REVIEW

The protein machinery of vesicle budding and fusion

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Abstract

A general protein machinery that buds and fuses transport vesicles is harnessed to generate the complex web of intracellular transport pathways critical for such diverse processes as cell growth, endocytosis, hormone release, and neurotransmission. With this appreciation, the challenge of understanding the precise molecular mechanisms of these many facets of cell biology has been reduced to a series of problems in protein structure and chemistry.

Keywords: budding and fusion mechanisms; endoplasmic reticulum; Golgi stack; soluble NSF attachment proteins, NSF protein, ARF proteins, coat proteins, SNARE proteins

Most of the organelles in the cytoplasm of eukaryotic cells are connected by a series of vesicle shuttles whose purpose is to transport proteins and other macromolecules among them. These transport processes are fundamental to cell growth as well as other critical physiological processes, such as endocytosis and secretion.

Newly made proteins that will ultimately reside in such diverse compartments as the cell surface or lysosomes, among many others, are first segregated into the endoplasmic reticulum (ER) by virtue of signal peptide-dependent translocation, and are then transported by a series of vesicle shuttles from the ER into and across the Golgi stack (where many posttranslational modifications, including modifications of their saccharide chains, occur). From the Golgi stack, other shuttles carry them to their final destinations. Such "biosynthetic" transport processes thus account not only for the replenishment and growth during cell division of all cellular membranes (except mitochondria and chloroplasts), but also provide the basis for the propagation and maintenance of the unique and specific protein composition of each organelle—and thus propagation of the compartments with preservation of their various assigned functions.

Certain receptors at the cell surface are captured in vesicles that fuse with endosomes, carrying along specific cargo, such as lipoprotein particles and iron, that is critical for cell growth. The receptors are then carried back to the cell surface by other vesicles, their cargo carried to lysosomes by still other vesicles for digestion and/or utilization by the cell.

Specialized transport vesicles bud from the Golgi and are programmed to fuse with the plasma membrane, but will not do so until a special, extracellular signal is received. Such secretory storage vesicles are critical in physiology, for they store and re-

lease during exocytosis a diverse array of mediators such as hormones, enzymes, and, in the nervous system, neurotransmitters.

It is a remarkable fact that these and many other transport processes utilize the same or very similar molecular machinery to enable the budding of the responsible transport vesicles and the fusion of each with a specific target compartment. The discovery of this important and unifying principle has come from biochemical studies that resulted in the isolation of protein machinery critical for budding and fusion, and from genetic and other studies that allowed these concepts to be generalized.

Now the problem of understanding the precise molecular mechanisms of vesicle budding and fusion has been reduced to a series of tractable but still challenging and unsolved problems in protein structure, protein-protein interactions, and in enzyme chemistry. It is my hope that this review article may help to attract to this exciting field protein scientists who may wish to address some of these issues.

The core protein machinery that has been discovered includes coat proteins such as clathrin and coatamer, to sculpt a vesicle out of a donor membrane; vesicle and target-specific identifiers v-SNAREs and t-SNAREs, to dock the vesicle; and NSF and SNAP proteins, to help fuse the docked vesicle. (SNARE is short for SNAP receptor; v- and t-SNAREs are membrane protein superfamilies that are either vesicle (v) or target (t) membrane specific.) Although the same coat proteins and fusion proteins can, in principle, be used at multiple transport steps without sacrificing specificity, different v- and t-SNAREs must be employed by each shuttle. Although important pieces of this machinery may well remain to be identified, enough has been learned for the general principles to be clear.

Cell-free reconstitution of vesicle transport

How did this story develop? The finding that transport between organelles can occur in cell-free extracts (Fries & Rothman,

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1980), even after the precise relationships among organelles that exist within cells are destroyed, established that transport operates according to intrinsic chemical specificities, and could thus be understood in terms of solution chemistry. Cell-free systems, of course, afford the unique means to establish molecular mechanisms, and can also be harnessed as functional assays to aid in the identification and/or purification of the required protein machinery and have been the key for understanding the basis of transport. When combined with immunocytochemistry at the electron microscopic level, the structures formed by these proteins can be readily identified in relation to function. Numerous cell-free systems that reconstitute virtually every avenue of biosynthetic and endocytic protein transport in animal and yeast cells have now been described (see chapters in Rothman, 1992). The isolation of secretion mutants in yeast (Novick et al., 1980, 1981) established that the overall organization of the secretory organelles is universal, and offered a powerful, reductionist approach complementary to biochemistry, capable of identifying required polypeptides and establishing their physiological relevance, but not in and of itself capable of establishing their precise function.

