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The Road Taken: Past and Future Foundations of Membrane Traffic

Review

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The greatest scientific advance of the last 1000 years was providing the evidence to prove that human beings are independent agents whose lives on earth are neither conferred nor controlled by celestial forces. Although it may be more conventional to measure scientific progress in terms of specific technological developments, nothing was more important than providing the means to release men and women from the hegemony of the supernatural.

Establishing human biological autonomy has been slow and is by no means completed. It began with advances in the physical sciences, notably the work of Copernicus and Galileo, who helped to establish that the earth, and thus humankind, did not occupy a unique or privileged position at the center of the universe. Despite the obvious difference in scale, as important were the contributions of the life sciences. Cell biology in particular provided the incontrovertible proof that humans as well as all other living beings consist of individual cells (utilizing the same genetic code) whose activities, inheritance, and ability to assemble into organisms can be understood in logical, if not always straightforward, biochemical terms. This fact demonstrated that however miraculous the existence of life on earth might be, it is not entirely mysterious. Life's mechanisms can be understood, regardless of whether one invokes spiritual or quantum mechanical reasons for its existence. As we stand at the end of the millennium, we probably understand, albeit superficially, nearly all of the basic principles that govern life, death, cognition, and reproduction. We are not yet at the Golden Age predicted some years ago by Gunther Stent (Stent, 1969), but it seems increasingly likely that the study of cells as integrated, functional units will be the vehicle that will finally bring us to a complete understanding of our physical existence.

Cell biology can be divided into a number of branches, which, with every advance, are becoming progressively intertwined. The study of biological membranes is the branch most responsible for this confluence, largely because it is a cell's system of membranes that provides the very boundaries within which life exists. A membrane separates the panoply of biochemical reactions that define a living cell from the extracellular world. Within the cell, membranes also organize and separate these biochemical reactions from each other, generating the compositionally and morphologically distinct compartments characteristic of eukaryotes. Transfer of material between many of these compartments (in particular, secretory or endocytic compartments) occurs by means of small, membrane-bound vesicles. Although vesicular traffic is extensive, it does not compromise the identity of the compartments involved. Arriving at a general understanding of how these events occur has been a major advance of the past half century. Accordingly, it is appropriate to begin with an appreciation of the intellectual foundations on which our current state of understanding rests.

Foundations from the Recent Past

Biology, particularly cell biology, is a quintessentially group effort, with each new concept growing from insights and experimental data contributed by many individual laboratories. Perhaps the reason for this is that single "definitive experiments" are exceedingly rare in cell biology. Progress has been achieved in a graded fashion including influences from ideas and data, which, ironically, may have later proved to be incorrect. It would thus be difficult to enumerate the many individuals responsible for bringing the field to its current state of sophistication. Nevertheless, acknowledging a few people and events is essential to illustrating some key observations and discoveries.

Prescient Early Observations

The study of membrane traffic dates back to a time before it was clear that membranes even existed. Undoubtedly the most widely known, if not necessarily the most important, observations were provided by the great Italian histologist Camillo Golgi. Golgi developed his silver nitrate–based cytochemical stains to explore the organization of the central nervous system but, in addition, revealed that neurons contain a distinctive internal reticular structure, which has borne his name ever since (Golgi, 1898). Though the existence of this structure in all eukaryotic cells was hotly debated for the next half century (Bentivoglio, 1998), the "Golgi apparatus" was in fact the first organelle of the secretory or endocytic pathways to be identified. Ironically, Golgi's main conclusion from his studies, namely that the brain consisted of a continuous syncitial network, turned out to be less influential in the long run (Henry, 1998).

Another critical if less widely appreciated observation of this era was provided by Elie Metchnikoff (Metchnikoff, 1887). Born in 1845, just two years after Golgi (he also won his Nobel Prize two years after Golgi), Metchnikoff's discovery of cellular immunity provided a similar cell biological by-product. Whereas the Golgi apparatus was an object without a clear function, the ability of individual cells to internalize extracellular particles by "phagocytosis" suggested a functional intracellular digestive tract. This suggestion was highlighted by the demonstration that ingested particles (e.g., blue litmus) were exposed to acidic pH and presumably degraded following uptake. Metchnikoff provided the first demonstration that cells have internal specializations that carry out specific functions.

Founding the Modern Era

By the middle of the twentieth century, the advent of electron microscopy (EM) combined with the development of fixatives (e.g., glutaraldehyde [Sabatini et al., 1963]) that permitted the preservation of biological

membranes marked the beginning of the modern era of cell biology. The impact was particularly great on the study of membrane traffic, as cells were found to contain an abundance of membrane-bound organelles that soon became associated with the processes of secretion and endocytosis. A great many groups contributed to this effort, but the ones that obviously must be singled out were headed by George E. Palade and Christian de Duve, who made the critical connection between the existence of these structures and individual cell functions (de Duve, 1975; Palade, 1975). This connection was aided by the development of cell fractionation and of assays to measure the enzymatic activities associated with subcellular fractions. Palade and de Duve used this approach, combined with EM, to prove the existence in eukaryotic cells of physically distinct organelles that each performed distinct, essential functions. This approach also provided the conceptual and experimental foundation on which virtually every advance in cell biology for the next 50 years was based.

