

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<sup>3</sup> 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)<sup>8</sup>. 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<sup>9</sup>.

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<sup>3</sup>. 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

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**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.**

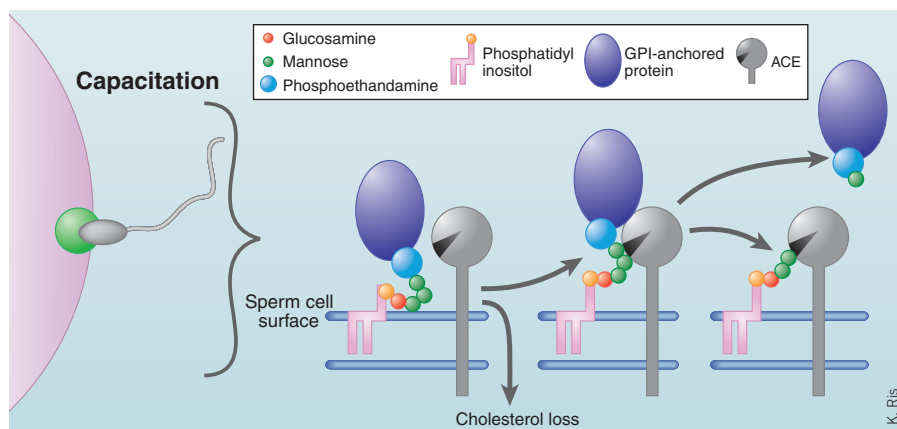
Fertilization is the process by which a sperm and an egg unite to form the first cell in the development of a multicellular organism. In mammals, the membrane properties of a viable and motile sperm undergo drastic alterations during the encounter with the mature oocyte, leading to binding and eventually 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  $\beta$ -hydroxysterols (cholesterol in mammals). This culminates in the initiation of the acrosome reaction<sup>2</sup>.

Kondoh *et al.*<sup>3</sup> 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

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**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)<sup>3</sup>. This enzyme catalyzes the cleavage of C-terminal dipeptides from several substrates including angiotensin I and bradykinin<sup>4</sup>. Coincidentally, testicular ACE had already been implicated in governing the fertility of sperm in transgenic mice experiments<sup>5,6</sup>. 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<sup>4</sup>. 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 func-

tions for the testis-specific ACE isoform in the fertility equation.

In concert with this observation, Kondoh *et al.*<sup>3</sup> 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<sup>7</sup>. 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<sup>8</sup>, there is little evidence for the involvement of this activity in any physiological process. If the result reported by Kondoh *et al.*<sup>3</sup> 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<sup>9</sup>. 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<sup>3</sup>. 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)<sup>2</sup>. 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<sup>10</sup>, or cholesterol-dependent structures termed 'rafts'<sup>11</sup>. 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.*<sup>3</sup> is one step toward understanding the fertilization process at the molecular level.

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