these experiments, the order of protein addition with respect to the nucleotide was critical. When GTP was added after all COPII components, no signal could be detected (data not shown). In this case, the assembly of the COPII coat was rate-limited by the slow spontaneous nucleotide exchange on Sar1p, and the GAP activity of the coat might counteract the accumulation of Sar1pGTP. To detect transient coating in the presence of GTP, we incubated liposomes with Sar1pGDP and GTP for a few minutes before adding Sec23/24p. COPII proteins were assayed at levels close to the stoichiometry suggested by previous experiments with GMP-PNP to ensure that the kinetics reflected as closely as possible a single round of coating and uncoating. Figure 2a shows that the addition of Sec23/24p onto major–minor mix liposomes preloaded with Sar1pGTP induced an instant increase in light scattering (~2 s) followed by a decay, which terminated in ~2 min and contrasted with the stable signal observed with Sar1pGMP-PNP. We then repeated these experiments in the presence of Sec13/31p to follow the kinetics of assembly and disassembly of the complete COPII coat (Fig. 2b). Sec13/31p was included at the beginning of the experiment so that its engagement in the coat was initiated by the later addition of Sec23/24p. Strikingly, in that case, a much more transient binding signal was observed, lasting only 10 s. During the first 2–3 s the signal coincided with the

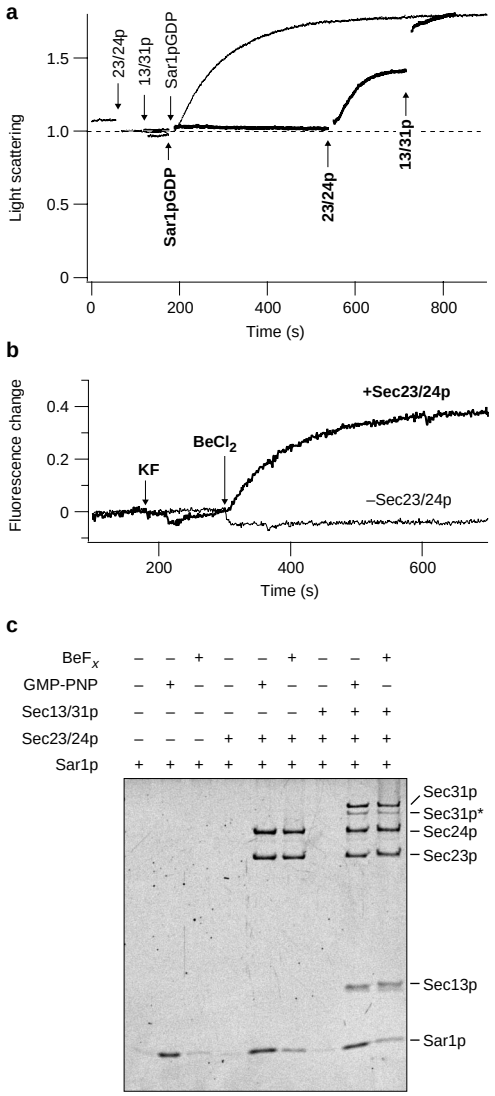

Figure 4 **Translocation of the COPII components in the presence of the phosphate analogue BeF$_x$. a**, Light scattering assay. The experimental conditions were as in Fig. 1a–c except that the buffer contained 250 μM $BeCl_2$ and 10 mM KF. Note the different order of protein additions between the thin trace and the bold trace. **b**, Tryptophan fluorescence change induced by the addition of KF (10 mM) and $BeCl_2$ (250 μM) on mixtures of Sar1pGDP (500 nM) with major–minor mix liposomes (100 μg ml^{-1}) and with or without Sec23/24p (250 nM). **c**, Flotation assay. Various combinations of COPII proteins were incubated with major–minor mix liposomes in the presence or absence of GMP-PNP (100 μM) or BeF$_x$ (250 μM $BeCl_2$, 10 mM KF) as indicated. Proteins bound to liposomes were recovered by flotation, resolved by SDS–PAGE and stained with Sypro red.

large binding signal observed with Sar1pGMP-PNP, which monitors the concomitant binding of Sec23/24p and Sec13/31p. However, the GTP signal then declined in ~5 s and reached the light-scattering level of protein-free liposomes. We conclude that with Sar1pGTP the COPII coat decomposes nearly immediately after assembly. Further, we suggest that Sec13/31p might accelerate this process by stimulating the GAP activity of Sec23/24p.