Electron Microscopy and Cell Fractionation. It was at this point that the field of membrane traffic was developed, largely due to the efforts of Palade and the impressive "school" of cell biologists he spawned either directly or indirectly. One of the most important experiments early in this period resulted in the functional elucidation of the secretory pathway: that secretory proteins are synthesized in the endoplasmic reticulum (ER), pass through the Golgi complex, and then are packaged into granules for exocytosis at the plasma membrane. The syllogism of ER to Golgi to plasma membrane became cell biology's equivalent of molecular biology's DNA to RNA to protein (both are also not always true!).

The secretory pathway's logic was best illustrated by the acinar cell of the exocrine pancreas, among the most professional secretory cells known. Palade and James D. Jamieson made use of the newly developed technique of EM autoradiography, in which newly synthesized secretory proteins were labeled by a pulse of radioactive amino acids and, after various chase periods, detected on EM sections overlaid with a photographic emulsion. Together with biochemical fractionation, these studies definitively demonstrated the initial appearance of secretory proteins over the ER, their transient association with elements of the Golgi complex, their concentration in post-Golgi secretory granules, and their secretagogue-stimulated release from the cell by granule fusion with the plasma membrane (Figure 1). There have been many embellishments of this scenario over the years, but these basic features remain the secretory pathway's most significant elements.

Implicit in the elucidation of these events was another fundamental principle of membrane traffic: namely, that transport of secretory proteins between these distinct organelle compartments occurs via vesicular carriers (Palade, 1975). In other words, transfer from the ER to the Golgi requires the formation of a transport vesicle by a budding event at the "donor" organelle (ER) and the subsequent fusion of the carrier at the "acceptor" organelle (the Golgi). The importance of this concept cannot be overestimated and is probably the single most important concept underlying the modern understanding of membrane traffic. However, it also created a vexing paradox that has occupied the field ever since.

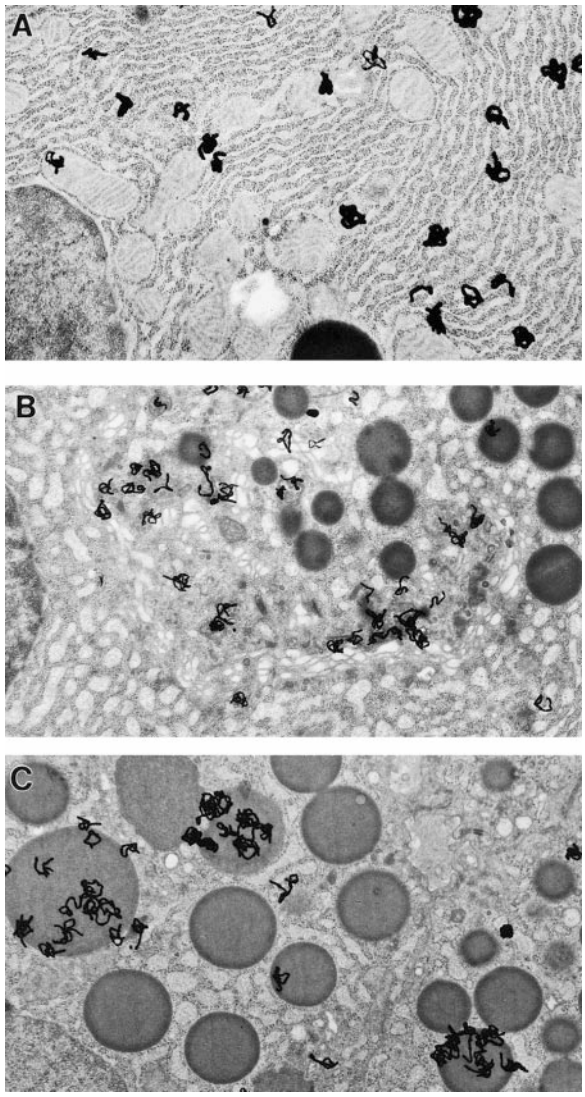

Figure 1. Exocytic Transport in Pancreatic Acinar Cells

Pancreatic slices were briefly pulsed with ^{3}H leucine, then the label was chased for 0 (A), 7 (B), and 80 (C) min before fixation and preparation for EM autoradiography. The autoradiographic grains representing newly synthesized secretory proteins were located first over the ER (A), then over the Golgi region (B), and finally over the secretory/zymogen granules (C). Data are from Jamieson and Palade (1967; 1971). All micrographs are X6,400.

