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The Mechanisms of Vesicle Budding and Fusion

Review

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Genetic and biochemical analyses of the secretory pathway have produced a detailed picture of the molecular mechanisms involved in selective cargo transport between organelles. This transport occurs by means of vesicular intermediates that bud from a donor compartment and fuse with an acceptor compartment. Vesicle budding and cargo selection are mediated by protein coats, while vesicle targeting and fusion depend on a machinery that includes the SNARE proteins. Precise regulation of these two aspects of vesicular transport ensures efficient cargo transfer while preserving organelle identity.

Like other landmark papers, the articles by Novick et al. (1980) and Balch et al. (1984) featured in the supplement to this 30th Anniversary issue of *Cell* introduce themselves right off the library shelves. The bound volumes spontaneously open to the right pages, which are half-detached, tattered, and inscribed with pencil markings—testaments to the countless times that these articles have been read and copied. Naturally, these papers have already been the subject of many reviews. Herein we will once more attempt to convey the enormous influence that these studies have had on the field of intracellular protein trafficking, this time by focusing on how they helped to bring about the current understanding of the molecular mechanisms of vesicle budding and fusion.

The Vesicular Transport Hypothesis

The stage for the discoveries discussed here was set over 30 years ago (that is, "B.C." or "before *Cell*") by the work of George Palade and colleagues on protein secretion (Palade, 1975). This work established that newly synthesized secretory proteins pass through a series of membrane-enclosed organelles, including the endoplasmic reticulum (ER), the Golgi complex, and secretory granules, on their way to the extracellular space (Figure 1). Proteins destined for residence at the plasma membrane, endosomes, or lysosomes share the early stations of this pathway (i.e., the ER and the Golgi complex) with secretory proteins. Importantly, the secretory proteins are often found within small, membrane-enclosed vesicles interspersed among the major organelles of the pathway. Such observations inspired the vesicular transport hypothesis, which states that the transfer of cargo molecules between organelles of the secretory pathway is mediated by shuttling transport vesicles. According to this hypothesis, vesicles bud from a "donor" compartment ("vesicle budding") by a process that allows selective incorporation of cargo into the forming vesicles while retaining resident proteins in the donor compartment ("protein sorting"). The vesicles are subsequently targeted to a specific "acceptor" compartment ("vesicle targeting"), into which they unload their cargo upon fusion of their limiting membranes ("vesicle fusion"). An updated representation of the steps of vesicular transport is shown in Figure 2. The processes of budding and fusion are iterated at the consecutive transport steps until the cargo reaches its final destination within or outside the cell. To balance this forward movement of cargo, organelle homeostasis requires the retrieval of transport machinery components and escaped resident proteins from the acceptor compartments back to the corresponding donor compartments ("retrograde transport"), a process that is also proposed to occur by vesicular transport. All of these steps are tightly regulated and balanced so that a large amount of cargo can flow through the secretory pathway without compromising the integrity and steady-state composition of the constituent organelles.

Genetic and Biochemical Dissection of the Secretory Pathway

In his 1974 Nobel Prize lecture, Palade stated "Further understanding of the secretory process is now becoming dependent on adequate information on the chemistry of these membranes and on the reactions involved in their interactions" (Palade, 1975). The challenge to find this information was taken on by Randy Schekman and Jim Rothman, who in the late 1970s independently set out to elucidate the molecular mechanisms that underlie vesicular transport. Inspired by Arthur Kornberg's molecular dissection of DNA replication, Schekman and Rothman embarked on the task of reducing vesicular transport to a set of elementary biochemical reactions. Toward this goal, each investigator initially pursued a different approach.

Schekman and colleagues had the foresight to choose for their studies the yeast *Saccharomyces cerevisiae*, at a time when it was not yet clear that yeast and mammals had similar secretory apparatuses. The ease of genetic manipulation of yeast allowed these researchers to isolate temperature-sensitive "*sec*" mutants that were defective in protein secretion. Twenty-three complementation groups, each corresponding to a different gene, were identified in the study by Novick et al. (1980). Strikingly, electron microscopy of the *sec* mutants at the nonpermissive temperature revealed the intracellular accumulation of various types of membrane-enclosed structures (Figure 3). Depending on the mutant strain, these structures appeared as (1) small vesicles of 60–100 nm diameter that presumably corresponded to the

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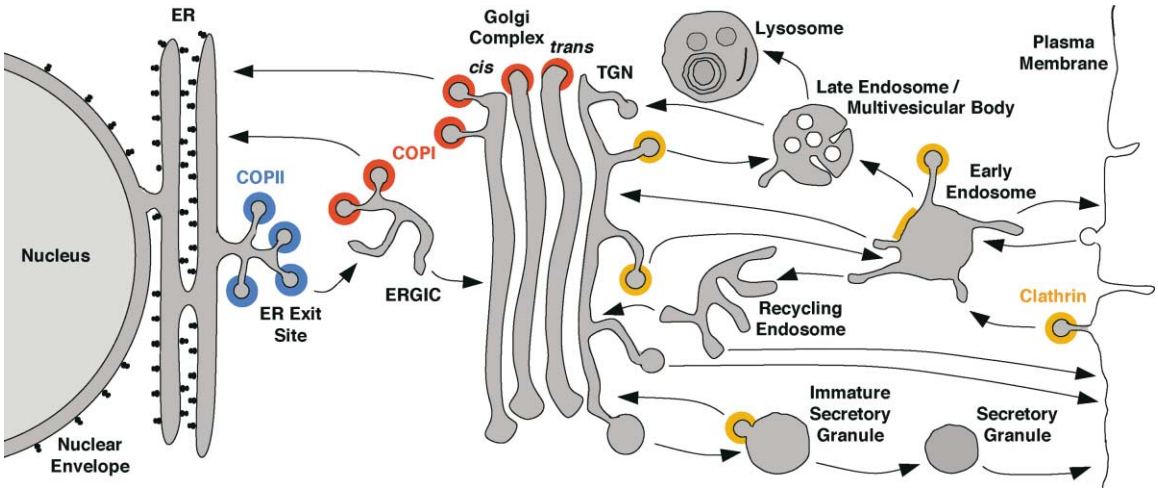

Figure 1. Intracellular Transport Pathways

The scheme depicts the compartments of the secretory, lysosomal/vacuolar, and endocytic pathways. Transport steps are indicated by arrows. Colors indicate the known or presumed locations of COPII (blue), COPI (red), and clathrin (orange). Clathrin coats are heterogeneous and contain different adaptor and accessory proteins at different membranes. Only the function of COPII in ER export and of plasma membrane-associated clathrin in endocytosis are known with certainty. Less well understood are the exact functions of COPI at the ERGIC and Golgi complex and of clathrin at the TGN, early endosomes, and immature secretory granules. The pathway of transport through the Golgi stack is still being investigated but is generally believed to involve a combination of COPI-mediated vesicular transport and cisternal maturation (Pelham and Rothman, 2000). Additional coats or coat-like complexes exist but are not represented in this figure.

transport carriers, (2) an enlarged ER network, or (3) a cup-shaped membranous organelle (the "Berkeley body"), which was later identified as an abnormal Golgi complex. Schekman and colleagues quickly recognized that each of these structures represented an exaggerated secretory pathway intermediate that had accumu-