With these approaches in hand, many pieces of the protein machinery responsible for transport vesicle budding, targeting, and fusion have been revealed during the last decade. Because this machinery was first elucidated in the context of intercisternal transport in the Golgi stack, this has served as a paradigm, and so it is appropriate to begin here and then generalize from this.

The Golgi stack is specialized for protein processing and transport, providing an ideal context for addressing basic questions about vesicle budding and fusion. It consists of a stack of 4–6 flattened and topologically separate membrane-bound cisternae. The stack receives protein from the ER at one face (termed the *cis*-face). A series of vesicle shuttles carry this protein across the stack, from cisterna to cisterna, until the last (*trans*) cisterna is reached. This entire process takes about 5 min in most cells.

Transport between Golgi cisternae is reconstituted when Golgi membranes are incubated with the cytosol fraction (100,000 g supernatant) and a source of energy, ATP in early experiments with crude cell extracts (Fries & Rothman, 1980; Balch et al., 1984a). A “donor” population of Golgi, containing the cargo to be transported (the VSV-encoded G protein), is incubated with an “acceptor” population of Golgi, containing a glycosylating enzyme (adding *N*-acetylglucosamine) lacking in the donor population. Transfer of G protein from donor to acceptor can be measured by the addition of radioactive *N*-acetylglucosamine to G protein’s oligosaccharide chain, and is a vectorial process that moves G protein unidirectionally forward between successive Golgi compartments (Dunphy et al., 1981; Rothman et al., 1984). Because the donor and acceptor Golgi stacks remain distinct and the glycosylated G protein resides only in the acceptor population (Braell et al., 1984), cell-free transport involves a diffusible (e.g., transport vesicle) intermediate.

Insight into the nature of the intermediate came from a combination of biochemistry with electron microscopy; ≈ 70 -nm diameter vesicles formed from Golgi cisternae when transport was reconstituted *in vitro* (Balch et al., 1984b). These vesicles contained the VSV G protein and only appeared when cytosol and ATP were both added (Orci et al., 1986). Most of the fully formed vesicles, and all of the vesicles still in the process of budding, had a distinct, approximately 18-nm thick “coat” on their

cytoplasmic surface (Orci et al., 1986) reminiscent of, but distinct from, the clathrin coat of endocytic vesicles (Goldstein et al., 1979; Pearse & Bretscher, 1981). The coat was only found in budding regions and was apparently lacking from Golgi membranes prior to incubation with cytosol (Orci et al., 1986). This suggested that the coat drives budding as it assembles on the Golgi membrane from dispersed cytosolic subunits, deforming the adjoining membrane to assume its own spherical shape.

Selective inhibitors revealed distinct stages in the maturation of these Golgi-derived coated vesicles and further established their role as carriers. Transport was blocked by nonhydrolyzable analogues of GTP, like GTP γ S (Melançon et al., 1987), and by low concentrations of the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) (Glick & Rothman, 1987), which reacts with cysteine in proteins. Electron microscopy revealed that such treatment accumulated VSV G protein-containing 70-nm vesicles; however, the vesicles accumulating with GTP γ S were coated (Melançon et al., 1987), whereas those accumulating after NEM treatment were not coated (Malhotra et al., 1988). Combined inhibition with both NEM and GTP γ S accumulated exclusively coated vesicles (Orci et al., 1989). Thus, the GTP γ S block precedes the NEM block, implying that coated vesicles become uncoated after budding.

This suggested that transport across the Golgi is due to these coated vesicles, which were termed COP (for Coat Protein)-coated vesicles, but are now referred to as COPI-coated vesicles. The coat would have to be removed after budding to allow fusion of the enclosed vesicle. The simplest explanation of the simultaneous inhibition of transport and accumulation of coated vesicles with GTP γ S is that uncoating is driven by hydrolysis of GTP. Because the uncoated vesicles accumulate bound to Golgi cisternae, there must be an NEM-sensitive protein required for their consumption by membrane fusion. Identification of the targets for GTP γ S and NEM inhibition has indeed led directly to the heart of the mechanisms for budding and fusion, as will now be described.