Fluidity, Topology, and Sorting. Concomitant with the explication of the secretory pathway, the nature and properties of biological membranes were also becoming apparent. It was originally thought, based on EM images, that a membrane was a "protein-lipid-protein sandwich." However, the efforts of many groups defined another critical insight: that membranes are lipid bilayers (Gorter and Grendel, 1925; Engelman, 1971) in which proteins exhibit considerable two-dimensional fluidity (Frye and Edidin, 1970). Lipids and transmembrane proteins are generally free to diffuse laterally within the plane of the bilayer, but due to unfavorable energetic considerations, proteins (and many lipids) could not "flip-flop" across the bilayer (Bretscher and Raff, 1975).

Thus, when two membranes fuse, their sidedness must be maintained: proteins facing the luminal side of an internal organelle or transport vesicle will remain facing the luminal side after budding or fusion. Indeed, the luminal surface of all vesicles and organelles of the secretory (and endocytic) pathway are topologically equivalent to the extracellular environment. Despite the fact that it is now clear that many cell types can organize their membranes into stable or dynamic microdomains, this general view of membranes as a "fluid mosaic" of conserved topology (Singer and Nicolson, 1972) remains a foundation of our understanding.

However, this view created a problem for rationalizing vesicular transport. Donor and acceptor organelles typically have biochemically distinct membrane compositions. As a result, membrane traffic between them would appear to be an invitation to randomness, an invitation that clearly cannot be accepted. At the conceptual level, this problem is solved by invoking two critical principles, deciphering the mechanisms of which represents a major focus of current effort.

First, there is the principle of "molecular sorting," the idea that membrane components are either selectively included within or excluded from nascent transport vesicles. Thus, only those components intended for forward transport need to be sorted from the donor's resident components and removed from the donor organelle. Sorting can occur either by allowing a transported component to interact with one of several known cytoplasmic coat components, by retaining a resident component due to interactions with an intraorganelle or cytoplasmic matrix, or by salvaging those few organellar proteins that inadvertently leave.

Second, there is the principle of "vesicle targeting." Vesicles emanating from a donor organelle were long predicted to bear address tags that permit them to interact and fuse with only the appropriate acceptor compartment. These tags are now known to include the organelle-specific family of SNARE proteins and ras-like GTPases of the Rab protein family. Together with proteins that tether vesicles to target membranes, these components help form target-specific protein complexes, which allow for vesicle acceptor compartment recognition and subsequent fusion.

Translocation across Membranes. Since protein synthesis (except the protein synthesis that occurs within organelles such as mitochondria and chloroplasts) occurs in the cytosol, it was clear from the outset that secretory and membrane proteins synthesized on cytoplasmic ribosomes somehow gained access to the ER. The mechanism was revealed by experiments showing that all such proteins contain distinctive "signal sequences" that program the energetically unfavorable process of protein translocation across the ER membrane (Blobel and Dobberstein, 1975a, 1975b; Blobel, 1980). Translocation of proteins into the ER of animal cells occurs concomitantly with translation and involves the attachment of polysomes producing a signal sequence–containing protein to the "rough" (or ribosome-studded) regions of the ER. Among the legions of cell biologists who have contributed to this fundamental concept, Günter Blobel—perhaps not surprisingly a direct product of the Palade School—has provided the longest and most influential stream of experimental insight, a view also held by the 1999 Nobel Prize committee.

The concept of signal-directed translocation has turned out to be far more robust, flexible, and applicable to a wider range of issues than originally thought. It occurs during secretion in yeast and even in bacteria in ways that are superficially distinct (in the sense that translocation can occur posttranslationally in these organisms) but in fact are remarkably similar in intent and mechanism (Lyman and Schekman, 1996; Duong et al., 1997). Mitochondria, peroxisomes, and chloroplasts also import nuclear-encoded proteins produced in the cytoplasms of their ancestral hosts, again in a posttranslational fashion (Neupert, 1997; Subramani, 1998; Koehler et al., 1999; May and Soll, 1999). Although a decidedly different translocation mechanism is used by these nonvacuolar organelles, the basic logic involving the use of signal sequences to specify entry is preserved. Import (and export) of proteins and nucleic acids into the nucleus uses yet another variation on this theme (Gorlich and Laskey, 1995).

In addition to defining a process of fundamental importance, work on translocation had an equivalently strong influence on the development of methods to study membrane traffic. Experiments in which the insertion of proteins into the ER and import into mitochondria were reconstituted represented the first true in vitro reconstitutions of complex activity related to membrane traffic. Such "in vitro assays" facilitated the stepwise dissection and identification of important protein components involved in these processes, components whose physiological relevance was confirmed by subsequent genetic analysis of the same processes in yeast (Novick et al., 1980). Today, in vitro reconstitution is among the most important and widely used strategies in studying membrane traffic. Now routinely combined with morphological, genetic, and molecular biological approaches, in vitro assays are being applied to increasingly complex problems and, as described below, are allowing further understanding, at the biochemical level, of processes such as membrane fusion, vesicle formation, organelle biogenesis, and protein sorting.