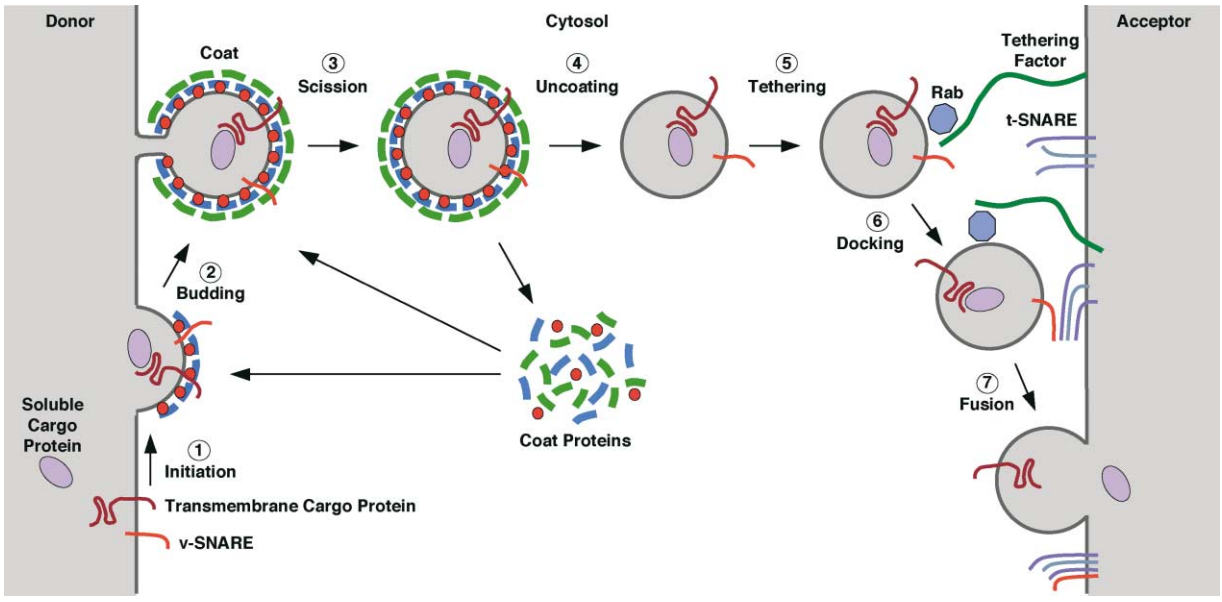

Figure 2. Steps of Vesicle Budding and Fusion

(1) Initiation of coat assembly. The membrane-proximal coat components (blue) are recruited to the donor compartment by binding to a membrane-associated GTPase (red) and/or to a specific phosphoinositide. Transmembrane cargo proteins and SNAREs begin to gather at the assembling coat. (2) Budding. The membrane-distal coat components (green) are added and polymerize into a mesh-like structure. Cargo becomes concentrated and membrane curvature increases. (3) Scission. The neck between the vesicle and the donor compartment is severed either by direct action of the coat or by accessory proteins. (4) Uncoating. The vesicle loses its coat due to various events including inactivation of the small GTPase, phosphoinositide hydrolysis, and the action of uncoating enzymes. Cytosolic coat proteins are then recycled for additional rounds of vesicle budding. (5) Tethering. The "naked" vesicle moves to the acceptor compartment, possibly guided by the cytoskeleton, and becomes tethered to the acceptor compartment by the combination of a GTP bound Rab and a tethering factor. (6) Docking. The v- and t-SNAREs assemble into a four-helix bundle. (7) This "*trans*-SNARE complex" promotes fusion of the vesicle and acceptor lipid bilayers. Cargo is transferred to the acceptor compartment, and the SNAREs are recycled as shown in Figure 7B.

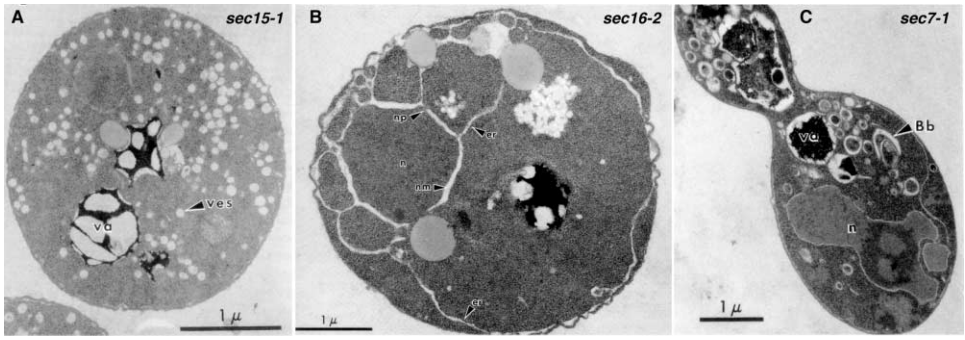

Figure 3. Electron Micrographs of *S. cerevisiae sec* Mutants Incubated at the Nonpermissive Temperature of 37°C

The mutant strains shown in this figure illustrate the accumulation of transport vesicles (A), the expansion of the ER (B), and the expansion of the Golgi complex ("Berkeley body") (C). Symbols: ves, vesicles; va, vacuole; er, endoplasmic reticulum; n, nucleus; nm, nuclear membrane; np, nuclear pore; Bb, Berkeley body. Pictures reprinted from Novick et al. (1980).

lated due to a specific block in protein transport. Subsequent identification of the genes that were defective in these *sec* mutants revealed a fascinating array of novel proteins involved at multiple stages of the secretory pathway (a list of the 23 original *SEC* genes and their protein products is shown in Table 1 of Schekman and Novick [2004]).

Rothman and colleagues addressed the same problem using a completely different strategy. Few had contemplated studying cell biological processes by in vitro reconstitution, but these researchers devised an ingenious cell-free assay to measure protein transport between cisternae of the mammalian Golgi complex (Balch et al., 1984; Balch, 2004) (Figure 4). This assay consisted of incubating (1) a "donor" Golgi fraction derived from Vesicular Stomatitis Virus (VSV)-infected cells lacking the enzyme N-acetylglucosamine (GlcNAc) transferase I, (2) an "acceptor" Golgi fraction prepared from uninfected wild-type cells, (3) UDP-^{3}H-GlcNAc as a radiolabeled substrate for GlcNAc transferase I, (4) cytosol, and (5) ATP. Transport between the donor and acceptor compartments was measured by the incorporation of ^{3}H-GlcNAc into the VSV-G glycoprotein, which was isolated by immunoprecipitation. Combining this cell-free assay with classical protein purification led to the identification of various components involved in vesicle budding and fusion.

Strikingly, these disparate methodologies converged with the discovery that two of the genetically identified Sec proteins were orthologous to biochemically identified proteins required for intra-Golgi transport (Wilson et al., 1989; Griff et al., 1992). The implication of this finding was profound: yeast and mammals share a conserved vesicular transport machinery, which can be dissected using both genetic and biochemical tools. A powerful synergy developed from the combined use of these approaches in many laboratories. The results have produced a detailed molecular picture of the mechanisms of trafficking in the secretory pathway and the related endocytic and vacuolar/lysosomal targeting pathways. Central to these mechanisms are the two most critical events in the lifetime of a transport vesicle, namely budding and fusion.

