Self-assembly of minimal COPII cages

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The small G-protein Sar1 and the cytosolic complexes Sec23/24 and Sec13/31 associate sequentially on endoplasmic reticulum membranes to form a protein coat named COPII, which drives the formation of transport vesicles. Using dynamic light scattering, we show that Sec23/24 and Sec13/31 can self-assemble in a stoichiometric manner in solution to form particles with hydrodynamic radii in the range of 40–60 nm. Self-assembly is favoured by lowering the pH, the ionic strength and/or the temperature. Electron microscopy reveals the formation of spherical particles 60–120 nm in diameter with a tight, rough mesh on their surfaces. We suggest that these structures, which represent a minimal COPII cage, mimic the molecular organization of the membrane-associated COPII coat.

EMBO reports 4, 419–424 (2003)

doi:10.1038/sj.embor.embor812

INTRODUCTION

Protein coats transiently cover the cytosolic faces of cellular membranes (Kirchhausen, 2000). They impose a strong curvature on the lipid bilayer, giving rise to small buds, which are precursors of transport vesicles. Through specific interactions with short cytosolic motifs, protein coats capture transmembrane proteins and associated extracellular or luminal proteins, thus ensuring efficient transport between cellular compartments.

Three protein coats have been described so far: COPI, COPII and clathrin/adaptor coats. COPI and COPII coats are involved in various transport steps between the endoplasmic reticulum (ER) and the Golgi apparatus. Clathrin/adaptor coats are involved in export from the trans-Golgi and in endocytosis. The architecture and principles of assembly of the clathrin coat were described 25 years ago from the comparison of electron microscopy images of the clathrin triskelion (the minimal unit of the clathrin

coat) with images of auto-assembled clathrin cages (Crowther & Pearse, 1981; Harrison & Kirchhausen, 1983). At low pH, clathrin triskelions self-assemble into spherical cages with diameters of 60–100 nm that show the characteristic lattice structure, consisting of hexagons and pentagons, that is found on biological membranes. Through a combination of cryo-electron microscopy (cryo-EM) studies on clathrin cages and X-ray diffraction studies on individual protein domains, a high resolution structure of the clathrin coat is emerging (Smith *et al.*, 1998; Musacchio *et al.*, 1999).

Our knowledge of the architecture of the COPII coat is less advanced. This coat forms by the sequential recruitment of the small G-protein Sar1 and two cytosolic complexes, Sec23/24 and Sec13/31, to the ER membrane (Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998, 2001). Sar1–GTP interacts directly with the bilayer through its amino terminus. Sec23/24 interacts with Sar1–GTP, with negatively charged lipids and with export motifs of transmembrane proteins. Sec13/31 binds to Sec23/24 and forms what is probably the most external layer of the coat. Isolated Sec23/24 and Sec13/31 complexes have been visualized by EM, and the atomic structure of Sec23/24 in a complex with Sar1–GTP has been determined (Lederkremer *et al.*, 2001; Matsuoka *et al.*, 2001; Bi *et al.*, 2002). Sec23/24 contains one Sec23 subunit (85 kDa) and one Sec24 subunit (104 kDa), which each form half of a bow-tie shape (dimensions 11 nm × 17 nm). Sec13/31 probably contains two Sec13 subunits (33 kDa) and two Sec31 subunits (139 kDa) and looks like a flexible string, 28–30 nm in length, made up of five globular domains. COPII complexes are therefore different from the clathrin triskelion. However, these proteins are able to form a coat, with an apparent lattice structure, on membranes, although a much less obvious one than that of the clathrin coat. This suggests a regular mode of assembly (Matsuoka *et al.*, 2001). Here, we show that under defined conditions of pH, temperature and ionic strength, Sec23/24 and Sec13/31 self-assemble in solution to form spherical particles that may mimic the polymerization state of COPII proteins on membranes.