Endocytosis and Molecular Sorting. Although endocytosis was the first form of membrane traffic to be appreciated, it did not emerge as a central topic in cell biology until it was suggested as a pathway by which secretory vesicle components were recovered (or "recycled") following their insertion into the plasma membrane during exocytosis (Heuser and Reese, 1973). It was the study of endocytosis in nonsecretory cells, however, that established the principle of recycling during membrane traffic. The first indication that membrane components are continuously reutilized for vesicular transport can be traced to the quantitative EM investigations of Zanvil Cohn and Ralph Steinman who showed that, every hour, tissue culture cells internalized amounts of plasma membrane that greatly exceeded their biosynthetic capacity (Steinman et al., 1976). Thus, endocytic vesicle components must be recycled back to the plasma membrane for reuse. This was in contrast to the extracellular material internalized as vesicle content, the bulk of which was accumulated intracellularly in lysosomes and degraded.

This concept was reinforced and greatly extended by the work of Joseph Goldstein and Michael Brown on the low-density lipoprotein (LDL) receptor (Goldstein et al., 1979). Biochemical techniques and EM were used to analyze defects in LDL uptake or processing exhibited by cells from patients with familial hypercholesterolemia. Not only did this work elucidate the cell biological basis for a major human genetic disorder, but also contributed four basic precepts of membrane traffic: that receptors exist to mediate the intercompartmental transport of specific ligands, that these receptors can be reutilized many times (i.e., recycled), that exposure to acidic pH was a basic mechanism used to dissociate ligand–receptor complexes upon arrival at the appointed destinations, and that receptors (and presumably other membrane proteins) can be selected for specific inclusion in nascent transport vesicles due to the interaction of cytoplasmic tail targeting sequences with cytosolic adaptors.

Selectivity in transport involves a unique, tyrosine-containing tetrapeptide sequence that permits the LDL receptor (and many others) to concentrate up to 100-fold at characteristic invaginations of the plasma membrane whose cytoplasmic surfaces were coated with the hexagonal-pentagonal arrays of the protein clathrin (Goldstein et al., 1979; Heuser and Evans, 1980). These coated pits pinched off to form endocytic coated vesicles, confirming genetically Palade's original predictions concerning the role of such vesicles in mediating macromolecular transport in cells.

Clathrin and clathrin-coated vesicles had already been identified in oocytes (Roth and Porter, 1964) and later in neurons (Heuser and Reese, 1973). These "vesicles in baskets" (Kanaseki and Kadota, 1969) were presumed to take up nutrients and to recover synaptic vesicle membrane following exocytosis during synaptic transmission. By the mid-1970's, Barbara Pearse had characterized their coat components (Pearse, 1975) and in the process provided the first biochemical characterization of a transport vesicle. This was a contribution that qualifies as a "foundation" since it set the standard for work on other transport steps and predated them by over a decade. This work also helped to initiate the incorporation of neurobiology into the study of membrane traffic, an addition that has had a profound effect on both fields.

The cell biological analysis of familial hypercholesterolemia was perhaps even more important for the enormous impact it had in shifting the intellectual tradition of cell biology. Together with similar studies on human lysosomal storage diseases (which revealed the critical role of mannose-6-phosphate receptors in targeting acid hydrolases from the Golgi to lysosomes) (Kornfeld and Mellman, 1989), this work completed the addition of genetics into the zeitgeist of mainstream cell biology. It is this change that, more than anything else, marks the transition from the era in which the field was founded to the present and to the future "post-Palade" periods in which genetics, genomics, and molecular biology will dominate the landscape.

The Advent of Molecular Cell Biology

Just as the previous generation of cell biologists presided over the origin of membrane traffic as a field, the current generation has been responsible for leading the field in a direction where understanding intracellular membrane transport at the molecular and biochemical level has become the predominant consideration. This transition, which began in ~1980, was characterized by the use of cell culture systems, molecular cloning, enveloped viruses, yeast genetics, and in vitro reconstitution of complex transport events. Although these approaches, at least initially, replaced mammalian tissues as the preferred mode of analysis, they did not supplant the reliance on subcellular fractionation and morphological analysis but rather were added to it. Moreover, these "traditional" strategies evolved in important ways, including the development of immuno-EM of ultrathin cryosections using colloidal gold-coupled protein A and the development of antibody probes as markers for intracellular compartments (Slot and Geuze, 1983). Together, these changes marked the beginning of molecular cell biology, a paradigm shift that affected all aspects of cell biology, particularly of membrane traffic. At the start of the new millennium, we find that most of the major problems identified during the past century, if not solved, at least have logical, biochemically defined frameworks. We now believe we know the fundamental principles underlying how individual membrane components are selectively transferred between organelles by vesicular transport, and how intercompartmental traffic of vesicles occurs without compromising the integrity of the participant organelles.

