Illuminating COPII coat dynamics

Wei Liu & Jennifer Lippincott-Schwartz

A FRET-based approach to monitor the assembly of membrane-associated COPII coat proteins provides new insights into how the COPII–cargo complex is maintained on the membrane during continuous rounds of Sar1p-GTP hydrolysis.

Within eukaryotic cells, membrane-based transport carriers play a central role in the directed movement of proteins between intracellular compartments as well as in the release and uptake of molecules from outside the cell. The formation of many of these carriers depends on a system of specialized 'coats' whose components are recruited individually to the membrane, where they assemble into a lattice[1,2]. This creates a sorting domain on the membrane into which cargo proteins concentrate. The domain deforms into a coated bud and then pinches off the membrane as a carrier. How coats on membranes sort cargo into a transport carrier remains unclear. A promising approach for addressing this issue, as well as for understanding other aspects of coat assembly and disassembly, is reported on page 167 of this issue of *Nature Structural & Molecular Biology*. There, Sato and Nakano[3] use fluorescence resonance energy transfer (FRET) to study the kinetic interactions between coat components during coat assembly and disassembly. Focusing on the COPII coat, which is necessary for vesicle trafficking out of the endoplasmic reticulum (ER), the authors dissect the temporal and spatial interactions between coat and cargo components, and further address the role of Sar1p-GTP hydrolysis in the generation and maintenance of the lattice.

The assembly and disassembly of COPII coat components on the membrane are regulated by the small GTPase Sar1p, which is active and membrane-associated in its GTP-bound state, and inactive and off-membrane in its GDP-bound state[4,5] (also see refs. 6,7). The transmembrane GDP-GTP exchange factor Sec12p initially recruits Sar1p to the membrane and converts it to a GTP-bound state. Because Sec12p strictly resides in the ER, COPII coat assembly is restricted to the ER. Activated Sar1p then recruits Sec23/24p to the membrane through binding to Sec23p, a GTPase-activating protein (GAP). This permits Sec24p to capture transmembrane cargo proteins, including SNARE proteins that regulate membrane fusion. The subsequent association of Sec13/31p with this complex completes the process of coat complex assembly. Self-association of the complexes into a lattice then leads to membrane deformation into a coated bud, which then transforms into a COPII-coated carrier. Disassembly of the lattice, which occurs after carrier budding, requires the GAP activity of Sec23p to enhance the GTP-to-GDP conversion at Sar1p.

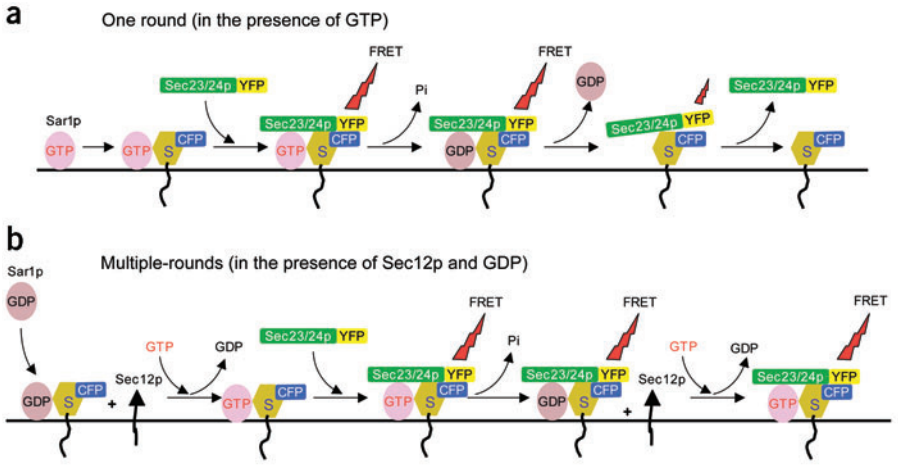

Figure 1 FRET-based assay for monitoring the kinetics of COPII coat complex assembly and disassembly in reconstituted liposomes. Association of Sec23/24p-YFP with SNARE-CFP within the COPII complex on membranes was detected by the presence of a FRET signal between CFP and YFP. (**a**) Without the Sar1p GDP-GTP exchange factor, Sec12p, the FRET signal increased and then decayed owing to one round of assembly and disassembly of the COPII complex. (**b**) In the presence of Sec12p, in which Sar1p can be reactivated after undergoing GTP hydrolysis, a persistent FRET signal was observed, indicating that in the continued presence of Sar1p-GTP, COPII coat components become stabilized on membranes.