Coatomer and ARF

The nature of the protein coat was established following the purification of Golgi-derived vesicles accumulated with GTP γ S (Malhotra et al., 1989; Serafini et al., 1991b). Eight polypeptides are known to compose the coat. One subunit is ARF (Kahn & Gilman, 1986), a GTP-binding protein that is found mainly as a monomer in cytosol. The other seven coat proteins (termed COPs; α -COP, β -COP, γ -COP, etc.) are associated with each other in an equimolar complex (Waters et al., 1991) termed “coatomer” (for coat protomer because this unit contributes most of the mass to the coat). The coatomer, like ARF, is mainly found in the cytosol, with only traces present in starting Golgi membrane fractions. ARF and coatomer therefore co-assemble on the Golgi surface from the cytosol to bud vesicles; their later release constitutes uncoating. Fusion-competent coated vesicles can be formed from Golgi membranes with only coatomer, ARF, and nucleotides (Orci et al., 1993a; Ostermann et al., 1993).

ARF, short for ADP Ribosylation Factor, was first described as a cofactor required for ADP-ribosylation of a trimeric G protein α -chain by cholera toxin (Kahn & Gilman, 1986). Clearly, cells do not have ARF for this purpose. The finding that ARF is a stoichiometric component in COP-coated vesicles (Serafini et al., 1991a) suggested that ARF functions in vesicle budding

and pinpointed ARF as the likely target for inhibition of transport by GTP γ S.

Steps in vesicle budding

Indeed, the finding of ARF protein in the coat (Serafini et al., 1991a) provided the key to understanding budding, because with this and the physicochemical properties of this small G protein, a simple mechanism by which ARF could trigger budding and uncoating via GTP binding and hydrolysis suggested itself. ARF protein is N-myristylated (Kahn et al., 1988) and is water-soluble in its GDP-bound (cytosolic) forms; in its GTP-bound form, however, ARF inserts into lipid bilayers in a myristic acid-dependent manner (Kahn et al., 1991). In other words, the GDP–GTP conformational “switch” (characteristic of all “G” proteins) appears to be used by ARF (at least in part) to control the exposure of its covalently attached fatty acid, and thus to modulate membrane insertion.

Specifically, we proposed (Serafini et al., 1991a) that ARF is activated for budding as cytosolic ARF[GDP] undergoes GTP–GDP exchange at the Golgi surface, resulting in Golgi-bound ARF[GTP], which then recruits coatamer from the cytosol to assemble the bud. Later hydrolysis of the bound GTP would release ARF and coatamer, corresponding to uncoating. According to this model, loading ARF with GTP γ S would prevent uncoating and therefore accumulate coated vesicles and block transport, as observed.

Recent studies (Fig. 1) have clearly established the essential correctness of this simple model. ARF binds to membranes in the absence of coatamer (Donaldson et al., 1992a; Palmer et al., 1993), indicating that ARF binds first in the budding pathway. Binding of soluble N-myristylated-ARF[GDP] requires GTP and involves nucleotide exchange catalyzed by an enzyme activity associated with Golgi membranes and which is inhibited by the anti-secretion drug Brefeldin A (Donaldson et al., 1992b; Helms & Rothman, 1992). GTP from the incubation medium

replaces the previously bound GDP in the membrane-bound population of ARF. The myristic acid group is required; non-myristylated ARF undergoes nucleotide exchange but does not remain bound to the membrane.

Binding of coatamer to Golgi membranes requires bound ARF protein (Donaldson et al., 1992a; Palmer et al., 1993). The amount of coatamer bound at saturation depends on the amount of ARF bound according to a stoichiometric relationship (Donaldson et al., 1992a; Palmer et al., 1993). These stoichiometric complexes consist of coated buds (Ostermann et al., 1993), virtually completely coated vesicles that are, however, not yet pinched off at their base. When only ARF is bound, cisternal membranes remain flat (Orci et al., 1993a). It is only when coatamer is added that buds form (Orci et al., 1993b). Most likely, the buds form as coatamer–ARF complexes self-assemble in a coatamer-dependent reaction to form the coat. This process resembles self-assembly in that only the coat proteins themselves are required from the cytosol.

Evidently, it is coatamer binding and assembly that drives budding. The role of ARF in budding is to initiate budding by providing stoichiometric binding sites for coatamer. Once the vesicle pinches off, the coat must be removed to enable contact with the target membrane for fusion. That hydrolysis of GTP bound to ARF is required for uncoating was established by employing a mutant version of ARF protein that binds but does not effectively hydrolyze GTP (Tanigawa et al., 1993). This GTPase mutant, just like GTP γ S, inhibits transport with concomitant accumulation of coated transport vesicles. Although the ARF mutant is competent for budding, the resulting coated vesicles cannot be uncoated, and are thus incompetent for fusion with acceptor Golgi (Ostermann et al., 1993), confirming that uncoating is a prerequisite for fusion. Uncoating can therefore be understood as a simple reversal of the pathway of coat assembly: Binding of coatamer to membranes is ARF[GTP] dependent; when ARF hydrolyzes its bound GTP, the myristic acid will be “retracted,” ARF will dissociate, and coatamer will follow suit.