Role of Protein Coats in Vesicle Budding and Cargo Selection

The budding of transport vesicles and the selective incorporation of cargo into the forming vesicles are both mediated by protein coats (Kirchhausen, 2000; Bonifacino and Lippincott-Schwartz, 2003) (Figure 2). These

Table 1. Components of the COPII ER Export Machinery

Yeast Proteins	Human Orthologs	Functions and/or Properties
Sar1p	Sar1a, Sar1b	Small GTP binding protein of the Ras superfamily
Sec23p	Sec23A, Sec23B	Sar1p•GTP binding subunit; GTPase-activating protein (GAP) for Sar1p
Sec24p, Lst1p, Iss1p	Sec24A, Sec24B, Sec24C, Sec24D	Cargo binding subunit
Sec13p	Sec13	Component of the membrane-distal layer of COPII coat; probably contains β-propeller domain
Sec31p	Sec31A, Sec31B	Component of the membrane-distal layer of COPII coat; probably contains β-propeller domain
Sec16p	Unknown	Scaffold protein at ER exit sites; large hydrophilic protein that is peripherally but tightly associated with the ER membrane
Sec12p	Sec12 (PREB)	Guanine nucleotide exchange factor (GEF) for Sar1p; type II transmembrane protein with probable β-propeller domain
Sed4p	Unknown	Sec12p homolog devoid of Sar1p-GEF activity; putative Sar1p GAP inhibitor

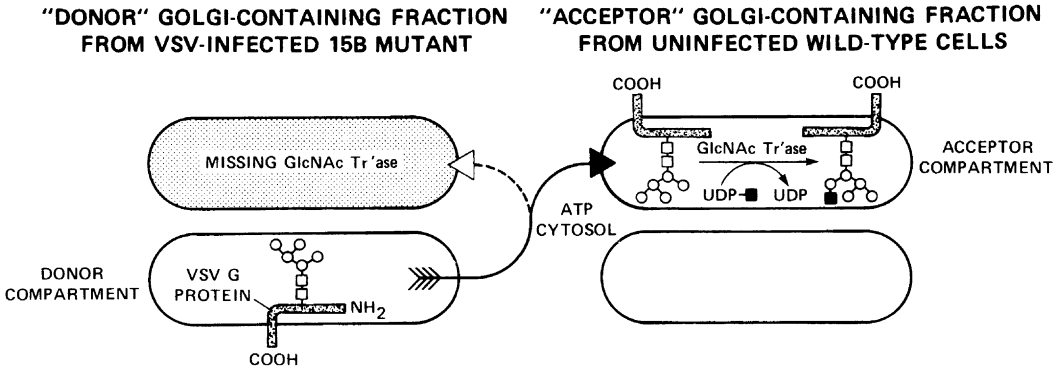

Figure 4. Schematic Representation of the In Vitro Assay for Protein Transport between Donor and Acceptor Golgi Compartments
See text for details. Reprinted from Balch et al. (1984).

coats are supramolecular assemblies of proteins that are recruited from the cytosol to the nascent vesicles. The coats deform flat membrane patches into round buds, eventually leading to the release of coated transport vesicles. The coats also participate in cargo selection by recognizing sorting signals present in the cytosolic domains of transmembrane cargo proteins. Vesicle budding and cargo selection at different stages of the exocytic and endocytic pathways are mediated by different coats and sorting signals. The first coats to be identified and characterized contained a scaffold protein, clathrin, as their main constituent (Roth and Porter, 1964; Pearse, 1975). Clathrin coats were initially assumed to participate in most, if not all, vesicular transport steps within the cell. However, later studies demonstrated that the function of these coats was restricted to post-Golgi locations including the plasma membrane, the *trans*-Golgi network (TGN), and endosomes. A major discovery by the Rothman and Schekman labs was the existence of non-clathrin coats that mediate vesicular transport in the early secretory pathway (Waters et al., 1991; Barlowe et al., 1994). One of these coats, COPII, is now known to mediate export from the ER to either the ER-Golgi intermediate compartment (ERGIC) or the Golgi complex (Barlowe et al., 1994), while another coat, COPI, is involved in intra-Golgi transport and retrograde transport from the Golgi to the ER (Letourneur et al., 1994). Of the various protein coats that have been identified to date, COPII is one of the best understood and the one that we will use as an example in our discussion of vesicle budding.

Composition of COPII

The identification and characterization of COPII are among the greatest achievements to emerge from the *sec* mutant screen. In *S. cerevisiae*, the core COPII components are the small Ras-like GTPase Sar1p, the Sec23p•Sec24p subcomplex, and the Sec13p•Sec31p subcomplex (Table 1) (Barlowe et al., 1994). Sar1p together with Sec23p•Sec24p form the membrane-proximal layer of the coat, while Sec13p•Sec31p forms a second, membrane-distal layer (Figure 5). Additional regulatory proteins (Table 1) participate in COPII assembly, including Sec16p, a putative scaffold protein (Espenshade et al., 1995), and Sec12p, a guanine nucleotide exchange factor (GEF) for Sar1p (Barlowe and Schekman, 1993). Sed4p, a Sec12p homolog that may function as an inhibitor of GTP hydrolysis by Sar1p (Gimeno et al., 1995; Saito-Nakano and Nakano, 2000), is likely to be specific to *S. cerevisiae* and closely related species (Payne et al., 2000). Orthologs of the other structural and regulatory COPII components exist in higher eukaryotes, including mammals (Table 1) (Bock et al., 2001). There are two additional paralogs of Sec24p in *S. cerevisiae* (Lst1p and Iss1p) and two or more paralogs of Sar1p, Sec23p, Sec24p, and Sec31p in humans (Table 1). This diversification of COPII subunits likely endows the coat with the ability to sort different cargo proteins and to be differentially regulated (Roberg et al., 1999; Shimoni et al., 2000). Apart from Sar1p, the subunits of the COPII coat are structurally distinct from those of the COPI and clathrin coats. The relative simplicity of COPII, as well as its unique role in ER export, have facilitated the analysis of its assembly and function.

Coat Assembly

The COPII coat assembles by the stepwise deposition of Sar1p•GTP, Sec23p•Sec24p, and Sec13p•Sec31p onto sites where newly synthesized proteins exit from the ER (Figure 5). These ER exit sites (also known as transitional ER sites) are generally devoid of ribosomes and range in complexity from discrete buds on the nuclear envelope to convoluted networks of tubules and vesicles (Bednarek et al., 1995; Orci et al., 1991; Bannykh and Balch, 1997). The more elaborate ER exit sites are long-lived membrane subdomains from which COPII vesicle budding occurs repeatedly (Hammond and Glick, 2000; Stephens et al., 2000). At present, it is unclear what marks these sites for COPII recruitment. A candidate for this role is Sec16p, a large peripheral ER membrane protein (Espenshade et al., 1995). Sec16p interacts with Sec23p, Sec24p, and Sec31p via different domains (Espenshade et al., 1995; Shaywitz et al., 1997) and may serve as scaffold for the nucleation or stabilization of the assembling coat (Supek et al., 2002). It is likely that Sec16p acts in conjunction with the transmembrane protein Sec12p to recruit GTP bound Sar1p to the ER membrane. Sar1p•GTP associates with the lipid bilayer through a hydrophobic amino-terminal extension and recruits its effector, the Sec23p•Sec24p subcomplex, through interactions with two "switch" regions characteristic of Ras superfamily proteins (Huang et al., 2001;