To study the behavior of key players involved in COPII coat protein assembly and disassembly, including Sec12p-GEF, Sar1p-GTPase, Sec24/23p-GAP and transmembrane cargo, Sato and Nakano[3] attached the FRET donor and acceptor pairs YFP and CFP to the N termini of Sec24/23p and SNAREs, respectively. The molecules were then reconstituted into liposomes—artificial lipid bilayers shaped into hollow spheres—with a lipid composition similar to that of ER membranes. Because the emission spectrum of CFP overlaps with the excitation spectrum of YFP, when CFP-SNAREs and YFP-Sec24/23p are in close proximity, as they are in the COPII protein complex, excitation of CFP should lead to fluorescence emission from YFP through resonance energy transfer.

The authors began by focusing on interactions between Sec24/23p-GAP and the transmembrane cargo SNARE protein, Bet1p, under conditions in which Sarlp was incubated with GMP-PNP, a nonhydrolyzable form of GTP. This results in Sarlp being in a constitutively active state, which keeps Sarlp on the membrane. Liposomes were reconstituted with CFP-Bet1p-SNARE plus Sar1p-GDP and incubated with YFP-Sec24/23p in the presence or absence of GMP-PNP. Notably, an increase in the basal FRET level was observed when GMP-PNP was added to the mixture. Because a different SNARE protein tagged with CFP did not produce an increase in FRET, the data indicate that nucleotide exchange at Sar1p leads to the formation of a specific complex between Bet1p-SNARE, Sec24/23p-GAP and Sar1p-GMP-PNP. Interestingly, when a mutation was introduced into the sequence responsible for interactions between Bet1p-SNARE and Sec24p, a weak Sar1p-GMP-PNP-dependent FRET signal was observed. This indicates that Sar1p-GMP-PNP binds to both Sec23/24p and Bet1p-SNARE

The authors are at the Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA.
e-mail: jlippin@helix.nih.gov

and that these interactions are sufficient to form a complex via binding to Sar1p without Sec24p-Bet1p interactions. Hence, the FRET assay was sensitive enough to dissect specific interactions among coat components in the COPII complex.

Sato and Nakano[3] then used the FRET assay to examine whether the complex of Sec24/23p bound to cargo (SNARE in this study) remains associated with the membrane after Sar1p-GTP is hydrolyzed (**Fig. 1a**). When YFP-Sec24/23p was added to CFP-Bet1p-SNARE liposomes preloaded with Sar1p-GTP, an initial increase in FRET signal (comparable to that of GMP-PNP) was observed, indicative of Sar1p-mediated formation of a complex between YFP-Sec24/23p and CFP-Bet1p-SNARE. Soon thereafter, the FRET signal declined owing to disassembly of the complex upon Sar1p-GTP hydrolysis. Because the decline was slower than for Sar1p-GTP hydrolysis (monitored by tryptophan fluorescence) the results indicate that the complex of Sec24p and Bet1p-SNARE is stable on membranes for a short period after hydrolysis of Sar1p-GTP. This behavior of COPII coat components is reminiscent of that of COPI components, which remain associated with Golgi membranes after Arf1-GTP hydrolysis (determined from photobleaching studies in living cells)[8]. Therefore, there seems to be a commonality in the mechanism(s) whereby coat components assemble into metastable coats on the membrane.

To determine whether the persistent association of Sec24/23p with membranes after Sar1p-GTP hydrolysis results from Sec24p interacting specifically with Bet1p-SNARE, the authors repeated the FRET assay using the mutant Bet1p-SNARE, which lacks the Sec24p-binding sequence. In this case, the signal declined at the same rate as Sar1p-GTP hydrolysis. Hence, the delay in membrane dissociation of Sec24/23p after Sar1-GTP hydrolysis requires a specific interaction between SNARE-cargo and Sec24p. This finding raises the possibility that SNAREs serve as scaffolds for Sar1p on the ER, directing Sec24/23p GAP activity toward Sar1p.

In the last set of experiments, the authors examined the behavior of YFP-Sec24/23p and CFP-Bet1p-SNARE on membranes under conditions of multiple rounds of Sar1p-GTP hydrolysis (**Fig. 1b**). Here, the reaction mix was supplemented with Sec12p, which catalyzes efficient GDP-GTP exchange at Sar1p. Notably, the FRET signal persisted through the exchange reaction, indicating that YFP-Sec24/23p association with Bet1p-SNARE is maintained, most likely because Sec12p continuously reactivates Sar1p to its GTP-bound form. This finding is important as it explains how kinetically stable COPII complexes can potentially form during Sar1p-GTPase cycles. In this scenario, the continuous interaction of Sec24/23p with cargoes on the membrane—owing to repeated cycles of Sar1p-GTP hydrolysis and reactivation—leads to the formation of 'coated' domains or buds that are metastable; that is, they exist as long as Sar1p-GTP is being supplied to the domain. Because Sec12p is strictly localized to ER membranes, when the coated domains pinch off of the membrane, their coat is rapidly shed owing to depletion of Sar1p-GTP in the absence of GDP-GTP exchange activity. Not all interactions between different types of cargo and Sec24/23p may be stabilized sufficiently on the membrane during Sar1p-GTPase cycles to produce such coated domains. The authors thus speculate that oligomerization of cargo proteins may create combined signals for high-affinity binding to Sec24/23p. This would lead to the formation of cargo oligomer–Sec24/23p complexes, which in the presence of continuous Sar1p-GTPase cycles would create 'coated' sorting domains containing more diverse types of cargo[9].