Fig. 1. Pathway by which a COP coat assembles on a Golgi cisterna to yield a transport vesicle. This reaction can be reproduced with ARF, coatamer, and Golgi membranes in the presence of GTP and fatty acyl-Coenzyme A, the latter required for vesicles to pinch off (Ostermann et al., 1993). BFA, Brefeldin A.

Altogether, four different kinds of coats have so far been characterized in molecular terms, with various degrees of insight into their budding mechanisms. They all employ coat proteins whose assembly is triggered by ARF (or a close relative) in its GTP-bound form, and are for the most part used at distinct regions of the cell. The previously described coatomer-ARF coat (now renamed the COPI coat) is intrinsically unstable and disassembles as soon as ARF dissociates. It is not yet known whether all of these COPI-coated vesicles have precisely identical coatomers, so it is possible that different subtypes mediate different transport steps. A clathrin-containing coat, historically the first to be discovered (Pearse, 1976), surrounds endocytic vesicles (Goldstein et al., 1979) budding from the plasma membrane and contains clathrin and another complex of proteins termed AP-2 (Ahle et al., 1988; Robinson, 1992). These clathrin-coated vesicles contain little or no ARF when isolated, even though ARF may well be required to assemble the coat (Zhang et al., 1994). In contrast to the COPI coat, this clathrin coat is intrinsically stable; thus, an uncoating enzyme system (most likely including the cytoplasmic constitutively expressed hsp70 ATPase) is needed to remove the remaining coat proteins (Rothman & Schmid, 1986). The third coat contains clathrin together with a distinct "adaptor" (Pearse & Robinson, 1990) complex (AP-1) from that of endocytic vesicles, and assembles at the *trans*-most Golgi cisterna to carry lysosomal precursors. ARF is required to assemble the AP-1 clathrin coat on Golgi membranes (Stamnes & Rothman, 1993; Traub et al., 1993). The fourth coat, COPII, buds vesicles from ER that are targeted to Golgi, and represents an intermediate case in which the coat persists but is unstable after the GTPase (in this case the ARF relative SAR1 [Nakano & Muramatsu, 1989]) dissociates (Salama et al., 1993; Yoshihisa et al., 1993; Barlowe et al., 1994). It seems likely that the catalogue of coats known to bud vesicles will have many new entries in the next few years.

In summary, budding employs a coat, most likely acting as a mechanical device that assembles at a membrane surface from dispersed cytoplasmic coat protein subunits (such as clathrin or coatomer) to deform the membrane into its own spherical shape. A small GTP-binding protein (ARF or a close relative) is the master molecule that controls coating and uncoating. Coat assembly is initiated when ARF is activated by binding GTP, and ARF is assembled into the coat bud. After the vesicle is pinched off, the coat disassembles when ARF hydrolyzes its bound GTP, making the enclosed vesicle available for fusion.

General fusion machinery: ATPase NSF and SNAPs

Understanding the machinery and mechanism of membrane fusion has posed an enduring challenge to cell biologists since the discovery of this process (Palade, 1959). A way to proceed was suggested by the selective accumulation of uncoated vesicles bound to acceptor Golgi cisternae when transport is blocked by mild NEM treatment (Malhotra et al., 1988; Orci et al., 1989). This implied that an NEM-Sensitive Fusion protein (NSF) must act in the process of fusion, defined as any and all steps that lead to content mixing following the binding of a transport vesicle to its target membrane. The finding that transport can be restored to NEM-inactivated reactions by adding back fresh cytosol (Glick & Rothman, 1987) meant that this fusion protein is cytosolic in origin and provided the functional assay used to guide the purification of NSF (Block et al., 1988).

NSF is an ATPase whose hydrolysis of ATP is necessary for vesicle fusion. It has two homologous domains, each containing an ATP binding site (Tagaya et al., 1993). Mutation of either ATP-binding site seriously compromises fusion activity and the slow intrinsic ATPase activity of NSF; mutating both sites eliminates fusion and ATPase activity (Whiteheart et al., 1994).

The sequence of NSF revealed (Wilson et al., 1989) a functionally interchangeable homologue in yeast, Sec18, previously known from genetics to be required for transport from ER to Golgi (Novick et al., 1980). This linkage provided important confirmatory evidence that the fusion pathway reconstituted in the cell-free system operates in living cells, and established that the same fundamental mechanism operates in yeast and animals—a theme of universality that has persisted ever since. NSF has now been shown to be required for fusion at many steps in transport from ER to plasma membrane and for endocytosis in animals and in yeast (Beckers et al., 1989; Diaz et al., 1989; Graham & Emr, 1991).