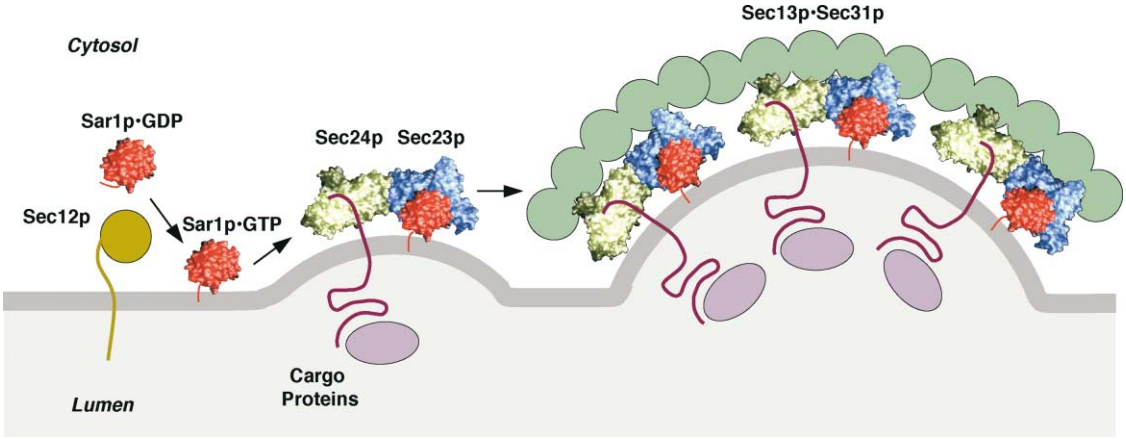

Figure 5. Assembly of COPII

Cytosolic Sar1p•GDP is converted to membrane bound Sar1p•GTP by the transmembrane protein Sec12p. Sar1p•GTP recruits the Sec23p•Sec24p subcomplex by binding to Sec23p, forming the "pre-budding complex". Transmembrane cargo proteins gather at the assembling coat by binding to Sec24p. The Sec13p•Sec31p subcomplex polymerizes onto Sec23p•Sec24p and crosslinks the pre-budding complexes. Cargo proteins are further concentrated. The depictions of Sar1p, Sec23p, and Sec24p are surface representations from the crystal structures of these proteins (Bi et al., 2002). The Sec13p•Sec31p complex is represented as an elongated, five-globular domain structure based on electron microscopy (Lederkremer et al., 2001). Sec16p and Sed4p also participate in the assembly of COPII, but are not represented here because their roles are less well understood. See text for additional details.

Bi et al., 2002). The initiation of COPII assembly thus involves both GTP-independent and GTP-dependent reactions that cooperate to deposit the coat at ER exit sites.

Sar1p•GTP together with Sec23p•Sec24p constitute the so-called "pre-budding complex," which has recently been analyzed by electron microscopy (Lederkremer et al., 2001; Matsuoka et al., 2001) and X-ray crystallography (Bi et al., 2002). This complex has the appearance of a bow tie with one side corresponding to Sec23p and the other to Sec24p (Bi et al., 2002) (Figure 6). Sec23p makes direct contact with Sar1p•GTP (Bi et al., 2002), while Sec24p participates in cargo recognition (see below). Once assembled onto membranes, the pre-budding complex recruits the Sec13p•Sec31p subcomplex, which consists of two Sec13p and two Sec31p subunits (Lederkremer et al., 2001). Sec13p•Sec31p appears by electron microscopy as a flexible, elongated structure that polymerizes to form a mesh-like scaffold (Lederkremer et al., 2001; Matsuoka et al., 2001). Sec23p stimulates the GTP hydrolysis activity of Sar1p (Yoshihisa et al., 1993) by contributing an "arginine finger" that pokes into the GTP binding site and aids catalysis (Bi et al., 2002). This activity of Sec23p as a GTPase-activating protein (GAP) is augmented approximately ten-fold by addition of Sec13p•Sec31p (Antonny et al., 2001). A paradoxical implication of this mechanism is that COPII coat assembly should trigger disassembly by promoting GTP hydrolysis. How can the COPII coat polymerize to cover a forming vesicle if the basic unit of the polymer is unstable? A possible explanation is that the kinetics of GTP hydrolysis might be slower than the kinetics of vesicle budding, in which case there would be time for a vesicle to form before the coat fell apart. Alternatively, GTP hydrolysis might cause Sar1p to be released from the coat while the other subunits remained assembled on the membrane. The polymeric nature of the coat could provide kinetic stability in the absence of Sar1p•GTP. In addition, the cytosolic domains of transmembrane cargo proteins could act as secondary membrane tethers or could modulate

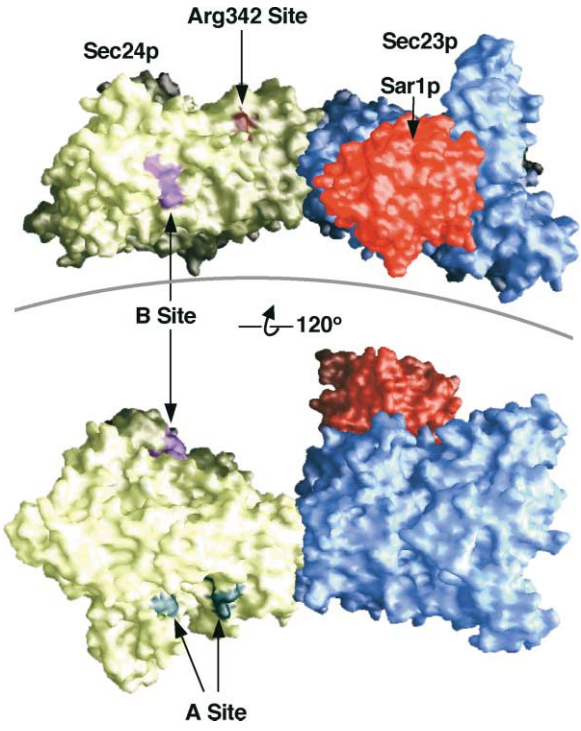

Figure 6. Surface Representation of the Crystal Structure of the Pre-Budding Complex

The locations of the A, B, and Arg342 sites for binding different ER export signals are indicated. The membrane apposed to the concave surface of the complex is represented by a curved line. The bottom image is rotated by 120° along a longitudinal axis relative to the top image. Adapted from Mossessova et al. (2003) and Miller et al. (2003). Images were generated using GRASP (Nicholls et al., 1991).

Table 2. ER Export Signals

Proteins	Functions	Signals
Sys1p	Golgi protein; high copy suppressor of *ypt6* mutants	DLE
Gap1p	General amino acid permease	DID
VSV-G	Envelope glycoprotein of vesicular stomatitis virus	YTDIE
Kir2.1	Inwardly rectifying potassium channel	FCYENE
Kir1.1	Inwardly rectifying potassium channel	VLSEVDETD
Prm8p	Pheromone-regulated membrane protein	FF
ERGIC53	Mammalian type I transmembrane lectin; ER export receptor for subset of glycoproteins; homologous to yeast Emp46p and Emp47p	FF
hp24δ_1	p24 family member; putative ER export receptor; homologous to yeast Erv25p and Emp24p	FF
Erv46p	ER vesicle transmembrane protein; part of a complex with Erv41p	FY
Erv41p	ER vesicle transmembrane protein; part of a complex with Erv46p	IL
Emp46p	Type I transmembrane lectin; cycles between the ER and the Golgi; homologous to Emp47p	YYM, LL
Emp47p	Type I transmembrane lectin; cycles between the ER and the Golgi; homologous to Emp46p	LL
Erv25p	p24 family member; putative ER export receptor; part of a complex with Emp24p	FF, LV
Emp24p	p24 family member; ER export receptor for Gas1p; part of a complex with Erv25p	FF, LV
Sed5p	Golgi t-SNARE	YNNSNPF, LMLME
Bet1p	ER-Golgi v-SNARE	LASLE
GalT2	Golgi enzyme	RR
GalNAcT	Golgi enzyme	RR

Information was obtained from Barlowe (2003) and Giraudo and Maccioni (2003). Underlining indicates known key residues.

the GAP activity of Sec23p. Any of these alternative explanations would imply that Sar1p•GTP is dispensable for the integrity of the central area of the coat and is required only to stabilize the coat edges (Antonny and Schekman, 2001).