Sec13/31p stimulates the GAP activity of Sec23/24p tenfold. We assessed directly the effect of Sec13/31p on the GAP activity of Sec23/24p. Tryptophan fluorescence was used to follow the transition of Sar1p from the GTP-bound to the GDP-bound state. In a first stage, Sar1pGDP was added to liposomes, and fast nucleotide exchange was achieved by the addition of an excess of GTP in the presence of EDTA to lower free Mg^{2+} to the micromolar range (Fig. 3a). After completion of nucleotide exchange, 1 mM free Mg^{2+} was restored and Sec23/24p was added. This induced a fluorescence decay, which was not observed with Sar1p-GTP-γS, and was correlated with the hydrolysis of GTP as shown by a classical assay with radioactive GTP (Fig. 3a, b). In these experiments, the ratio between Sec23/24p and Sar1p was one-sixth of that used in the light-scattering assay, and the concentration of liposomes was higher to allow more complete GTP loading of Sar1p. The overall time course of the reaction should reflect several rounds of Sec23/24p-mediated GTP hydrolysis on Sar1p. Strikingly, in the presence of Sec13/31p, the GTPase reaction was strongly accelerated, with a tenfold increase in the rate at saturation (Fig. 3b, c). Sec13/31p had no effect on the GTPase reaction in the absence of Sec23/24p, in agreement with the sequence of binding events between the three COPII components.

Assembly of the COPII coat in the presence of beryllium fluoride (BeF$_x$). Ionic complexes between fluoride and aluminium or beryllium act as phosphate analogues[10]. On G proteins, fluoride complexes bind next to the bound GDP and mimic the γ-phosphate of GTP, thereby promoting the switch to a GTP-like conformation[10,11]. In all cases reported so far, the fluoride complex is coordinated to an Arg residue, which participates directly in the hydrolysis of the γ-phosphate and is provided either by the G protein itself (for heterotrimeric G proteins) or by a GAP (for Ras-like G proteins)[11]. Therefore, whereas heterotrimeric G proteins are direct targets of fluoride complexes, small G proteins such as Ras-GDP bind fluoride complexes only in the presence of their corresponding GAPs[10,11]. Thus fluoride complexes can serve as a tool with which to explore the mechanism of GTP hydrolysis on G proteins.

Next, we added Sec23/24p, Sec13/31p and Sar1pGDP sequentially to major–minor mix liposomes in a buffer supplemented with BeF$_x$ (Fig. 4a). With Sec23/24p and Sec13/31p present in the sample, an increase in light scattering was observed on the addition of Sar1pGDP (thin trace). The amplitude of the signal was less than the COPII binding signal observed with GMP-PNP (compare Fig. 4a with Fig. 1a). Changing the order of protein additions indicated that the mechanism by which BeF$_x$ promotes the binding of the COPII components differed from that observed with GTP analogues. First, with only Sar1pGDP present, BeF$_x$ promoted no binding signal (Fig. 4a, bold trace). This contrasted with the slow Sar1p-binding signal observed with GTP or nonhydrolysable analogues (see Figs 1d, 2). Second, a slow increase in signal was observed when Sec23/24p was added to incubations containing Sar1pGDP and BeF$_x$, which contrasted with the instantaneous binding signal observed on Sar1p preloaded with GTP analogues. BeF$_x$ might act only when both Sar1pGDP and Sec23/24p are present. This possibility was addressed more directly by tryptophan fluorescence measurements. Whereas BeF$_x$ induced no fluorescence change on Sar1pGDP alone, a increase in fluorescence was observed in the presence of Sec23/24p (Fig. 4b). Aluminium did not substitute for beryllium in promoting Sar1p activation, in contrast to other G proteins (data not shown).