NSF itself does not bind to Golgi membranes, but does so when a crude cytosol fraction is added (Weidman et al., 1989). This defined the Soluble NSF Attachment Proteins (SNAPs), which were purified from cytosol both according to this assay and according to their requirement for transport at the fusion step (Clary & Rothman, 1990). Three species of SNAP were thus obtained (Clary et al., 1990). α -SNAP and γ -SNAP have been found in all cell types and tissues examined. β -SNAP has only been found in brain, and is very similar in sequence and interchangeable with α -SNAP in many assays. γ -SNAP is only weakly homologous to α/β -SNAPs (Whiteheart et al., 1993).

How do NSF and SNAP interact with each other and with target membranes? Although NSF will not bind Golgi membranes in the absence of SNAPs, SNAP proteins will bind to membranes in the absence of NSF, implying that SNAPs bind first and NSF binds second (Whiteheart et al., 1992). This binding site operationally defined a SNAP Receptor (abbreviated SNARE). SNAP proteins do not discernibly interact with NSF in solution, but once themselves bound to SNARE sites, now allow saturable binding of stoichiometric amounts of NSF to Golgi membranes. Upon binding to SNAP-SNAP receptor complexes, NSF hydrolyzes ATP and releases itself (Wilson et al., 1992). The complex of NSF, α -SNAP, γ -SNAP, and the SNAP receptor sites can be extracted intact from Golgi membranes with nonionic detergent. This "fusion particle" sediments at 20S, and has been suggested to form the core of a general "fusion machine" (Malhotra et al., 1988; Clary et al., 1990; Wilson et al., 1992). SNAREs (like NSF and SNAP) are expected to be important for fusion because of their presence in this particle. How this particle and cytoplasmically derived proteins like the ATPase NSF and SNAP may participate in fusion will be discussed later.

In summary, the ATPase NSF and SNAP are general components of an intracellular membrane fusion machinery. Following specific vesicle docking, NSF and SNAP assemble to initiate fusion according to a stereotyped, compartment-independent mechanism that is powered by the hydrolysis of ATP.

Vesicle docking by SNAREs

Whatever the precise role of NSF and SNAPs in fusion, such general and cytoplasmically derived machinery cannot provide

the needed specificity; maintaining the identity of membrane-bound compartments in the face of a massive flux among them indeed requires a fusion mechanism of exquisite specificity. For example, a vesicle coming from the ER must fuse with the *cis* face of the Golgi stack, rather than a lysosome or a mitochondrion, and so on; otherwise compartmentation will be lost within minutes. Certainly, a general fusion machinery can be called into play without loss of specificity provided that the transport vesicle has already made the correct choice of target, but how is this specific docking achieved? Do already-linked docking proteins from vesicle and target membrane come together to create the scaffold on which NSF and SNAPs can assemble to form a general fusion machine?

Because NSF and SNAP must be able to bind to many different intracellular membranes to promote fusion at each of many locations, the SNAREs must comprise a family of fusion proteins, each member being sufficiently distinct so as to allow residence in a distinct location, yet, sufficiently similar to allow SNAP and NSF to assemble on it. Given *this* need for a family of compartmentally specific SNAREs, together with the requirement that the general fusion machinery should only be activated after specific vesicle docking, SNAREs might be expected to provide an important interface between the processes of vesicle docking and fusion. The simplest possibility would be for SNAREs (the abbreviation also connotes a device for capturing small game) to do double duty by participating in fusion (minimally by anchoring SNAPs and NSF) and also in specific vesicle docking.

The likely basis for targeting specificity became apparent when the identity of SNAREs was revealed after they were purified (Söllner et al., 1993b). A total particulate fraction of brain was chosen as a source because of the extreme specialization of this tissue for membrane fusion in the context of neurotransmission at synapses: it has been estimated that the synaptic vesicles, containing neurotransmitters, account for more than 7% of the total protein of brain (Südhof & Jahn, 1991). The genes encoding most major proteins of synaptic vesicles, and many of the proteins of the pre-synaptic plasma membrane with which these vesicles are programmed to fuse, have been cloned and sequenced and their protein products localized (Südhof & Jahn, 1991; Bennett & Scheller, 1994), but the functions of these many proteins are naturally largely unknown given the means of their description. This wealth of structural information is the result of the intensive focus of molecular neurobiologists on the central process of synaptic transmission, and had been waiting for functional biochemistry.