Mechanisms of Vesicle Targeting and Membrane Fusion

Perhaps the most fundamental aspect of membrane traffic relates to how vesicular carriers identify and fuse with their intended targets. Given the vast array of membrane systems within eukaryotic cells, understanding the specificity of fusion events is critical to understanding membrane traffic and is a difficult challenge. It was long presumed that vesicle–membrane targeting events are controlled by specific interactions of cognate receptor proteins at the cytoplasmic faces of interacting membranes (Palade, 1975). Although critical issues remain to be solved, a remarkable synthesis has been achieved based on the convergence of three distinct lines of investigation dating back some 20 years.

Reconstitution of Membrane Fusion In Vitro. The first of these story lines begins with the application of enveloped animal viruses to the study of membrane transport (Lodish et al., 1981; Simons, 1993). Although professional secretory cells, such as the pancreatic acinar cell, served well for initial descriptions of the secretory pathway, tissues did not lend themselves to the types of manipulations that would be needed to solve questions at the molecular level. Enveloped animal viruses such as vesicular stomatitis virus (VSV), Semliki Forest virus, and influenza virus turn almost any tissue culture cell into a factory committed to the synthesis of viral proteins. Since these viruses express membrane proteins that must be transported to the plasma membrane to permit budding of progeny virions, the infected cells became professional secretory cells for viral envelope glycoproteins.

The ability to provide a synchronous pulse of a single type of membrane protein allowed detailed kinetic descriptions of transit through secretory organelles and

correlation of their localization (by immuno-EM or cell fractionation) relative to glycosylation state. As a result, transit through the stacked cisternae of the Golgi complex was confirmed to have a distinct polarity, with entry of membrane (and secretory) proteins exported from the ER at the "*cis*" face and exit at the "*trans*" face (Bergmann et al., 1981). Moreover, two previously unappreciated Golgi compartments were identified. One was the *trans*-Golgi network (TGN), a system of tubules emanating from the *trans*-most Golgi cisterna (Griffiths and Simons, 1986). The TGN proved to be the exit site of completed glycoproteins from the stack. The other was a similar array of tubules identified at the *cis*-face and whose function we will consider later. Though controversial, these data suggested that intercisternal transport occurred via the formation and transit of vesicular carriers from one to the next.

How did all of this help us understand the mechanism of vesicle targeting and fusion? By designating biochemical signposts for different stages of the secretory pathway, James Rothman and colleagues were able to develop conditions that reconstituted intercisternal transport in vitro (Fries and Rothman, 1980). Early Golgi or ER-derived ("donor") vesicles from VSV-infected CHO (Chinese hamster ovary) cells (deficient in oligosaccharide processing) were incubated together with ("acceptor") Golgi membranes from wild-type cells. Upon addition of ATP and cytosolic components, the immature G protein in the donor vesicles was found to acquire complex oligosaccharides indicative of Golgi processing. This event could only have occurred after fusion with the wild-type acceptor membranes that contained the necessary glycosyltransferases. Hence, at a minimum, the assay reconstituted the fusion of Golgi-derived membranes.

The reason that the Golgi transport/fusion assay was so important was that it provided the means to identify, for the first time, protein components required for vesicle recognition and fusion (Block et al., 1988). Since the assay was sensitive to the alkylating agent NEM (N-ethylmaleimide), it was possible to isolate a cytosol-derived NEM-sensitive factor or "NSF," an ATPase that played an essential role in fusion. Several more essential cytosolic proteins were also isolated in this way, such as soluble NSF attachment proteins or "SNAPs" (Clary et al., 1990). A variation of this approach also enabled the identification of the NSF-SNAP receptors, a family of proteins (termed "SNAREs") (Söllner et al., 1993) whose significance was highlighted by the cell biological analysis of neurons.

Membrane Proteins Required for Synaptic Vesicle Fusion. For years, a cadre of neurobiologists labored to apply the techniques of cell biology to the nervous system. One very fruitful area of investigation involved the characterization of synaptic vesicles (SVs). Despite the brain's cellular complexity, SVs are common to virtually all neurons and, importantly, lend themselves spectacularly well to isolation by cell fractionation. The highly purified fractions were subjected to painstaking biochemical analysis, an effort that yielded an impressive catalog of proteins whose functions, however, remained unknown (Fernandez-Chacon and Sudhof, 1999). This intense devotion was motivated by the knowledge that SVs represent a profoundly important element of synaptic transmission. As it turned out, the analysis of these proteins provided fundamental insight into the mechanism of membrane fusion in neuronal and nonneuronal cells alike (Ferro-Novick and Jahn, 1994). Arguably, this work also provided one of the most tangible advances in neurobiology of the past decade.