Cargo Selection

The majority of cargo proteins are actively concentrated in COPII-coated buds and vesicles prior to export from the ER (Balch et al., 1994; Malkus et al., 2002). Most transmembrane cargo proteins exit the ER by binding directly to COPII (Kuehn et al., 1998; Aridor et al., 1998; Votsmeier and Gallwitz, 2001), but some transmembrane and most soluble cargo proteins bind indirectly to COPII through transmembrane export receptors (Table 2) (Appenzeller et al., 1999; Muniz et al., 2000; Powers and Barlowe, 2002). Export receptors leave the ER together with their ligands, unload their cargo into the acceptor compartment, and recycle back to the ER.

The sorting signals recognized by the COPII coat are found in the cytosolic domains of transmembrane cargo proteins. These signals are quite diverse (Table 2) (Barlowe, 2003). Some consist of di-acidic motifs fitting the consensus [DE]X[DE] (where D is aspartate, X is any amino acid, and E is glutamate) (Nishimura and Balch, 1997; Votsmeier and Gallwitz, 2001), whereas others are based on short hydrophobic motifs such as FF, YYM, FY, LL, or IL (F is phenylalanine, Y is tyrosine, M is methionine, L is leucine, and I is isoleucine) (Kappeler et al., 1997; Nakamura et al., 1998). In addition, a di-basic [RK]X[RK] (R is arginine and K is lysine) motif has recently been shown to promote ER exit of Golgi glycosyltransferases (Giraudo and Maccioni, 2003). Yet other signals consist of longer sequences, folded determinants, or combinations of any of the above (Table 2). The involvement of so many different signals in the same sorting step implies the existence of either multiple binding sites on the same recognition protein or a family of recognition proteins. Both of these solutions have evolved for COPII. Genetic, biochemical, and structural analyses have demonstrated that most ER export signals bind to Sec24p (Miller et al., 2002, 2003; Mossessova et al., 2003). Sec24p displays at least three distinct signal binding sites termed "A," "B," and "Arg342" (this latter site is named after an arginine residue that is critical for binding of Sec22p) (Miller et al., 2003; Mossessova et al., 2003) (Figure 6). The Sec24p paralogs Lst1p (Roberg et al., 1999) and Iss1p (Kurihara et al., 2000) may interact with export signals different from those recognized by Sec24p. In addition, Sar1p may participate in signal recognition either by direct binding to the signals or by allosteric modulation of Sec24p (Springer and Schekman, 1998; Aridor et al., 1998; Giraudo and Maccioni, 2003). In this regard, it is interesting that mutations in one of two human Sar1p homologs (Sar1b) results in a specific defect in chylomicron export from the ER (Jones et al., 2003). This diversity of signals and recognition modes explains the ability of COPII to package a wide variety of exported proteins.

Vesicle Budding

How do the properties of the COPII proteins lead to vesicle formation? A mixture of purified Sec23p•Sec24p, Sec13p•Sec31p and GTP-locked Sar1p is sufficient to generate coated vesicles from liposomes, indicating that these proteins are intrinsically capable of deforming the membrane and pinching off a vesicle (Matsuoka et al., 1998). An important clue to the genesis of the curvature of COPII-coated buds comes from the crystal structure of the pre-budding complex, which has a positively charged, concave surface that likely apposes the membrane and induces membrane bending (Bi et al., 2002) (Figure 6). The contribution of the Sec13p•Sec31p subcomplex to membrane deformation is still not clear, although this subcomplex might stabilize the curvature generated by the pre-budding complex. The final stage in vesicle formation is scission of the neck of the bud. To date, no proteins have been identified as being specifically involved in this process. One possibility is that coat polymerization itself may drive membrane scission by closing the spherical COPII cage.

Comparison with Other Coats

It is now clear that other vesicle coats follow the basic COPII paradigm, but with variations (Kirchhausen, 2000; Bonifacino and Lippincott-Schwartz, 2003). For example, the initiation step for COPI assembly involves activation and membrane recruitment of Arf GTPases that are closely related to Sar1p (Donaldson et al., 1992; Helms and Rothman, 1992). But unlike Sar1p, which has an exclusive relationship with COPII, Arf proteins have many effectors, including COPI and other coats (see below) as well as lipid-modifying enzymes (Nie et al., 2003). Many different GEFs and GAPs activate and inactive Arf, respectively, in an effector- or compartment-specific fashion (Nie et al., 2003). During COPI coat assembly, Arf•GTP simultaneously recruits the membrane-proximal $\beta\gamma\delta\zeta$ and the membrane-distal $\alpha\beta'\epsilon$ subcomplexes (Hara-Kuge et al., 1994), in apparent contrast to the stepwise assembly of COPII. Like COPII, COPI recognizes specific signals in the cytosolic domains of transmembrane cargo proteins, although in this case the signals function to retrieve proteins from the ERGIC or the Golgi complex to the ER (Cosson and Letourneur, 1994; Bremser et al., 1999).

Clathrin coats are considerably more complex than COPII and COPI. Arf•GTP and/or specific phosphoinositides (e.g., phosphatidylinositol 4,5-bisphosphate, and phosphatidylinositol 4-phosphate) recruit a variety of clathrin "adaptors" from the cytosol to membranes (Bonifacino and Lippincott-Schwartz, 2003; Wang et al., 2003). Examples of adaptors are the heterotetrameric AP-1, AP-2, and AP-3 complexes and the monomeric GGA, Hrs, Epsin 1, and ARH proteins, specific combinations of which form a heterogeneous membrane-proximal layer onto which clathrin is subsequently deposited. The adaptors also bind to transmembrane cargo proteins by recognizing cytosolic sorting signals that contain either critical tyrosine or di-leucine residues or conjugated ubiquitin (Bonifacino and Traub, 2003). Indeed, the tyrosine-based signal present in the cytosolic domain of the low-density lipoprotein receptor was the first cytosolic sorting signal to be identified, a finding that was reported in another landmark paper by Mike Brown and Joe Goldstein (Davis et al., 1986). This particular signal is now known to interact with the ARH adaptor (He et al., 2002; Mishra et al., 2002). Clathrin and clathrin-adaptor complexes can polymerize into spherical, cage-like structures (Kirchhausen and Harrison, 1981), as can COPII (Antonny et al., 2003), indicating that these proteins have an intrinsic ability to sculpt buds and vesicles from membranes. Thus, the clathrin-adaptor complexes appear to perform the same basic functions as the COPII coats: cytosolic signal recognition and membrane deformation. However, the clathrin vesicle cycle involves additional classes of proteins that do not seem to operate during COPII vesicle formation. Clathrin vesicle assembly is regulated by an ensemble of kinases, phosphatases, and other accessory proteins (Lafer, 2002). In addition, clathrin vesicle scission depends on accessory factors such as dynamins (Sever, 2002). Finally, clathrin vesicle uncoating is mediated by the cytosolic chaperones Hsc70 and auxilin (Rothman and Schmid, 1986; Ungewickell et al., 1995). Why does clathrin utilize all of this extra machinery? Part of the answer may lie in the participation of clathrin in multiple post-Golgi sorting events, each of which requires a specific set of adaptors and regulators.