An affinity purification was employed (Söllner et al., 1993b) that took advantage of the properties and ATPase activity of NSF. SNAREs were assembled into 20S particles from a crude detergent extract of total brain membranes by adding recombinant NSF and SNAPs in the presence of nonhydrolyzable ATP. Three kinds of polypeptides were thereby isolated in a stoichiometric complex with the NSF and SNAPs. Protein sequencing revealed all of them to be synaptic proteins (Fig. 2) that had been cloned and localized previously, but whose precise functions had been unknown previously for lack of functional assays or correlates.

Two of these proteins, Syntaxin (Bennett et al., 1992) and SNAP-25 (an acronym for Synaptosome-Associated Protein-25 kDa, coincidentally similar to the acronym SNAP for Soluble NSF Attachment Protein; the proteins are unrelated in sequence [Oyler et al., 1989]) are localized to the pre-synaptic plasma

Fig. 2. Localization of the three membrane proteins identified as SNAP receptors (SNAREs) in brain. VAMP (alternately termed synaptobrevin), syntaxin, and SNAP-25 (Synaptosomal Associated Protein, 25 kDa) are all located in the presynaptic nerve terminal at a synapse. VAMP is found in the synaptic vesicles that store neurotransmitter, releasing this content into the synaptic cleft following an action potential. This triggers the entry of calcium into the nerve ending, which is the immediate signal for membrane fusion. Syntaxin and SNAP-25 are located in the presynaptic plasma membrane. VAMP and syntaxin have most of their mass (including their amino termini) in the cytoplasm, whereas SNAP-25 is entirely cytoplasmic, anchored to the plasma membrane by fatty acid chains.

membrane. The other protein, VAMP (Vesicle-Associated Membrane Protein, also termed synaptobrevin), is found only in synaptic vesicles (Trimble et al., 1988; Baumert et al., 1989), and was previously implicated in transmitter release (Schiavo et al., 1992) because it was identified as the target for the proteolytic activity of tetanus and botulinum B toxins, agents that cause paralysis by blocking neurotransmitter release (Fig. 3). Evidently, the 20S fusion particle involved in synaptic vesicle exocytosis consists of NSF, SNAPs, VAMP, syntaxin, and SNAP-25 in a complex, without other stoichiometric components.

VAMP and syntaxin are both integral membrane proteins with similar topographies, consisting of an amino-terminal cytoplasmic domain, a single presumed membrane-spanning segment, and only a handful of mainly polar residues at their carboxy termini. This topography seems ideally suited for docking. Although SNAP-25 is intrinsically a water-soluble protein, it is anchored to the presynaptic plasma membrane, having the solubility properties of an integral membrane protein. SNAP-25 is fatty-acylated, most likely explaining its membrane association; its four cysteine residues are closely clustered in the sequence and are the likely acylation sites (Hess et al., 1992).

The finding that 20S fusion particles contain both a membrane protein derived from the synaptic vesicle (VAMP) and two proteins derived from the pre-synaptic membrane (syntaxin and SNAP-25) implied that the synaptic vesicle must dock when this complex (Fig. 3) forms (Söllner et al., 1993b). These particular proteins (VAMP, syntaxin, and SNAP-25) are limited in their distribution to synapses and certain related tissues; yet, SNAP and NSF presumably interact with SNAREs in many subcellular membranes in a standard fashion. Therefore, we proposed (Söllner et al., 1993b) that each membrane involved in an NSF-dependent fusion process must have homologues of these synaptic proteins that would be similarly involved with SNAP and

Fig. 3. Diagram of the 20S docking and fusion particle containing VAMP, syntaxin, SNAP-25, NSF, and SNAP proteins (the latter indicated by the black disk). VAMP, syntaxin, and SNAP-25 have each been identified as select targets for various neurotoxins causing botulism and tetanus (Huttner, 1993). These toxins cleave the indicated target protein and block release of neurotransmitter, establishing the requirement for these subunits of the docking and fusion particle in synapses. That SNAP and NSF are also required for transmission *in vivo* has also been confirmed (DeBello et al., 1995; Pallanack et al., 1995).

NSF in docking and fusion. Because NSF and SNAP cannot contribute the needed specificity to this docking mechanism, we were then obliged to propose (Söllner et al., 1993b) that the specificity in this complex would reside in the partnering of compartment-specific cognate transport vesicle-localized and target membrane-localized SNAREs.