Many other aspects of COPII coat assembly and its modulation by cargo could potentially be studied using the FRET-based approach of Sato and Nakano[3]. For example, how does Sec13/31p interact with Sec23/24p to crosslink adjacent Sec23/24p complexes into a structural scaffold? Does Sec13/31p play a role in accelerating Sec23p-mediated GAP activity? As the FRET-based assay can be used to dissect recruitment and cargo-capture events during the assembly of other coat systems on membranes, including COPI and clathrin coats, this assay offers a general new approach for clarifying protein-protein interactions and their kinetics on the membrane.

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ACEing GPI release

Satyajit Mayor

A recent study shows that the testicular angiotensin converting enzyme (ACE) has a new unexpected activity: it is responsible for cleaving GPI off from proteins anchored on the sperm cell membrane.

Fertilization is the process by which a sperm and an egg unite to form the first cell in the development of a multicellular organism. In mammals, the membrane properties of a viable and motile sperm undergo drastic alterations during the encounter with the mature oocyte, leading to binding and eventually the fusion with the mature oocyte (for a recent review on the fertilization process, see ref. 1). Many glycosylphosphatidylinositol (GPI)-anchored proteins, such as CD52, PH20 and TESP2, in the sperm are involved in triggering the maturation of the sperm and in oocyte recognition events. Most of these proteins are released in the medium surrounding the sperm, some during epididymal maturation, and others after a specific membrane altering process termed 'sperm capacitation' (**Fig. 1**), in which a drastic reorganization of the membrane of the sperm takes place, partially due to efflux of β-hydroxysterols (cholesterol in mammals). This culminates in the initiation of the acrosome reaction[2].

Kondoh *et al.*[3] reported, in a recent issue of *Nature Medicine*, that testicular ACE specifically cleaves GPI-anchored proteins from the membrane tether. This observation unites three seemingly disparate facets of the fertilization process: (i) the requirement for GPI-anchored sperm membrane proteins, (ii) a specific requirement for ACE in germ cells and (iii) a role for cholesterol removal. Furthermore, their

The author is in the Cellular Organization and Signaling Group, National Centre for Biological Sciences, Bellary Road, Bangalore 560 065, India. e-mail: mayor@ncbs.res.in

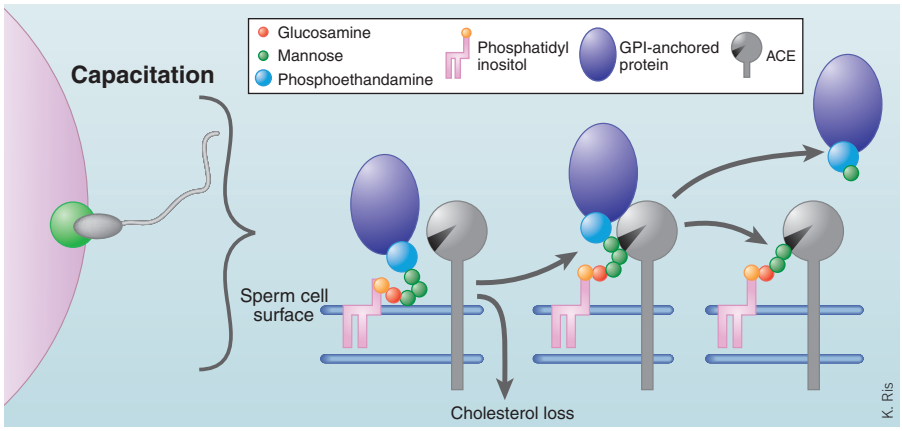

Figure 1 Release of GPI-anchored proteins during sperm cell capacitation. The sperm-egg interaction (left) begins after alterations of the sperm cell surface, including loss of cholesterol from the sperm cell surface. ACE seems to be required at this time to facilitate the release of GPI-anchored proteins and other associated molecules thought to act as inhibitors of sperm-egg binding.

results should keep structural biologists busy in predicting function of an enzyme based on structure, as the GPI-cleavage activity is unexpected for this well characterized and evolutionarily conserved protein.