The critical finding was that in detergent solution, a characteristic class of SV membrane protein (VAMP/synaptobrevin) (Trimble et al., 1988; Baumert et al., 1989) formed large complexes with a related presynaptic membrane protein (syntaxin; Bennett et al., 1992) and, importantly, NSF and SNAPs (Söllner et al., 1993). VAMP/synaptobrevins and syntaxins were thus identified as SNAP and NSF attachment receptors, or "SNAREs," a finding that linked them functionally to the activities of NSF and SNAP as defined in the in vitro Golgi fusion assay. Given the localization of VAMP/synaptobrevin to SVs (vesicles) and syntaxin to the presynaptic plasma membrane (target), it became common to distinguish between two types of SNARES, v-SNAREs and t-SNAREs.

The functional link between SNAREs and membrane fusion was extended by the discovery that the SNAREs themselves are substrates for cleavage by potent neurotoxins such as botulinim toxin, which block SV exocytosis (Montecucco and Schiavo, 1995). Since related members of the SNARE family were found in nonneuronal cells and localized to specific subcellular compartments on both the secretory and endocytic pathways, it seemed likely that SNAREs, NSF, and SNAPs played a general role in vesicle recognition or fusion (Rothman, 1994). This interpretation has received direct support from reconstitution studies in which SNARE-containing liposomes were made to fuse in vitro (Weber et al., 1998). Structural analysis has revealed that v-/t-SNARE complexes form helical coiled-coil assemblies that may bring interacting membranes in close enough apposition to facilitate if not complete bilayer fusion (Hanson et al., 1997; Sutton et al., 1998). NSF, rather than being needed for the fusion process per se, appears to act as a chaperone that dissociates the SNARE complex following fusion (Mayer et al., 1996).

By this view, SNARE-mediated fusion proceeds via a mechanism directly analogous to that demonstrated for viral fusion proteins (Skehel and Wiley, 1998). Following virus endocytosis, proteins such as the influenza HA mediate fusion of the viral envelope with the limiting membrane of the endosome due to a low pH-induced conformational change involving a helical coiled-coil domain inserting a fusion-active peptide into the target bilayer.

Although viral spike proteins are necessary and sufficient for fusion, a variety of other components contribute to the presumed fusogenic function of the SNAREs. Help almost certainly occurs at the level of vesicle docking (Pfeffer, 1999), since it is apparent that the specificity of SNARE interactions alone is insufficient to ensure the fidelity of vesicle interactions. While other components may similarly participate in the fusion step, it is clear that the SNAREs represent an aspect of the machinery that lies at the core of the mechanisms controlling vesicular transport.

Genetics of Membrane Fusion. Concomitant with the

development of the in vitro biochemical approach, Randy Schekman and his colleagues developed a novel genetic strategy to study the mechanisms of membrane traffic. This genetic approach, using the budding yeast *S. cerevisiae*, confirmed and extended the biochemical results in such a dramatic fashion that "the awesome power of yeast genetics" became a pejorative catch phrase. The first screens were designed to isolate mutants with conditional defects in the secretory pathway and were based on the assumption that secretion mutants, unable to discharge secretory products, would be denser than wild-type cells (Novick et al., 1980). They were indeed denser, and for a remarkable range of reasons. Dozens of complementation groups were isolated, and each affected single or multiple steps or events involved in secretion (thus referred to as "sec" mutants). Among the mutations found to act throughout the secretory (and endocytic) pathways were mutations of *SEC18*, the gene encoding the yeast homolog of NSF, and of *SEC17*, the gene encoding the yeast homolog of α-SNAP. Importantly, SNARE mutants were also isolated, and individual alleles often affected only transport steps related to the organelles with which a particular SNARE was associated (Nichols and Pelham, 1998).

One of the most important advantages of using yeast genetics was the ability to identify important gene products regardless of their involvement in a particular in vitro assay. Thus, this approach facilitated the identification of a large array of proteins that are involved together with the SNAREs in the vesicle recognition/fusion process. The most important of these is the large family of Ras-like GTPases of the Rab family (Novick and Zerial, 1997). The first member to be so characterized was Sec4p, a gene product that was required for secretory vesicle fusion with the plasma membrane (Salminen and Novick, 1987). In yeast, and particularly in mammalian cells, a large family of related Rab proteins exists, each with characteristic organelle distributions (Chavrier et al., 1990). Under normal conditions in intact cells, they are associated with specific transport steps, linking GTP hydrolysis with the onset of membrane fusion (Pfeffer, 1996). Moreover, their ability to control fusion has been documented in vitro, particularly using cell-free assays that reconstitute the fusion of endocytic vesicles (Mayorga et al., 1989). Currently, it is thought that Rab proteins work synergistically with the SNAREs via large protein complexes (perhaps also specific to each fusion step), which allow the integration of the specificity and catalytic functions associated with each class of protein (Pfeffer, 1999).