RESULTS

Sec23/24 and Sec13/31 can self-assemble in solution

By dynamic light scattering, it is possible to determine the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination. An 'intensity' histogram gives the distribution in hydrodynamic radius (R_h) that best describes the contribu-

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Received 24 January 2003; revised 26 February 2003; accepted 27 February 2003

tion of the diffusing particles to the scattering of light. Importantly, this distribution emphasizes the presence of large objects, as they diffuse more light than small objects (see Methods).

We first conducted dynamic light-scattering experiments on separate concentrated solutions of Sec23/24 and Sec13/31, which were purified in high-salt buffers. At 27 °C, the hydrodynamic radii of Sec23/24 and Sec13/31 were 7.4 nm (± 1 nm) and 11 nm (± 1.5 nm), respectively (Fig. 1A,B, left panels). These values are far higher than the calculated radii of hypothetical spherical proteins of the same molecular weight ($r = 3.9$ nm for Sec23/24 and 4.8 nm for Sec13/31, where $r = ((3M)/(4\pi v))^{1/3}$ and $v = 750$ Da nm³), but are in good agreement with the actual dimensions of these elongated complexes as observed by EM (Lederkremer *et al.*, 2001).

We then mixed the two stock solutions to obtain a 1:1 molar ratio of Sec23/24 to Sec13/31 (Fig. 1C). Because Sec13/31 is probably a tetramer, the actual ratio between the four Sec subunits was Sec23:Sec24:Sec13:Sec31 = 1:1:2:2. For simplification, the molar concentration of Sec subunits, rather than the molar concentration of the complex, will be referred to hereafter. At 27 °C, the distribution of R_h in the mixture was close to that observed for the two individual complexes, suggesting that no significant assembly had occurred (Fig. 1C, left panel). By contrast, when the temperature was lowered to 4 °C, the mean R_h increased up to fivefold within a few minutes (Fig. 1C, middle and right panels). This effect was reversible and reproducible; rounds of aggregation/disaggregation were observed by submitting the COPII mixture to consecutive tempera-

ture ramps. After equilibration at 4 °C, the intensity histogram of R_h in the Sec23/24 plus Sec13/31 mixture showed a monomodal distribution centred at 50.5 nm, with a low dipersity (± 8.5 nm), indicating the formation of homogeneous aggregates (Fig. 1C, right panel). Almost no effect of temperature on the separate COPII components was observed (Fig. 1A,B).

On lipid membranes, the COPII coat contains approximately equimolar amounts of Sec23, Sec24, Sec13 and Sec31 subunits (Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998; Antonny *et al.*, 2001). To determine the optimal stoichiometry for the interaction of Sec23/24 with Sec13/31 at low temperature in solution, we changed the molar ratio of the two complexes. As judged by the distribution of R_h and the intensity of the scattered light, the optimal stoichiometry was ~1 mole of each Sec subunit, although we could not distinguish between similar proportions (Sec23:Sec24:Sec13:Sec31 = 1:1:2:2, 1:1:1:1, or 2:2:1:1; data not shown).

Sec23/24 and Sec13/31 self-assemble at low pH

The experiments described above were carried out using stock solutions of Sec23/24 and Sec13/31. These solutions contained high levels of potassium acetate (790 mM and 700 mM, respectively), which was used as a counter-ion for purification by ion-exchange chromatography. We found that as the COPII mixtures were diluted further in similar high-salt buffers, the temperature-induced aggregation disappeared abruptly (data not shown). This suggested that aggregation occurred only above a protein concentration threshold. However, we

Fig. 1 | Effect of temperature on the assembly of COPII aggregates. Stock solutions of (A) Sec23/24 (1.35 μ M), (B) Sec13/31 (4.15 μ M) or (C) a mixture of the two solutions (Sec23 and Sec24 at 0.82 μ M; Sec13 and Sec31 at 1.64 μ M) were analysed by dynamic light scattering. The panels on the left show the initial intensity histograms of hydrodynamic radius (R_h) at 27 °C. The middle panels show the evolution of the change in the mean R_h (bold trace) when the temperature of the sample was decreased from 27 °C to 4 °C. The temperature ramps are shown as thin traces. The panels on the right show the final intensity histograms of R_h at 4 °C.

found that COPII aggregates of $R_h = 40\text{--}80\text{ nm}$ formed when the proteins were diluted or dialysed in low-salt and low-pH buffers. This effect was also observed at 25 °C.