When studying the distribution of GPI-anchored GFP protein (GFP-GPI) in transgenic mice, Kondoh and colleagues noticed that there was a significant amount of GFP-GPI protein released into seminal plasma surrounding seminal vesicles. To pinpoint the source of the releasing activity, they purified the releasing factor from germ cell membranes and discovered an unusual candidate: ACE (EC 3.4.15.1)[3]. This enzyme catalyzes the cleavage of C-terminal dipeptides from several substrates including angiotensin I and bradykinin[4]. Coincidentally, testicular ACE had already been implicated in governing the fertility of sperm in transgenic mice experiments[5,6]. The single gene for ACE is alternatively spliced to encode both a somatic and testis-specific form. The testis-specific isozyme is only found in developing spermatids and in mature sperm. While both isoforms are synthesized as transmembrane-anchored proteins, the somatic isoform has two catalytic domains and is often found as a soluble circulating protein, whereas the testis isoform encodes a single catalytic domain and is exclusively a membrane-anchored protein[4]. Although the exact roles of the ACE isozymes in male reproduction are unknown, experiments with mice deleted of somatic ACE produce viable fertile sperm while mice deleted of both isoforms produce sperm that are severely impaired in their ability to reach the uterus and fertilize the oocyte (at least in mice). However, the canonical substrates of ACE, angiotensin or bradykinin do not appear to be required for fertility of the sperm, suggesting other functions for the testis-specific ACE isoform in the fertility equation.

In concert with this observation, Kondoh *et al.*[3] find that the GPI-anchor-releasing activity of ACE is required for sperm fertility. Even when the peptidase activity is abolished by either mutation or inactivation, the enzyme could still cleave GPI-anchored proteins and restore fertility to ACE-deficient sperm. This activity is not protein-specific because it cleaves a variety of GPI-anchored proteins including GFP-GPI. An analysis of the fragments released from cells treated with purified ACE revealed that the cleavage site is within the GPI anchor, between the second and third residues of the conserved mannose core. This observation indicates that ACE is an endomannosidase.

Specific cleavage of GPI to release tethered proteins from the cell surface has long been proposed as an important function of the GPI-anchor[7]. Notwithstanding a flurry of interest in this subject after the identification of phospholipase D as an enzyme capable of releasing GPI-anchored proteins from the cell surface[8], there is little evidence for the involvement of this activity in any physiological process. If the result reported by Kondoh *et al.*[3] is indeed borne out by further experimentation with test substrates, this would be the first hydrolytic enzyme specific for the GPI-anchor that cleaves within the evolutionarily conserved GPI-glycan core.

The GPI-cleavage activity is, however, humbling from the point of view of structure-function prediction, since ACE is an extraordinarily well-studied molecule. Its endopeptidase activity is the target of many hypertension treatments available on the market. In addition, its crystal structure has been determined at a very high resolution[9]. This indicates that there is no foolproof prediction of enzymatic activity or function based on structures plus the bioinformatics tools available today. As pointed out by the authors, ACE does not seem to have obvious similarity with known glycosidases or lectins. This now raises the issue of how ACE recognizes its sugar substrate.

Given that purified soluble ACE has GPI-cleaving activity, the similarity between the testis and somatic-specific forms would suggest that somatic ACE is likely to be involved in the release of GPI-anchored proteins *in vivo*. This is certainly borne out in tissue culture cells as shown by the authors[3]. ACE knockout mice that are already available would be useful tools to examine this issue in relevant tissues, such as in hepatic ducts.

GPI-anchor-releasing activity of germ cell ACE requires removal of cholesterol from cell membranes (a process that is also expected to occur during sperm cell capacitation)[2]. Because somatic ACE is present on the surface of many tissues, it is likely that cholesterol depletion may be necessary as a trigger to release GPI-anchored proteins from the surface of these cells, possibly by disrupting nanoscale cholesterol-dependent structures of GPI-anchored proteins[10], or cholesterol-dependent structures termed 'rafts'[11]. This may provide access to the mannose core of these molecules for hydrolytic attack with physiological implications; the release of GPI-anchored proteins during specific cellular events could be regulated by cholesterol levels in cell membranes.

A simple model that stitches the different strands of this sperm-and-egg story together must include membrane-localized GPI-anchored proteins on the sperm as direct or indirect inhibitors of the oocyte-sperm interaction. Epididymal maturation and/or capacitation-triggered ACE activity would then act as a mechanism of release from this inhibition (**Fig. 1**). This inhibition could be either in the form of a physical barrier made up of GPI-anchored proteins or specific molecules that bind to these proteins. The study of Kondoh *et al.*[3] is one step toward understanding the fertilization process at the molecular level.

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