The binding of Sar1pGDP and Sec23/24p promoted by BeF$_x$ was functional because the light-scattering signal increased rapidly on addition of Sec13/31p (Fig. 4a, bold trace). This was confirmed by sucrose-gradient flotation experiments in which proteins bound to liposomes were recovered at the top of a sucrose cushion (Fig. 4c).

We compared the morphology of liposomes incubated with all COPII proteins and with BeF$_x$, GMP-PNP or no activator by thin-section electron microscopy (Fig. 5). As we observed previously with GMP-PNP[4], BeF$_x$ promoted the formation of a dense coat at the surface of liposomes and the appearance of COPII-like vesicles. We observed coated buds of variable length on some liposomes. The shape of some of these long coated profiles indicates that fragmentation into small vesicles can occur not only by single constric-

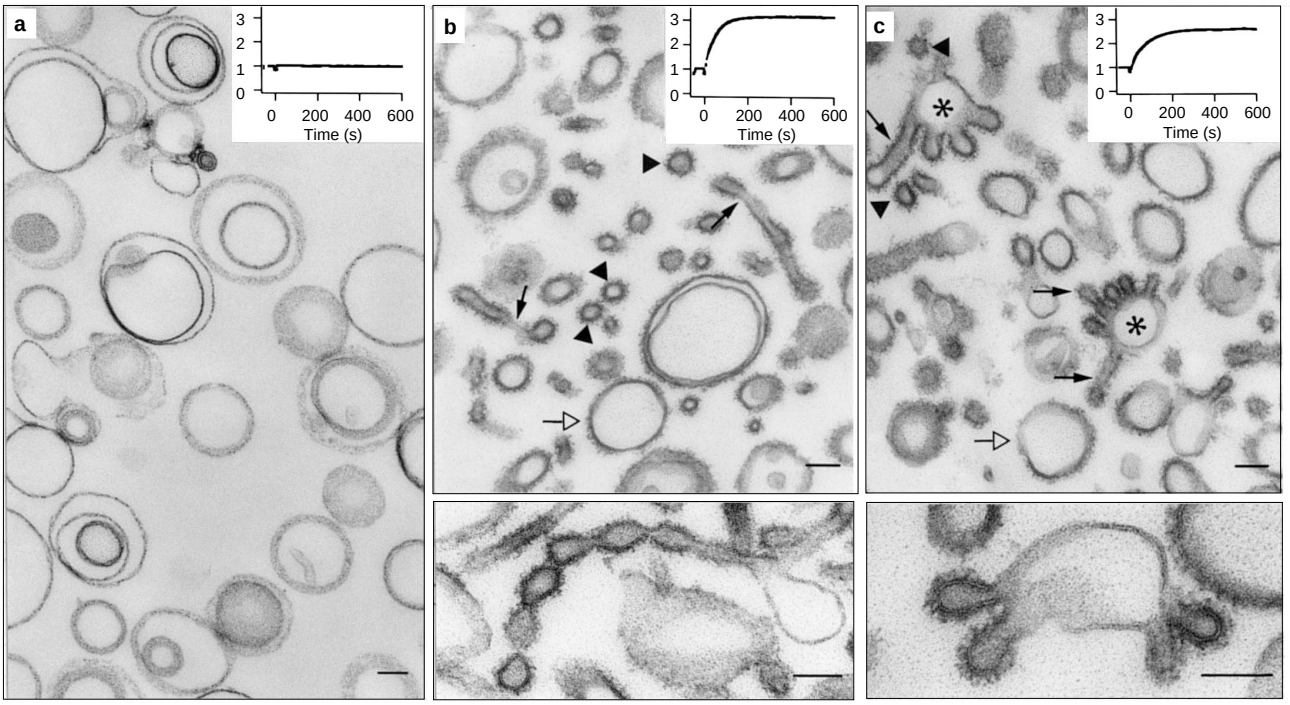

Figure 5 **Morphology of major–minor mix liposomes incubated with COPII proteins and with no activator (a), GMP-PNP (b) or BeF_x (c).** The reaction was initiated at zero time by the addition of Sar1pGDP. The insets show the changes in light scattering observed. After 20 min the samples were processed for electron microscopy. Characteristic profiles observed with GMP-PNP (**b**) or BeF_x (**c**) are indicated: partly coated liposomes (white arrows), liposomes with coated buds (asterisks), elongated coated structures (black arrows) and small COPII-like vesicles (arrowheads). The multiply-budded liposome profiles were more abundant in the BeF_x sample, whereas elongated profiles with multiple constrictions were observed more frequently in the GMP-PNP sample. However, both kinds of profile were observed in the two samples. Examples are shown at higher magnification at the bottom of panels **b** and **c**. Scale bars, 100 nm.

tion of small coated buds but also by multiple constrictions of more elongated coated membrane structures. In mammalian cells, a balance between stretching and constriction of ER membrane has been observed[12].