Figure 2A,B shows the intensity distribution of R_h at 27 °C in Sec23/24 plus Sec13/31 mixtures that were prepared by dilution in buffers of varying pH and ionic strength. Fig. 2A shows the results obtained when we fixed the final potassium-acetate concentration at 225 mM and varied the pH from 6.45 to 7.45 using a MES (2-(*N*-mor-

pholino) ethanesulphonic acid)/HEPES buffer system. Fig. 2B shows the results when we fixed the pH at 6.45 and varied the final potassium-acetate concentration from 225 to 455 mM. In both cases, a change in the balance between two peaks, one centred at 10 nm and one centred at 40–80 nm, was observed. Self-assembly, as detected by the second peak, was favoured by lowering the concentration of potassium acetate from 455 mM to 225 mM, and by lowering the pH from 7.45 to 6.45. Note that the relative amplitude of the two peaks

Fig. 2 | Effect of pH and ionic strength on the assembly of COPII aggregates at 27 °C. Mixtures of Sec23/24 and Sec13/31 were made by dilution of stock solutions in buffers of various pHs and potassium-acetate concentrations. After 40 min at 27 °C, the distribution of hydrodynamic radius (R_h) was determined by dynamic light scattering. In all experiments, the Sec subunit concentration was 0.30 μM . (A) Stoichiometric mixtures of Sec23/24 plus Sec13/31 in MES (2-(*N*-morpholino) ethanesulphonic acid)/HEPES buffers. The final pH is indicated. The final concentration of potassium acetate was 225 mM. (B) Stoichiometric mixtures of Sec23/24 plus Sec13/31 in Mes buffer containing increasing amounts of potassium acetate. The final concentration of potassium acetate is indicated. The final pH was 6.45. (C) Sedimentation analysis of Sec23/24 plus Sec13/31 mixtures prepared as in (A) in a final volume of 250 μl and centrifuged at 37,000 r.p.m. (50,000g) for 30 min at 25 °C. The percentage of Sec31, Sec23 and Sec24 in the pellet was determined by densitometry after SDS–polyacrylamide gel electrophoresis (10% acrylamide) and Coomassie-blue staining. (D) Dynamic light-scattering analysis of isolated Sec23/24, isolated Sec13/31 or a mixture of the two complexes after dilution in Mes buffer. When only one COPII complex was present, the missing complex was replaced by the same volume of the corresponding purification buffer. The final pH was 6.45 and the final potassium-acetate concentration was 225 mM.

Fig. 3 | Visualization of COPII aggregates by electron microscopy. (A) Negative staining of a Sec23/24 plus Sec13/31 mixture that was prepared by dilution in MES (2-(*N*-morpholino) ethanesulphonic acid) buffer and incubated at 25 °C. The sample contained each Sec subunit at a concentration of 0.3 μ M. The final pH was 6.45 and the final potassium-acetate concentration was 230 mM. (B) An isolated Sec23/24 complex under the same conditions. (C) The same mixture as in (A) after concentration by centrifugation and fixation with glutaraldehyde. The black arrow indicates an elongated particle. (D) Thin-section image of a similar preparation embedded in Epon resin. (E) Close-up view from part of panel (C), as indicated by the dashed box and arrow. (F) Distribution of the diameters of 151 aggregates prepared as in (C). Scale bars, 100 nm.

does not reflect directly the relative amounts of assembled and unassembled material. The contribution of the COPII aggregates is overestimated by approximately two orders of magnitude (see Methods), making this analysis more qualitative than quantitative. To quantify the proportions of COPII subunits that were assembled into aggregates, the COPII mixtures were centrifuged at 50,000g for 30 min (Fig. 2C). At pH 7.45, ~5% of Sec23/24 and Sec13/31 was found in the pellet. By contrast, at pH 6.45, 70–90% of Sec23/24 and Sec13/31 was pelleted. Therefore, the transition between assembly and disassembly was almost complete within one pH unit.