The Emergent Framework for Understanding Targeting and Fusion. Thus, three independent story lines—the reconstitution of Golgi transport in vitro, the biochemical analysis of synaptic vesicle membranes, and the genetic dissection of secretion in yeast—each of which began in the late 1970's to early 1980's, have converged to yield a general view of how vesicle targeting and recognition work. As diagrammed in Figure 2, the mechanism is deceptively simple, involving basically three steps. First, a transport vesicle bearing a v-SNARE is "tethered" to a potential target membrane by a Rab GTPase associated with filamentous coiled-coil proteins and perhaps other large protein complexes (Terbush et al., 1996; Sacher et al., 1998). Second, if the vesicle

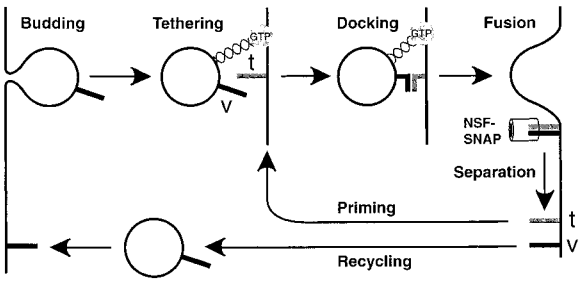

Figure 2. General Logic of Vesicle Targeting and Fusion

Budded vesicles containing v-SNAREs are tethered to acceptor membranes by protein complexes that include Rab GTPases and fibrous proteins. SNARE pairing follows, leading to membrane fusion, perhaps with the aid of downstream factors. The v-t-SNARE complexes are separated by the NSF ATPase in association with SNAPs. The v-SNARE is recycled to the donor membrane whereas the t-SNARE is primed for further rounds of fusion.

v-SNARE is a suitable match for the target's t-SNAREs, and the tethering complex is properly recognized, the loosely tethered vesicle becomes tightly "docked." Finally, after engaging both recognition elements, fusion is facilitated by the SNAREs themselves. The SNARE complex is then disrupted by the action of NSF and SNAP and is reutilized by recycling of the v-SNARE back to the donor compartment and repriming the t-SNARE at the acceptor compartment.

Molecular Sorting during Vesicular Transport

In addition to vesicle targeting and fusion, the other principle that has come to define our understanding of membrane traffic is molecular sorting. Molecular sorting refers to two related events: first, the ability to selectively include or exclude individual membrane and content proteins during the formation of nascent transport vesicles, and second, the ability to segregate the vesicular container from its cargo after vesicle fusion. This segregation permits the recycling or salvage of essential components involved in vesicle formation and targeting, which must be returned to the donor compartment to allow continued transport. Thus, vesicle targeting/fusion, vesicle budding, and vesicle recycling (Figures 3 and 4) can be considered the Trinity of membrane traffic.

Coat Proteins Mediate Vesicle Budding. The initial formation of transport vesicles at a donor membrane

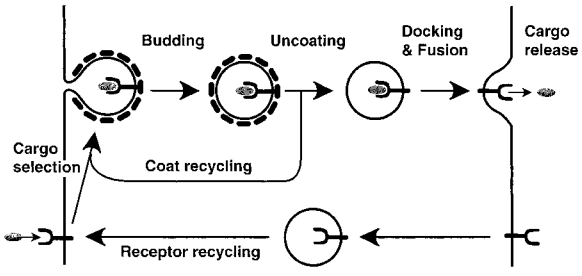

Figure 3. Vesicle Budding and Cargo Selection

Stepwise assembly of coat subunits deform the membrane into a bud and incorporate receptors that bind cargo. Uncoating (and recycling of coat subunits) is followed by docking, fusion, and release of the cargo to the next compartment. The empty receptors are then recycled back to the donor compartment.

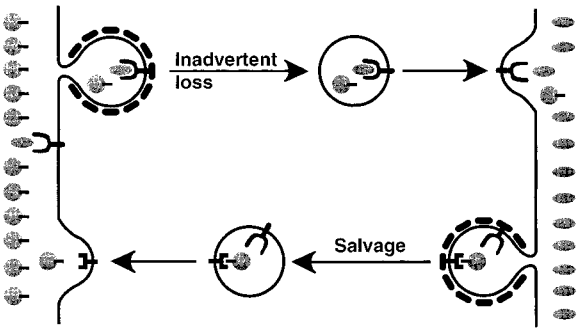

Figure 4. Salvage Mechanisms Compensate for Missorting during Cargo Transport

Cargo-containing vesicles sometimes incorporate residents of the donor compartment that are then wrongly delivered to the acceptor compartment. Specific salvage receptors recognize these "lost" proteins and return them to the donor compartment.

typically involves the assembly of characteristic protein complexes, or coats, at the site of vesicle formation (Figure 3). Coats are derived from soluble, cytosolic precursors and bind to specific organelle membranes. Their recruitment to organelles is regulated by monomeric GTPases of the Arf (ADP-ribosylation factor) or Sar1 families (Springer et al., 1999). In general, coats are thought to assist in the physical deformation of planar membranes into sharply curved buds, and also to act in the selective inclusion of proteins intended for forward transport. Shortly after formation, the coats dissociate from the newly formed transport vesicle, freeing the vesicle to dock and fuse with its target, and permitting the recycling of the coat components themselves.