Analysis of the COPII coat with Sar1pGTP by electron microscopy. We wished to analyse by electron microscopy the transient coating of liposomes when GTP was used in place of nonhydrolysable analogues. At 27 °C, after a single round of COPII assembly and disassembly (see Fig. 2b), few coat remnants were observed (data not shown). Light scattering experiments revealed a marked decrease in the rate of COPII coat disassembly with decreasing temperature, indicating that the coat reached a metastable state (Fig. 6a). Under these conditions most liposomes were covered by a coat exhibiting two distinctive features compared with the coat observed with GMP-PNP (Fig. 6b, c): first, the 'GTP' coat was ~60% thicker (32 ± 0.5 nm instead of 20 ± 0.4 nm; mean ± s.e.m.); second, most liposomes displayed a single coated area that covered a limited area of the liposome surface.

Discussion

We have shown previously that the COPII subunits form a stable coat and bud coated vesicles in a synthetic reaction containing liposomes and GMP-PNP[4]. GTP seemed not to sustain stable coat assembly because one of the coat subunits, Sec23p, is a Sar1p GAP[6]. Thus, in the absence of a membrane protein that could retard GTP hydrolysis, the coat would disassemble too quickly to be detected in membrane fractionation experiments. The real-time assay presented here allows the analysis of transient intermediates in coat formation.

Light scattering provides excellent temporal resolution and a level of sensitivity that ensures simple quantitative measurements. Because the sequence of events that lead to the formation of the COPII coat has been well defined[3,4], it was possible, by changing the order of protein and nucleotide additions, to assign each light scattering signal unambiguously to a binding event (Fig. 1a–c). It is less clear whether the changes in light scattering include a component that corresponds to the fragmentation of coated liposomes into small COPII-like vesicles. Neither the amplitude nor the sign of such a 'fragmentation' signal can be predicted. The signal that we observed in a suspension of liposomes obtained by extrusion through a polycarbonate filter with a pore size of 0.4 μm increased by 20% after a subsequent extrusion through a 0.05-μm filter (data not shown). Thus, even if all liposomes were to fragment into small COPII-like vesicles, the contribution of this reaction to the overall increase in light scattering (~150%; Fig. 1) would be small. It seems that the changes in light scattering that we observe are dominated by 'mass' effects, namely liposome coating, rather than by 'shape' effects, namely liposome fragmentation.