Role of SNARE Proteins in Vesicle Fusion

After a vesicle sheds its coat, it must be targeted to the appropriate acceptor compartment. The final step in a vesicle's existence is fusion with the acceptor membrane. Remarkably, the targeting and fusion reactions both rely on the same class of proteins, which were identified in a biochemical *tour de force*.

Discovery of the SNAREs

An early contribution of the cell-free intra-Golgi transport assay (Figure 4) was the identification of an "N-ethylmaleimide-Sensitive Factor" (NSF), which could exist in cytosolic or membrane bound forms (Glick and Rothman, 1987). Electron microscopy by Lelio Orci demonstrated that when NSF was inactivated, uncoated vesicles accumulated on Golgi membranes, implying that NSF is required for membrane fusion (Malhotra et al., 1988). By treating Golgi membranes with N-ethylmaleimide, the intra-Golgi transport reaction was converted into a specific assay that allowed for the purification of NSF (Block et al., 1988). Cloning of the corresponding gene revealed that NSF was the mammalian ortholog of yeast Sec18p, which had been implicated in ER-to-Golgi transport (Wilson et al., 1989; Eakle et al., 1988). It soon became apparent that NSF acts in a wide range of membrane fusion steps in the secretory and endocytic pathways (Beckers et al., 1989; Diaz et al., 1989).

Despite the obvious importance of NSF, its role in membrane fusion was initially unclear. NSF forms a hexameric ring (Whiteheart et al., 2001) and is a founding member of the AAA protein family ("*A*TPases *a*ssociated with diverse cellular *a*ctivities"), a group of enzymes that catalyze the structural remodeling of protein complexes (Lupas and Martin, 2002). A crucial step toward understanding NSF function came from identifying a partner protein called α-SNAP ("*s*oluble NSF *a*ssociation *p*rotein"), which binds NSF to membranes (Clary et al., 1990). α-SNAP turned out to be the mammalian ortholog of yeast Sec17p (Griff et al., 1992). At this point, it was evident that NSF and α-SNAP formed a complex with additional, unidentified membrane proteins. Using NSF/α-SNAP as an affinity reagent to fractionate a brain lysate, Thomas Söllner and colleagues identified a set of three membrane-associated "SNAP Receptors," or SNAREs (Söllner et al., 1993). These same membrane proteins had previously been implicated in linking synaptic vesicles to the plasma membrane (Walch-Solimena et al., 1993). One of the proteins, known as VAMP or synaptobrevin, was known to be associated with synaptic vesicles, whereas the other two proteins, syntaxin and SNAP-25 (no relation to α-SNAP!), had been localized to the presynaptic plasma membrane. From today's perspective, it may seem obvious that synaptic vesicle exocytosis is mechanistically related to other vesicular transport steps, but until 1993, most researchers assumed that these processes were distinct. The discovery of the link between NSF, α-SNAP, and SNAREs revolutionized the analysis of both intracellular transport and synaptic transmission and brought these two fields together in a spectacular collision.

Properties of the SNAREs

The product of this collision was the SNARE hypothesis, which proposed that each type of transport vesicle carries a specific "v-SNARE" that binds to a cognate "t-SNARE" on the target membrane (Rothman, 1994). This idea fits with the observations that cells contain families of proteins related to the synaptic SNAREs and that various SNAREs localize to different intracellular compartments (Bennett and Scheller, 1993; Weimbs et al., 1998; Chen and Scheller, 2001). Most SNAREs are C-terminally anchored transmembrane proteins, with their functional N-terminal domains facing the cytosol. Each of these proteins contains a heptad repeat "SNARE motif" of 60–70 amino acids that can participate in coiled-coil formation (Bock et al., 2001). An exception is SNAP-25, which contains two SNARE motifs and binds to the membrane via covalently linked palmitate groups attached to the central part of the protein. Structural and biochemical studies showed that the SNARE complex generated by the pairing of a cognate v- and t-SNARE is a very stable four-helix bundle, with one α helix contributed by the monomeric v-SNARE and the other three α helices contributed by the oligomeric t-SNARE (Fasshauer et al., 1997; Sutton et al., 1998) (Figure 7A). The t-SNARE usually consists of three separate polypeptides, although in the synaptic SNARE complex, two of the SNARE motifs are supplied by SNAP-25. All of the SNARE complexes in the cell appear to fit this general pattern in which the four SNARE motifs are contributed by a protein related to synaptobrevin, a protein related to syntaxin, a protein or protein domain related to the N-terminal part of SNAP-25, and a protein or protein domain related to the C-terminal part of SNAP-25 (Misura et al., 2002).

In some cases, the distinction between vesicles and target membranes is not meaningful—for example, during the homotypic fusion of organelles—but the general classification scheme of v-SNAREs (one α-helix) and t-SNAREs (three α helices) remains useful. An alternative scheme uses the terminology R- or Q-SNAREs, reflecting the presence of an arginine or a glutamine, respectively, at a characteristic position within the SNARE motif (Fasshauer et al., 1998). In each SNARE complex, three glutamines and one arginine form a central ionic layer in the otherwise hydrophobic core of the four-helix bundle (Sutton et al., 1998). Although the two classification schemes are based on different principles, there is a rough correspondence of R-SNAREs with v-SNAREs and of Q-SNAREs with t-SNAREs.

A major insight from structural analysis of the SNARE complex was that v- and t-SNAREs pair in a parallel fashion (Hanson et al., 1997; Lin and Scheller, 1997; Sutton et al., 1998). Therefore, v- and t-SNAREs in separate membranes can pair to form a *trans*-SNARE complex, or v- and t-SNAREs in the same membrane can pair to form a *cis*-SNARE complex. A *trans*-SNARE complex persists throughout the fusion reaction to become a *cis*-SNARE complex in the fused membrane (Figure 7B). α-SNAP then binds along the edge of the SNARE complex (Rice and Brunger, 1999) and recruits NSF. ATP hydrolysis by NSF dissociates the *cis*-SNARE complex (Mayer et al., 1996), possibly by exerting rotational force to untwist the four-helix bundle (May et al., 1999; Yu et al., 1999). Thus, NSF and α-SNAP do not participate directly in the fusion reaction, but instead act to recycle the SNAREs for another round of complex formation.

What Exactly Do SNAREs Do?

SNAREs seem to perform two major functions. One function is to promote fusion itself. In all transport reactions that have been examined, the formation of *trans*-SNARE complexes is essential for fusion. Assembly of the four-helix bundle is thought to supply the free energy needed to bring apposing membranes close enough to fuse (Hanson et al., 1997; Weber et al., 1998; Chen and Scheller, 2001). This model is appealing because a *trans*-SNARE complex, also known as a "SNAREpin," has hydrophobic segments in two apposing membrane bilayers and is therefore structurally analogous to the activated form of viral fusion proteins (Jahn et al., 2003). Support for the idea that SNAREs act as fusogens came from reconstitution experiments showing that purified recombinant SNAREs can promote the fusion of liposomes, provided that v- and t-SNAREs are in different liposomes (Weber et al., 1998). In an elegant extension of this work, Rothman and colleagues recently demonstrated that the fusion of natural biological membranes can be driven by SNAREs in the absence of accessory proteins (Hu et al., 2003). Cells were engineered to produce "flipped" SNAREs that faced the outside of the cell rather than the cytoplasm. When cells containing a flipped v-SNARE were mixed with cells containing the cognate flipped t-SNARE, efficient fusion occurred. The combined data leave little doubt that SNAREs form the conserved, essential core of the fusion machinery.