This constitutes the SNARE hypothesis for vesicle targeting (Fig. 4), which proposes that each kind of transport vesicle has as its own unique address marker one or more v-SNAREs (generally but not necessarily VAMP homologues) obtained from its parental membrane during budding. Further, each target membrane is proposed to be endowed with one or more t-SNAREs (generally consisting of a syntaxin homologue, or a SNAP-25 homologue, or a complex of the two). Specificity in targeting

Fig. 4. Each transport vesicle is endowed with one or more unique v-SNAREs (generally related to VAMP), which pair with cognate t-SNAREs (generally related to syntaxin and/or SNAP-25), thereby docking the vesicle to the correct target. Other proteins may be involved in regulating the assembly of this core docking complex, which then serves as a scaffold for assembly of the general fusion machinery yielding a 20S docking and fusion particle.

would be achieved by pairwise binding of v-SNAREs with matching t-SNAREs.

The SNARE hypothesis made many clear-cut and testable predictions (Söllner et al., 1993b), all of which have now been confirmed. Eukaryotic cells would be predicted to have a family of v-SNAREs and a family of t-SNAREs. These proteins would be expected to fall into cognate pairs on the basis of binding specificity, and should be essential for vesicle docking *in vivo*. The hypothesis also requires that the overall program of vesicle traffic be pre-ordained by the intracellular localization pattern of cognate v-SNAREs and t-SNAREs (Fig. 5). For example, the t-SNARE cognate to the ER-derived v-SNARE would have to be in *cis* Golgi, whereas the t-SNARE cognate to the v-SNARE of *trans*-Golgi-derived vesicles would have to be concentrated in the plasma membrane. Endocytic vesicles should harbor yet other v-SNAREs programmed to attach to t-SNAREs in endosomes. The v-SNAREs would have to be regularly included in budding coated vesicles, but t-SNAREs excluded. Cognate v-SNAREs and t-SNAREs should bind each other specifically, and should create a scaffold for assembly of SNAPs and NSF. All of these predictions have now been confirmed by biochemical, genetic, and morphological tests in animal cells, yeasts, and synapses (Aalto et al., 1992; Bennett et al., 1992; Hardwick & Pelham, 1992; Bennett & Scheller, 1993; Huttner, 1993; Lian & Ferro-Novick, 1993; McMahon et al., 1993; Protopopov et al., 1993; Söllner et al., 1993a; Calakos et al., 1994; Pevsner et al., 1994; Søgaard et al., 1994).

In summary, the pairing of v-SNAREs from transport vesicles with t-SNAREs from target membranes provides a core docking interaction, and erects a scaffold on which general fusion machinery such as NSF and SNAPs can assemble.

Fig. 5. According to the SNARE hypothesis, the road map of vesicular transport is determined by the pattern of localization of SNAREs among compartments. Cognate v-SNAREs and t-SNAREs (v's and t's having the same color) shown correspond to only a few of the many vesicle shuttles known to occur in cells.

Bilayer fusion and its regulation in exocytosis

The precise mechanism of membrane fusion remains unknown, and is perhaps the greatest challenge to protein biochemists in this field. Although it is clear from a wealth of genetic and biochemical evidence that SNAPs and NSF are required for many fusion processes, there is no proof that they act directly to fuse bilayers, or that they act alone. Such evidence can only come from reconstitution experiments, which have not yet been reported. However, available evidence is consistent with this simple possibility. It is known that when NSF hydrolyzes ATP, it dissociates, and in the process releases SNAPs from the SNARE complex and disrupts the SNARE complex, separating v-SNARE from t-SNARE (Söllner et al., 1993a). It is also known that the hydrolysis of ATP by NSF that drives these events is necessary for the fusion process (Whiteheart et al., 1994). It is easy to imagine that the conformation of one or more of the subunits of the 20S particle is switched to a fusogenic state during the NSF-ATPase-dependent disruption of the particle.

Although many intracellular vesicle fusion events utilize the same species of NSF and SNAPs, homologues may well perform their functions in other varieties of fusion, such as homotypic fusion. Homotypic fusion is a process in which one copy of a compartment fuses with another identical copy and is important for maintaining the integrity of organelles that are organized as a network, including the ER, Golgi, and endosomes, and for single-copy organelles like the nucleus (Rothman & Warren, 1994). A homologue of NSF, p97 (Peters et al., 1990), is employed in homotypic fusion of nuclear envelope, ER, and Golgi membranes, as required for budding in yeast and the reassembly of organelles after mitosis in animal cells (Acharya et al., 1995; Latterich et al., 1995; Rabouille et al., 1995). Another homologue is required for fusion of membranes within chloroplasts (Hugueney et al., 1995). It has been suggested that NSF is not required for fusion of Golgi-derived transport vesicles with apical plasma membrane (Ikonen et al., 1995). The same fusion machinery used for vesicle transport, generally SNAPs and NSF, is also employed for triggered exocytosis, as exemplified by synaptic transmission (Söllner et al., 1993b; DeBello et al., 1995; Pallanck et al., 1995), in which release of a neurotransmitter or a secreted protein or a hormone occurs only in response to a signal. The use of the same fusion mechanism for triggered exocytosis (which must be constitutively "off") and vesicle transport generally (which is constitutively "on") requires that the exocytic vesicles be "clamped" (Söllner et al., 1993a) by one or another mechanism, in order to allow such vesicles to be activated for fusion only in response to stimulation by second messengers such as calcium ion.