On pure liposomes, the rate of assembly of the COPII coat is limited by the spontaneous exchange of GDP for GTP on Sar1p (Fig. 1a–d). However, *in vivo* it is likely that Sec12p achieves rapid (subsecond) catalysis of nucleotide exchange to position Sar1p on the ER membrane once Sar1p has acquired GTP. The recruitment of Sec23/24p and Sec13/31p occurs within the time limit of our assay (~2 s; Fig. 2). This fast assembly is driven by protein–protein interactions and protein–anionic-lipid contact (Fig. 1e). *In vivo*, interactions with the cytosolic domains of proteins might also contribute to coat recruitment[13–15].

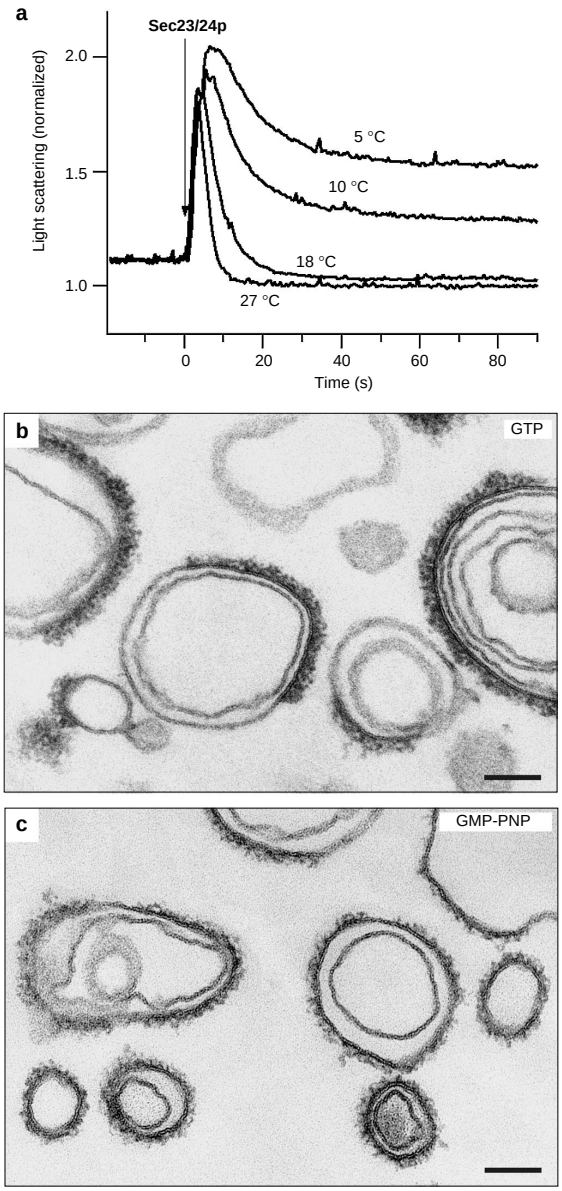

Figure 6 **Assembly and disassembly of the COPII coat with Sar1pGTP at low temperature. a**, Effect of temperature on the transient assembly of the COPII coat in the presence of Sar1pGTP. In a preliminary stage, major–minor mix liposomes were incubated with Sar1pGDP and GTP at room temperature. After 5 min, Sec13/31p was added and the sample was maintained at the indicated temperature. At zero time, Sec23/24p was added and light scattering was continously monitored. The other experimental conditions were as in Fig. 2b. **b**, An experiment similar to that at 5 °C in **a** was processed for EM 5 min after the addition of Sec23/24p. **c**, As in **b**, with GMP-PNP instead of GTP. Note the segmental distribution of the coat in **b** and its thicker appearance than the coat in **c**, which covers most of the liposome surface. Scale bars, 100 nm.