Control experiments were performed on isolated Sec23/24 or Sec13/31 complexes (Fig. 2D). Sec13/31 did not aggregate within the ranges of temperature, pH and ionic strength tested. By contrast, Sec23/24 underwent some aggregation in low pH and low ionic-strength buffers. However, the aggregates formed by isolated Sec23/24 were large and highly polydisperse ($R_h = 100$ –1,000 nm), and thus contrasted with the small aggregates formed by Sec23/24 plus Sec13/31.

Electron microscopy

Guided by the results of the dynamic light-scattering experiments, we prepared various solutions of Sec23/24 and Sec13/31 and visualized them by EM. We used salt and pH conditions that induced the most striking distributions of R_h (see Fig. 2). In preliminary experiments, the particles were directly adsorbed onto EM grids and

stained with uranyl acetate. In samples containing stoichiometric amounts of Sec23/24 and Sec13/31, at pH 6.45 and with 230 mM potassium acetate, spherical particles with diameters ranging from 40 to 100 nm were produced (Fig. 3A). Under the same conditions, but in the absence of Sec13/31, Sec23/24 formed large, heterogeneous aggregates (Fig. 3B). No aggregates were observed at pH 7.25 (data not shown).

To increase the particle density on the EM grid and reduce the background due to unassembled proteins, we fixed the spherical aggregates formed by Sec23/24 and Sec13/31 with glutaraldehyde and concentrated them by centrifugation (Fig. 3C,E). By negative staining, we observed numerous spherical aggregates (Fig. 3C,E). Their size distribution was relatively narrow, with more than 90% of the particles having a diameter of 60–85 nm (Fig. 3F). Occasionally, elongated particles were observed (Fig. 3C, black arrow). Many of the particles had a polygonal outline with 6–8 sides (Fig. 3E). Although no obvious lattice structure was observed, a rough surface, with some repetitive features of sizes in the range of 10 nm, was observed (Fig. 3E). On thin sections, the particles showed a dense pattern, again with no obvious lattice structure (Fig. 3D).

We observed a small effect of pH on the size of the COPII aggregates (Fig. 4). Dialysis against MES/HEPES at pH 6.85 with 230 mM potassium acetate promoted the formation of small aggregates (diameter = 70 nm), surrounded by unassembled or partially assembled material, as observed by negative staining (Fig. 4A) or

Fig. 4 | Effect of pH on the diameter of COPII aggregates. Concentrated solutions of Sec23/24 and Sec13/31 were mixed to give a final concentration of 0.9 μ M of each Sec subunit, dialysed at 25 $^{\circ}$ C against solutions containing 230 mM potassium acetate and 1 mM MgCl₂, and buffered at pH 6.85 (A,D), pH 6.33 (B) or pH 6.00 (C) using different ratios of MES (2-(*N*-morpholino) ethanesulphonic acid)/HEPES. After dialysis, aliquots were analysed by dynamic light scattering (DLS). The remaining solutions were fixed with glutaraldehyde and processed for negative staining (A–C) or thin-section electron microscopy (EM) (D). (E) Comparison of the average diameter of the spherical aggregates, as observed by EM, with the hydrodynamic diameter ($2 \times$ hydrodynamic radius) of the aggregates, as measured by DLS. The error bars indicate the s.d. from the mean. Scale bars, 100 nm.

thin-section EM (Fig. 4D). As the pH was decreased to 6.00, the proportion of unassembled material decreased, and the diameter of the aggregates increased from 70 nm to 110 nm, suggesting flexibility in the self-assembly process (Fig. 4A–C). However, it seems that there is a lower limit in the size of the COPII aggregates, as we did not observe well-formed spherical aggregates of less than 60 nm in diameter (Fig. 4E).