Much evidence suggests that the exocytic vesicle calcium-binding membrane protein synaptotagmin is the primary calcium receptor in exocytosis (De Bello et al., 1993; Elferink et al., 1993; Geppert et al., 1994; Südhof, 1995). Synaptotagmin has a special affinity for the brain-specific homologue of SNAP, β -SNAP, and can assemble with β -SNAP and NSF onto the SNARE complex to form a specialized version of the general 20S particle that has been proposed to be an obligatory intermediate in exocytosis (Schiavo et al., 1995). Exocytosis entails an ATP-dependent step (independent of calcium) that "primes" vesicles to fuse following the entry of calcium (Bittner & Holz, 1992; Hay & Martin, 1992; Thomas et al., 1993). It has been speculated that the ATP-dependent priming step encompasses the assembly of this

specialized docking and fusion particle and its ensuing disruption (Schiavo et al., 1995). As with the general 20S particle, disruption could create a fusogenically competent state; however, unlike the general (constitutive) case, this state could be "frozen" until calcium enters when produced from a particle specialized for exocytosis. Possibly, a complex of β -SNAP and synaptotagmin (Schiavo et al., 1995) is involved at this latter phase.

A large number of proteins are known including rab proteins, sec1 protein, among others (Südhof, 1995) that may regulate the core machinery of exocytosis and as well provide additional prior docking steps to improve specificity and/or allow additional regulation of exocytosis, preceding the general docking step afforded by SNAREs. Their precise roles are not yet well understood.

Summary

A coherent conceptual framework (Fig. 6) can now be provided to explain transport between membrane-bound compartments. Although many important details remain to be established, the core machinery and the outline of the mechanisms governing budding and fusion have greatly clarified:

Cytoplasmic coats act as mechanical devices to shape membranes into vesicles

The coat assembles at a membrane surface from dispersed cytoplasmic coat protein subunits and, in the process, appears to deform the membrane to assume its spherical shape. The coat must be removed before fusion can occur. Coat assembly is initiated when a small GTP-binding protein (like ARF) is activated by binding GTP; the GTP-binding protein is assembled into the coat. The coat later disassembles after the bound GTP is hydrolyzed.

Specific cognate SNAREs on vesicles and target membranes dock vesicles

Transport vesicles are tagged with address markers, termed v-SNAREs, members of a family of related integral membrane proteins, obtained during budding. When v-SNAREs are paired with cognate t-SNAREs (present in the target membrane), the vesicle is docked, prior to fusion.

A general fusion machinery then assembles at the site of vesicle docking using the SNARE complex as a scaffold

Much, if not all, of this general machinery consists of cytoplasmic proteins that are readily available to all cellular membranes, including the ATPase NSF and SNAPs. SNAPs bind to the SNARE complex, enabling NSF to bind. ATP hydrolysis by NSF is essential for fusion, separating v-SNAREs from t-SNAREs, and perhaps switching one or more of these proteins into a fusogenic conformation.

A similar protein machinery operates at multiple compartments in all cells from yeast to man

Regulation of exocytosis can be achieved by superimposing inhibitory "fusion clamps" on the general pathway of cytoplasmic

Fig. 6. Steps in a round of vesicular transport. See text for details. Acyl-CoA, fatty acyl-Coenzyme A. Periplasmic fusion, initiated on the luminal side of the bud, is needed as part of the process of pinching off (Rothman & Warren, 1994) and this step requires fatty acyl-Coenzyme A in the case of COPI-coated vesicles (Ostermann et al., 1993).

mic fusion; the clamps are removed in response to second messenger stimulation.

This protein machinery is abundant in cells and thus readily purified, and, in addition, most of these components can be made in a recombinant and functionally active form in bacteria, allowing unlimited quantities to be easily obtained. Thus, there are many ripe opportunities for protein scientists to flesh out our present outline of budding and fusion mechanisms, and we can now look forward to rapid developments and new insights at the level of protein structure and chemistry.

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