Liposome fusion with purified SNAREs is much slower than in vivo fusion reactions (Weber et al., 1998), implying that additional components cooperate with SNAREs to "tickle" the membranes and accelerate fusion. The best documented example is the yeast vacuolar ATPase V_0 subunit, which has been reported to act downstream of the SNAREs in vacuolar fusion (Bayer et al., 2003). Under some conditions, fusion can apparently proceed even if the *trans*-SNARE complex has already dissociated (Szule and Coorssen, 2003). The meaning of these observations is still being debated, but they suggest that assembly of a *trans*-SNARE complex is not always temporally coupled to membrane fusion. This point may be particularly relevant for the reversible "kiss-and-run" fusion that occurs during regulated exocytosis (Palfrey and Artalejo, 2003). Despite these complexities, it is likely that in all of the transport steps in the secretory and endocytic pathways, SNAREs perform the same function of overcoming the energy barrier to fusion.

The second major function of SNAREs is to help ensure the specificity of membrane fusion. Different v-/t-SNARE complexes form at different steps of intracellular transport. Surprisingly, purified SNAREs can pair promiscuously in vitro. But when purified SNAREs were tested in the liposome fusion assay, the formation of productive *trans*-SNARE complexes was almost exclusively restricted to physiologically relevant v- and t-SNARE combinations (McNew et al., 2000). As a result, the biophysical fusion assay actually has predictive power for identifying SNARE complexes that form in vivo (Parlati et al., 2002).

SNAREs cannot, however, be the only specificity determinants for membrane fusion because a given v-SNARE

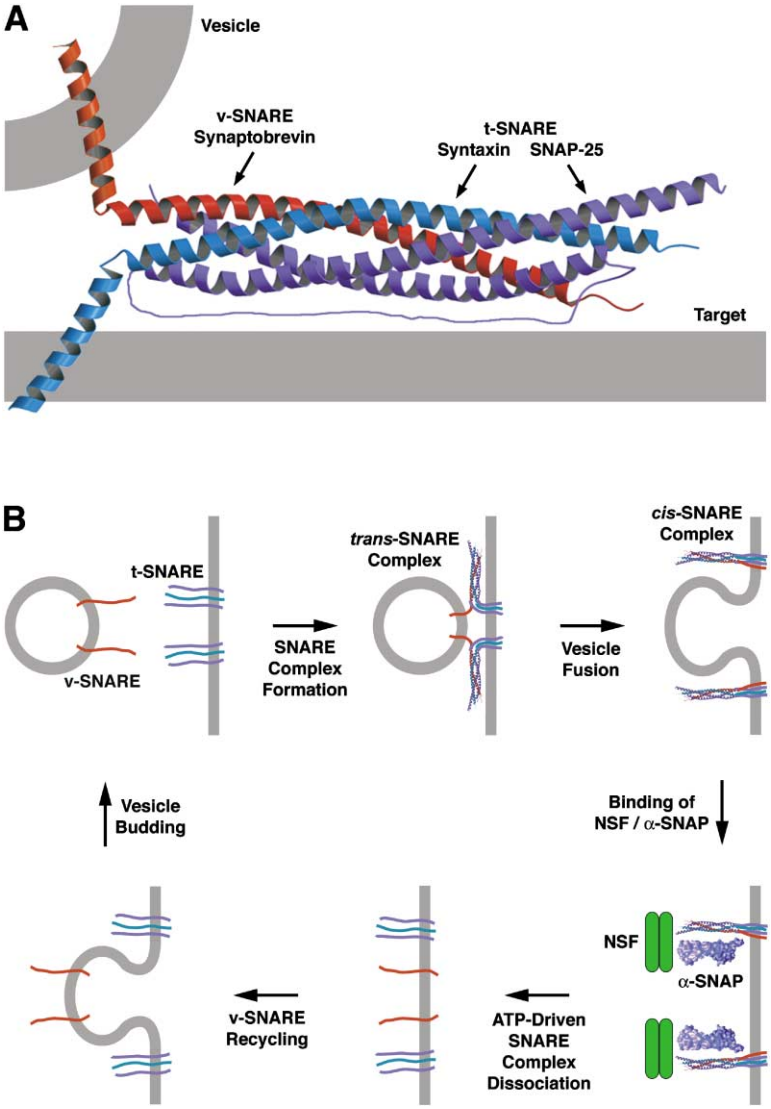

Figure 7. Structure and Function of SNAREs

(A) Crystal structure of a synaptic *trans*-SNARE complex drawn after Sutton et al. (1998). The structures of the two membrane anchors and of the peptide that links the two SNAP-25 α helices are hypothetical.

(B) The SNARE cycle. A *trans*-SNARE complex assembles when a monomeric v-SNARE on the vesicle binds to an oligomeric t-SNARE on the target membrane, forming a stable four-helix bundle that promotes fusion. The result is a *cis*-SNARE complex in the fused membrane. α-SNAP binds to this complex and recruits NSF, which hydrolyzes ATP to dissociate the complex. Unpaired v-SNAREs can then be packaged again into vesicles. The depictions of the SNARE complex and α-SNAP are from Sutton et al. (1998) and Rice and Brunger (1999), respectively. A complete crystal structure of NSF is not yet available, but the protein is known to form a double-barreled hexameric ring that binds to the end of the SNARE complex (Lupas and Martin, 2002).

recycles and is therefore present in both anterograde and retrograde vesicles (Figure 7B). Additional specificity is provided by tethering proteins that link the apposing membranes prior to SNARE complex formation. These tethering proteins come in several flavors (Whyte and Munro, 2002). The heteromeric "quatrefoil" tethers are exemplified by the exocyst, which links secretory carriers to the plasma membrane (Guo et al., 1999). Six of the original *sec* mutants defined different subunits of the exocyst (Schekman and Novick, 2004). Related quatrefoil tethers function in Golgi traffic. For example, the COG complex is believed to mediate the tethering of COPI vesicles to Golgi cisternae and was identified by several approaches (Whyte and Munro, 2002), including biochemical purification using the cell-free intra-Golgi transport assay (Ungar et al., 2002). A different type of tether is EEA1, a long coiled-coil protein that promotes the homotypic fusion of early endosomes (Christoforidis et al., 1999). Similar coiled-coil tethers called golgins are present in the Golgi (Barr and Short, 2003). These various tethers assemble with the aid of Rab family GTPases (known as Ypt proteins in yeast) to promote the initial association of two membranes (Segev, 2001; Jahn et al., 2003). Multiple Rab proteins operate at different steps of transport. Rabs, tethers, and SNAREs collaborate to ensure that membranes fuse at the correct time and place. Thus, like many biological processes, membrane fusion employs sequential, partially redundant mechanisms to achieve high fidelity.