DISCUSSION

The self-assembly process described here shows some analogy with the formation of clathrin cages. As is the case for clathrin, Sec23/24 and Sec13/31, the main components of the COPII coat, self-assemble in solution at low pH, low ionic strength and/or low temperature, to form spherical aggregates with diameters close to those of the coats that shape transport vesicles. These aggregates probably correspond to those observed previously in control incubations of COPII proteins at low temperature (Matsuoka *et al.*, 1998). Sec23/24 and Sec13/31 are highly charged molecules, and it is likely that their self-assembly is driven mainly by electrostatic interactions. The sensitivity to pH in the range of 6.0–7.0 may indicate the involvement of histidine residues (Fig. 2). The key question is whether the COPII aggregates mimic the organization of the COPII coat that forms on lipid membranes. The cages formed by clathrin contain large lattices that are similar to the lattice observed on transport vesicles (Crowther & Pearse, 1981). Such cages are therefore relevant to structural studies. For COPII, lattices cannot be observed easily by conventional EM, either for the protein coat on membranes (Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998) or for the protein aggregates in solution (this study). However, two arguments suggest a structural relevance of the COPII aggregates observed here. First, they formed with stoichiometric amounts of Sec23/24 and

Sec13/31. Second, they are spherical, and their diameter, which ranges from 60 to 120 nm, is close to the diameter of COPII-coated vesicles. The variation in diameter suggests that, as for clathrin, there is some flexibility in the way that the proteins assemble into a shell, to accommodate various curvatures. Accordingly, the lower limit for the diameter (60 nm) would correspond to a limit in the curvature that a COPII shell could adopt (Fig. 4E). Interestingly, the Sec23/24 complex has a concave surface that matches that of a 60-nm vesicle (Bi *et al.*, 2002). However, we cannot rule out the possibility that the COPII aggregates are solid spheres, rather than empty shells, and form by the addition of proteins around a nucleus. This hypothesis, however, would not be consistent with the observation that the COPII aggregates have a minimum diameter (Fig. 4E).

The assembly of the COPII coat on lipid membranes is strictly dependent on the presence of Sar1–GTP at the bilayer surface (Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998). By contrast, the COPII spherical aggregates formed in the absence of Sar1. In addition, we did not observe any influence of a soluble, truncated form of Sar1 on COPII self-assembly (B.A., unpublished data). Similarly, clathrin cages can form in the absence of the adaptors that link the clathrin lattice to the membrane. We conclude that if Sar1–GTP tethers the Sec23/24 complex to the lipid surface, it is not directly engaged in the lateral contacts that drive the two-dimensional polymerization of the COPII coat. This may be important for the dynamics of the coat. Sec23 is a GTPase activating protein for Sar1, the activity of which is increased tenfold when the Sec13/31 complex is incorporated (Antonny *et al.*, 2001). Thus, the proper assembly of all COPII components promotes GTP hydrolysis by Sar1. Immuno-EM showed that COPII vesicles isolated in the presence of GTP contain a low ratio of

Sar1 in relation to the other COPII components, as compared with vesicles isolated in the presence of a non-hydrolysable analogue (Barlowe et al., 1994). Thus, although GTP hydrolysis leads ultimately to coat disassembly, a coated structure may persist when some Sar1 molecules are lost. The fact that Sec23/24 and Sec13/31 can self-assemble in solution suggests that the lateral contacts between these complexes may be strong enough to transiently preserve the structure of the coat, provided that the GTP hydrolysis reaction is properly organized in time and in space (Antonny & Schekman, 2001).