The fast assembly of the COPII coat is challenged by its very efficient intrinsic GAP activity. We show here that Sec13/31p accelerates the GAP activity of Sec23/24p on Sar1pGTP by one order of magnitude (Fig. 3). The phosphate analogue BeF_x allowed us to dissect the roles of Sec23/24p and Sec13/31p in Sar1p GTP hydrolysis. BeF_x promoted the formation of a complex between Sar1pGDP and Sec23/24p, which in turn recruited Sec13/31p (Fig. 4). Therefore, by analogy with Ras-GTPase-activating protein (GAP) and Rho-GAP[11], Sec23/24p might supply residues directly involved in GTP hydrolysis. In contrast, Sec13/31p did not interact directly with Sar1pGTP or with Sar1pGDP and BeF_x, and its effect on Sar1p GTP hydrolysis was mediated by Sec23/24p. We suggest that Sec13/31p might orientate the catalytic residues of Sec23/24p better within the GTP-binding site of Sar1p. This effect might result either from a conformational change in Sec23/24p induced by Sec13/31p or from the optimization of all protein–protein contacts when the coat has achieved a polymeric state. Although Sar1p belongs to the family of Ras-like proteins, it possesses distinctive attributes such as a histidine residue (His 77) instead of the canonical glutamine involved in γ-phosphate hydrolysis (Gln 61 in Ras) and an absolute preference for BeF_x over AlF_x in combination with its GAP, Sec23p.

The effect of BeF_x on the COPII coat (Fig. 5) is reminiscent of the effect of AlF_x on the COPI coat, whose assembly is controlled by the small G protein ARF1 (refs 16–18). Because Sar1p and ARF1 are close homologues, it is reasonable to assume that ARF1GDP, like Sar1pGDP, might be the target of a fluoride complex, provided that its corresponding GAP is present. Unlike the COPII coat, the COPI coat has no intrinsic GAP activity, and a distinct ARF-GAP has been identified[19]. Coatomer, the large preformed protomer of the COPI coat, has been shown to increase the activity of an ARF-GAP domain markedly in solution[20], although the relevance of this interaction to the normal activity of ARF-GAP has been challenged[21]. Nevertheless, the coatomer–ARF-GAP synergy and the enhancement of the GAP activity of Sec23p by polymerization of the COPII coat seem to be formally analogous. A dissection of the AlF_x effect on a reconstituted system should reveal whether ARF1-GAP or coatomer supplies catalytic residues for the GTPase reaction.

The very efficient intrinsic GAP activity of the complete COPII coat triggers disassembly in a few seconds (Fig. 2b). This raises a central question: does this fast disassembly compromise the capture of cargo molecules and the shaping of lipid membranes? Previous studies have suggested that cargo capture occurs during the formation of prebudding complexes between Sar1pGTP and Sec23/24p (refs 13, 14, 22, 23). These complexes then diffuse laterally to form a complete coat through the bridging action of Sec13/31p. Our experiments suggest that the lifetime of the Sar1pGTP–Sec23/24p complex (~30 seconds; Fig. 2a) might be long enough to allow the formation and diffusion of prebudding complexes. Rapid GTP hydrolysis on Sar1p would occur once a prebudding complex had been included with Sec13/31p into the complete coat. In such a two-gear mechanism, GTP hydrolysis would be tuned so as to be simultaneous with or subsequent to the polymerization of the coat. As a consequence, the centre of the coat, formed by 'old' coat units, should contain less Sar1pGTP molecules than the periphery, where new coat units assemble. Interestingly, the COPII coat observed at low temperature on liposomes with Sar1pGTP was thicker than that formed with Sar1pGMP-PNP (Fig. 6). We suggest that this difference arises from the non-uniform distribution of Sar1p in the GTP coat, which might favour structural rearrangements of the coat lattice.

It is likely that COP coat dynamics display additional levels of complexity. In some circumstances, cargo might dampen the GTPase reaction to prevent the premature disassembly of the coat and to enhance the probability of selective membrane protein capture[24]. Alternatively, GTP hydrolysis might have an active role, allowing a relay between the small G protein and cargo for their interaction with the coat[25–27]. The combination of kinetics and morphological studies of the COPII coat on model membranes should allow us to investigate these issues. □

Methods

Proteins.