Accessory and Regulatory Proteins

Not surprisingly, a plethora of accessory components and regulatory reactions modulate the action of SNAREs (Gerst, 2003) (Table 3). This modulation is important to prevent inappropriate events of SNARE complex formation. For example, after two membranes fuse and the *cis*-SNARE complex is dissociated by NSF/α-SNAP, the SNAREs need to be kept inactive until the next round of fusion. Cytosolic factors such as GATE-16 and LMA1 bind the individual v- and t-SNAREs and help to keep them separate (Elazar et al., 2003). In some cases, SNARE complex formation is regulated by phosphorylation of SNAREs or of interacting components (Gerst, 2003). Key regulatory elements for SNARE complex assembly are present in the SNAREs themselves, many of

Table 3. Selected Protein Families Implicated in Vesicle Targeting and Fusion[a]

Family Names or Representative Family Members	Functions
VAMP/Synaptobrevin	Monomeric v-SNARE contributing a single R-SNARE helix
Syntaxin	t-SNARE subunit contributing one Q-SNARE helix
SNAP-25	t-SNARE subunit contributing two Q-SNARE helices, which can be present in a single polypeptide (as in SNAP-25) or in two separate polypeptides
NSF/Sec18p	ATPase that promotes dissociation of *cis*-SNARE complexes
α-SNAP/Sec17p	Links NSF/Sec18p to SNAREs
Sec1P, Munc-18	Bind to syntaxin family proteins, and perform diverse essential functions regulating SNARE complex assembly
GATE-16	Small ubiquitin-related mamamlian protein that binds and shields unpaired SNAREs
LMA1	Yeast protein, a complex of thioredoxin and the protease inhibitor IB2, that seems to be functionally analogous to GATE-16
Synaptotagmins	Putative Ca^{2+} sensors for regulated exocytosis
Vacuolar ATPase V_0 subunit	Promotes a late step of vacuolar fusion in yeast
Quatrefoil tethers	Heteromeric tethering factors that act at various transport steps
EEA1	Long coiled-coil tethering factor involved in early endosome fusion
Golgins	Coiled-coil proteins that mediate vesicle tethering and cisternal stacking in the Golgi apparatus
Rab/Ypt GTPases	Perform multiple regulatory functions in vesicle budding, vesicle tethering, interaction of vesicles with cytoskeletal motors, and membrane subdomain formation

[a] Additional regulators of SNARE complex assembly and disassembly are described by Gerst (2003), and further details about tethering proteins are given by Whyte and Munro (2002).

which contain extensions upstream of the SNARE motif (Misura et al., 2002; Dietrich et al., 2003). For example, syntaxins have an N-terminal three-helix bundle, which binds internally to the SNARE motif to generate a "closed" conformation that cannot bind to partner SNAREs; and certain members of the synaptobrevin family have an N-terminal "longin" domain that may have a similar autoinhibitory function (Dietrich et al., 2003). In some cases, *trans*-SNARE complex assembly seems to be arrested at an intermediate stage, with accessory proteins preventing the complete "zipping up" of the four-helix bundle until a fusion signal is received (Chen and Scheller, 2001). The best candidate for such an accessory protein is the putative Ca^{2+} sensor synaptotagmin, which interacts with SNAREs and promotes synaptic vesicle fusion in response to Ca^{2+} influx (Jahn et al., 2003).

An intriguing group of SNARE-interacting proteins is the SM family, whose founding members are yeast Sec1p—the product of the first gene identified by Novick and Schekman (1979)—and neuronal Munc-18. The SM proteins can be viewed as comparable in importance to the SNAREs because each membrane fusion step requires a specific SM protein (Toonen and Verhage, 2003; Gallwitz and Jahn, 2003). However, the function of SM proteins is still enigmatic. These proteins bind to syntaxins, but the mode of binding is not conserved, and various SM proteins either stimulate or inhibit SNARE complex assembly. Thus, much remains to be learned about the regulatory aspects of SNARE-dependent membrane fusion.

Intracellular Targeting of SNAREs

A typical SNARE is a transmembrane protein with an N-terminal cytosolic domain and a single membrane-spanning sequence near the C terminus. Such "tail-anchored" proteins insert into the ER membrane post-translationally and reach their final destinations by traversing the secretory pathway (Borgese et al., 2003). Little is known about how SNAREs are targeted to specific organelles. For the few SNAREs that have been examined, targeting determinants are present in the transmembrane sequence, the cytosolic domain, or both (Joglekar et al., 2003). An important mechanism for SNARE localization is interaction with vesicle coats. For example, SNAREs involved in ER-to-Golgi transport must be packaged into COPII vesicles during ER export and then into COPI vesicles during retrieval from the Golgi (Springer and Schekman, 1998; Rein et al., 2002).

Recent biochemical and structural studies have illuminated the process by which three *S. cerevisiae* SNAREs involved in ER-to-Golgi transport—Sed5p, Bet1p, and Sec22p—interact with the COPII coat (Miller et al., 2003; Mossessova et al., 2003). These SNAREs bind to distinct sites on the Sec24p subunit: a YNNSNPF (N is asparagine, S is serine, and P is proline) signal from Sed5p binds to the A site, a LXX[LM]E signal from Sed5p and Bet1p binds to the B site (as does a di-acidic signal from the Golgi protein Sys1p), and an unidentified determinant on Sec22p binds to a site that includes Arg342 (Miller et al., 2003; Mossessova et al., 2003) (Figure 6). Sec24p apparently cannot bind an assembled SNARE complex, but instead selects for the uncomplexed, fusogenic forms of the SNAREs (Mossessova et al., 2003). Thus, vesicle budding is mechanistically integrated with vesicle fusion.

Perspectives

Over the past 30 years, we have progressed from the classic morphological description of the secretory pathway to the present molecular understanding of vesicular transport. The experimental approaches introduced by Novick et al. (1980) and Balch et al. (1984) were crucial to this endeavor and remain among the most powerful tools available to probe the workings of the protein trafficking machinery. The advent of genomics and proteomics, the application of crystallographic and biophysical methods, and the development of fluorescent live-cell imaging technologies have further contributed to mak-

ing protein trafficking one of the most vibrant areas of modern cell biology.

In the near future, we can expect progress toward a better understanding of the structure, assembly, regulation, and function of vesicle coats. It is remarkable that to date the only coats with well-established functions are COPII (export from the ER) and plasma membrane clathrin coats (endocytosis). The exact roles of other coats, including COPI and TGN/endosomal clathrin coats, are less clear. Moreover, a look at Figure 2 reveals that the arrows representing single transport steps far outnumber the known coats. Is each of these steps mediated by a different coat? If so, there must be many coats yet to be discovered. A more likely alternative is that some transport steps occur by mechanisms other than coat-mediated budding. For example, transport from the TGN to the plasma membrane is not known to be concentrative or to involve conventional coats.

We also anticipate progress in elucidating the role of lipids in protein trafficking. Certain lipids (e.g., phosphoinositides) regulate the membrane recruitment of clathrin coats and of other trafficking components (De Matteis et al., 2002; Wang et al., 2003), while other lipids (e.g., glycosphingolipids and cholesterol) may help to partition proteins into specific membrane domains (Munro, 2003). How are local variations in lipid composition established and maintained? How do lipid modification and localization regulate protein trafficking? What lipid rearrangements occur during membrane fission and fusion? The fundamental nature of these questions illustrates that lipids are a new research frontier.

Finally, we can expect to learn more about the transport vesicles themselves. When and how do they lose their coats? How do they interact with the cytoskeleton? Do all transport vesicles have a uniformly small size and spherical shape? Regarding this last question, the best-characterized transport intermediates are indeed small, spherical coated vesicles. But emerging evidence from mammalian cells points to an alternative form of transport by large pleiomorphic intermediates (Bonifacino and Lippincott-Schwartz, 2003). A well-documented example of such a transport intermediate is the COPI-containing vesicular tubular carriers that move from ER exit sites to the Golgi complex (Aridor et al., 1995; Presley et al., 1997; Mironov et al., 2003). There are undoubtedly similarities between the transport mechanisms employed by large pleiomorphic intermediates and those employed by small vesicles, but there are probably significant differences as well. Understanding this variation on the vesicular transport paradigm is an important goal for future studies.

The expectations and questions listed above highlight only some of the many avenues of inquiry set in motion by the original genetic and biochemical analyses of vesicular transport. What can we expect to see ten years from now, when *Cell* turns 40? All of us will be older, some of us will be grayer, and the pathways of intracellular transport will be clearer.

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