In conclusion, we propose that the spherical COPII aggregates described here correspond to minimal COPII cages that mimic the basic molecular organization of the COPII coat on lipid membranes. Cryo-EM studies of such cages should help to solve this molecular organization.

METHODS

Proteins and buffers. A detailed protocol for the purification of the yeast Sec23/24 and Sec13/31 complexes has been published (Shimoni & Schekman, 2002). Sec23/24 and Sec13/31 were purified by nickel and ion-exchange chromatography using high-salt buffers. The final salt concentration in the protein fractions was estimated from conductivity measurements. Stock solutions of Sec23/24 (0.3–0.4 mg ml⁻¹) contained 50 mM HEPES, pH 7.0, 790 mM potassium acetate, 0.1 mM EGTA, 50 mM imidazole, 1.4 mM 2-mercaptoethanol and 10% glycerol. Stock solutions of Sec13/31 (0.6–0.7 mg ml⁻¹) contained 20 mM Hepes, pH 7.4, 700 mM potassium acetate, 0.1 mM EGTA, 1.4 mM 2-mercaptoethanol and 10% glycerol. Dynamic light-scattering experiments and EM experiments were performed either directly on these stock solutions or after dilution or dialysis (using mini-dialysis units; Pierce) against various buffers. Our two standard buffers contained 50 mM HEPES-KOH, pH 7.5, or 50 mM Mes-KOH, pH 6.0, 1 mM MgCl₂ and potassium acetate at varying concentrations. For intermediate pH values, the two buffers were mixed. All buffers were filtered and degassed before use.

Dynamic light scattering. All measurements were made using a Dynapro MSX instrument (Protein Solutions) equipped with a Peltier temperature controller. A 15-μl solution containing the amount of protein indicated in the figures was placed in a quartz cuvette. After equilibration at the desired temperature, ten autocorrelation functions of the scattered light were determined at the optimal laser intensity, each for 10 s. From multiexponential fits of the autocorrelation functions, an intensity histogram was produced (Dynamic V 5.0 software; Protein Solutions). These histograms show the contribution to the intensity of the scattered light of hard spheres that have the same diffusion coefficient as the particles being analysed. These histograms emphasize the contribution of large objects. The intensity of light scattered by a particle (A) in solution is proportional to $M^2 \times [A] = M \times C$ where [A] is the molar concentration of A, C is the weight concentration and M is the molecular weight. In a solution containing small (r_1 , M_1 , C_1) and large (r_2 , M_2 , C_2) hard spheres, for which the intensity distribution gives two peaks of equal size, $M_1 \times C_1 = M_2 \times C_2$. M is proportional to r^3 . Therefore, the weight concentration of the small spheres (C_1) is $(r_2/r_1)^3$ times higher than that of the large spheres (C_2). If $r_2/r_1 = 5$ (the average factor obtained when the

COPII components aggregate), the difference in weight concentration is 125.

For experiments performed at varying temperatures (Fig. 1, middle panels), the autocorrelation functions were fitted using a mono-exponential function (that is, by assuming a monomodal distribution), and the mean R_h was plotted over time.

Electron microscopy. COPII/buffer mixtures were prepared by dialysis or dilution under the same conditions as those used for dynamic light scattering. The solutions were examined by negative staining, either immediately or after concentration by centrifugation (at 100,000g for 30 min at 22 °C) and fixation with 0.5–1.0% glutaraldehyde. Formvar- or carbon-coated grids were applied to a drop of the protein solution, stained with 1% uranyl acetate and examined using a Phillips electron microscope at 80–100 kV. For transmission EM, the pellet of fixed material was processed as for COPII vesicles (Matsuoka et al., 1998).

ACKNOWLEDGEMENTS

We thank C.F. Chan for the preparation of COPII proteins. This work was supported by the Centre National de la Recherche Scientifique/Action Concertée Incitative and the European Molecular Biology Organization/Young Investigator Programme (B.A.), Howard Hughes Medical Institute (R.S.) and by the Swiss National Science Foundation (L.O.).

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