The expression and purification of Sar1p, Sec23/24(His_6)p and Sec13/31(His_6)p, have been described elsewhere[3,28].The Sec23/24p and Sec13/31p complexes contain equivalent numbers of subunits. Thus, for simplicity and although multimeric forms are likely to form in solution, the concentrations of complexes are given in molar units of heterodimer (relative molecular masses (M_r) 85 and 104 kDa (85K and 104K) for Sec23/24p, and 33K and 136K for Sec13/31p).

Liposomes.

Liposomes were prepared essentially as described[4]. A lipid film of the desired composition was formed in a glass tube or a pear-shaped flask in a rotatory evaporator, hydrated in 20 mM HEPES–KOH, pH 7.0, 160 mM potassium acetate (Buffer A) and the lipid suspension was then extruded 19 times through a polycarbonate filter with a 0.4 μm pore size. All liposomes contained 20% (w/w) ergosterol and 2% (mol/mol) fluorescent lipid 7-nitrobenz-2-oxa-1,3-diazole-phosphatidylethanolamine (NBD-PE) for the determination of lipid recovery in flotation experiments. Major–minor mix liposomes contained (in mol%): phosphatidylcholine, 50; phosphatidylethanolamine, 21; phosphatidylserine, 8; phosphatidic acid, 5; phosphatidylinositol, 9; phosphatidylinositol-4-phosphate, 2.2; phosphatidylinositol-4,5-bisphosphate, 0.8; cytidine-diphosphate-diacylglycerol, 2. Major mix liposomes contained phosphatidylcholine, 55; phosphatidylethanolamine, 21; phosphatidylserine, 8; phosphatidic acid, 5; phosphatidylinositol, 9. 'PC/PE/PI' liposomes contained phosphatidylcholine, 68; phosphatidylethanolamine, 21; phosphatidylinositol, 9. We used equimolar mixtures of 1,2-dioleoyl and 1-palmitoyl-2-oleoyl derivatives of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidic acid except in Figs 4 and 5, where only 1,2-dioleoyl-derivatives were used. Other lipids were of biological origin.

Light scattering measurements.

The scattering of light (λ = 350 nm) was measured at 90° in a fluorimeter (Fluorolog 3; Jobin-Yvon) equipped with a thermostatically controlled cell holder. Unless stated otherwise, the temperature was 27 °C. Most experiments were performed in a 100-μl quartz cuvette in which the reactants were added and mixed manually at the indicated times. Typically, the duration required for injection and mixing was ~10 s. For better temporal resolution, some experiments (Figs 1d, 2 and 6) were scaled up and performed in a cylindrical cuvette (sample volume 550 μl) in which mixing was ensured by a magnetic stir bar, and injections were done with Hamilton syringes through a guide in the cover of the fluorimeter. With this device, the recording was not interrupted by the injections and the mixing time was <2 s, allowing kinetic measurements in the range of a few seconds. The sampling rate was two measurements per second with a time constant of 0.3 s. For experiments at low temperature (Fig. 6), air streams were directed towards the faces of the cuvette to prevent condensation.

All experiments were performed in the following buffer (buffer B): 20 mM HEPES–KOH, pH 7.0, 160 mM potassium acetate, 1 mM $MgCl_2$ supplemented with 1 mM dithiothreitol. To minimize light-scattering artefacts due to air bubbles and dust particles, we filtered the buffer through a 0.22-μm filter and then degassed it before use. In a typical experiment, the cuvette initially contained a suspension of liposomes (90 μl, lipid concentration 100 μg ml^{-1}) in buffer B. Sar1pGDP, Sec23/24p, Sec13/31p and GTP or a nonhydrolysable analogue were added at the indicated times from concentrated stock solutions (the total volume after all injections was ~100 μl).

Special care was taken to keep the ionic strength of the sample within the range 180–240 mM throughout the experiment. Sec23/24p aggregates in solutions below 160 mM potassium acetate, resulting in an increase in light scattering that does not depend on the presence of liposomes and Sar1pGTP. At concentrations of salt of more than 240 mM, coated liposomes tended to cluster as revealed by electron microscopy. In this situation the increase in light scattering, indicating COPII binding to liposomes, was followed by a large descending phase, possibly reflecting coated liposome aggregation. Because high salt concentrations (~600 mM potassium acetate) were inherent in the purification of some COPII components (especially the Sec23/24p complex), we used highly concentrated (20–50-fold) stock solutions of proteins to minimize both sample dilution and changes in ionic strength on protein addition. In typical experiments we checked the sample conductivity before and after all additions.

Tryptophan fluorescence.

An assay similar to that previously described for ARF1 (ref. 29) was used to follow the transition of Sar1p between GDP-bound and GTP-bound forms. Tryptophan fluorescence was recorded at 340 nm (bandwidth 20 nm) upon excitation at 297.5 nm (bandwidth 1.5 nm). All experiments were performed at 27 °C in buffer B in a cylindrical 550-μl cuvette with the same injection and mixing facilities as used for the light scattering experiments at high temporal resolution.

GTPase assay.

GTPase assays were performed as described, with modifications[30]. Sar1pGDP (2 μM) was incubated in 45 μl reactions at 27 °C for 10 min with or without 60 nM purified Sec13/31p and 30 μM GTP, 100 nCi [α-^{32}P]GTP, 100 μM ATP, 300 μg ml^{-1} major–minor mix liposomes in buffer B supplemented with 2 mM EDTA to promote fast nucleotide exchange. The free concentration of $MgCl_2$ was then adjusted to 1 mM, and 2 μl of the reaction was withdrawn and mixed with an equal volume of ice-cold 100 mM EDTA to serve as the zero time point. Hydrolysis was initiated by the addition of purified Sec23/24p to 55 nM, and samples were withdrawn and quenched as before at the times indicated. One-μl aliquots were spotted onto polyethyleneimine cellulose plates (Selecto Scientific) and developed in 1 M LiCl, 1 M formic acid. The plates were dried and exposed overnight to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). Conversion to picomoles of GDP was determined by the fraction of GDP divided by the total GTP and GDP in each lane, multiplied by the number of picomoles of GTP included in each reaction. The zero time point of each reaction was subtracted as background.

Liposome binding assay.

Liposome binding assays were preformed as described, with modifications[4]. Reactions were incubated at 27 °C for 25 min with 700 nM Sar1pGDP, 105 nM Sec23/24p, 175 nM Sec13/31p, 100 μM GMP-PNP and 100 μg ml^{-1} major–minor mix liposomes in buffer B (total volume 150 μl). Where indicated, BeF_x complexes were formed by the inclusion of 10 mM KF and 250 μM $BeCl_2$. Reactions were placed on ice, and the concentration of sucrose in each reaction was adjusted to 1 M by the addition of 100 μl buffer B containing 2.5 M sucrose. Aliquots (220 μl) of sucrose-adjusted reactions were transferred to 11 mm × 34 mm polycarbonate centrifuge tubes and overlaid with 200 μl of buffer B containing 0.75 M sucrose and 25 μl of buffer B (where BeF_x complexes were used, sucrose solutions also contained 10 mM KF and 250 μM $BeCl_2$). The resulting step gradient was centrifuged at 55,000 rev. min^{-1} in a Beckman TLS-55 rotor for 2 h at 2 °C. Samples (50 μl) were collected from the top of each tube and transferred to a microtitre plate. The recovery of NBD-PE was determined by recording the fluorescence with a STORM 860 image analyser. After normalization for lipid recovery, proteins were separated by SDS–PAGE, stained by SYPRO Red, and detected with a STORM 860 image analyser.

Electron microscopy.

The experimental conditions were the same as that used for the light scattering experiments. After incubation, samples were processed for thin-section EM as described[4].

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