Three basic types of coat complexes have been extensively characterized. The first of these is clathrin, mentioned earlier, which binds to its target membranes in conjunction with one of several adaptor complexes (Hirst and Robinson, 1998). Distinct adaptors bind to the plasma membrane, to the TGN, or to endosomes and then recruit soluble clathrin, which assembles into a "clathrate" lattice. The adaptors also decode targeting signals in the cytoplasmic domains of plasma membrane receptors or other transmembrane proteins intended for selective inclusion in clathrin-coated vesicles. These coated pit signals often contain critical tyrosine (YXXΦ) or dileucine motifs (Bonifacino and Dell'Angelica, 1999) that signal rapid endocytosis from the plasma membrane or transport from the Golgi to endocytic organelles. In the case of coated vesicles forming at the plasma membrane, another GTPase (dynamin) is required to facilitate the final scission step (Takei et al., 1995; Marsh and McMahon, 1999). Interestingly, this function for dynamin was first identified as a *Drosophila* mutant (*shibire*) that exhibited a profound temperature-sensitive paralytic phenotype indicative of a block in neuromuscular transmission (Kosaka and Ikeda, 1983).

COPI coats generally function early in the secretory pathway (Oprins et al., 1993), although they may also act at the level of endosomes (Whitney et al., 1995). These coats are derived from cytosolic precursors (coatomer) and bind to ER-derived and Golgi membranes that form vesicles containing membrane proteins bearing the KKXX or RRXX class of targeting signals. Since these signals are characteristic of many resident ER membrane proteins, COPI is likely to mediate a retrieval or salvage operation that returns ER proteins that had escaped to the Golgi complex during normal ER-to-Golgi transport back to the ER (Letourneur et al., 1994). However, it also possible that COPI coats participate in the intercisternal transport of cargo through the Golgi stack in the anterograde (*cis* to *trans*) direction (Orci et al., 1997). Identified first biochemically and morphologically (Malhotra et al., 1989), a critical role for COPI coats in maintaining transport between the ER and the Golgi, and indeed their role as salvage operators, was dramatically illustrated by yeast mutants (Gaynor and Emr, 1997).

COPII coats also act early in the secretory pathway but do so at a single site: they are responsible for forming ER transport vesicles, which then proceed toward *cis*-Golgi elements. Genetic experiments in yeast and biochemical experiments involving in vitro ER budding assays showed that COPII coats are essential for this initial ER budding step (Bednarek et al., 1995). Interestingly, COPII vesicle formation seems to be spatially restricted to distinctive budding sites on the ER (Presley et al., 1997), presumably equivalent to the "transitional elements" observed many years earlier by Palade and colleagues in secretory tissues (Merisko et al., 1986). COPII coats may interact with at least some cargo molecules at the time of ER export (Nishimura and Balch, 1997; Kuehn et al., 1998). However, the evidence in support of this possibility is far less robust than the evidence for clathrin adaptor- or COPI-mediated selection of analogous cargo. Thus, cargo selection in the case of ER export may involve events that act upstream of COPII vesicle formation. As will be described below, these events may include the process of protein folding, which renders newly synthesized proteins competent for ER export.

Cargo Selection, Sorting, and Recycling during Endocytosis. Endocytosis provides the clearest example of how cargo selection and sorting work to organize intercompartmental membrane transport. Two basic forms of endocytosis exist. The first involves the receptor-mediated uptake of soluble macromolecular ligands (e.g., hormones, nutrients, viruses) that bind to specific cell surface receptors. As we have seen, these receptors selectively accumulate at clathrin-coated pits resulting in their internalization via coated vesicles. Coated vesicles also mediate the bulk phase, nonselective uptake of solutes in the extracellular fluid that is up to 1000-fold less efficient than receptor-mediated uptake.

A second form of endocytosis accommodates the uptake of large extracellular particles (dead or apoptotic cells, bacteria) or large droplets of fluid by phagocytosis (cell eating, a term first coined by Metchnikoff) or macropinocytosis (cell drinking, a mechanistically related process). These modes of uptake do not involve clathrin but require an ordered assembly of actin. Phagocytosis plays critical roles in tissue remodeling during development and in the uptake of antigen for priming immune responses. Both phagocytosis and receptor-mediated endocytosis result in the delivery to and degradation of internalized material in hydrolase-rich, acidic lysosomes.

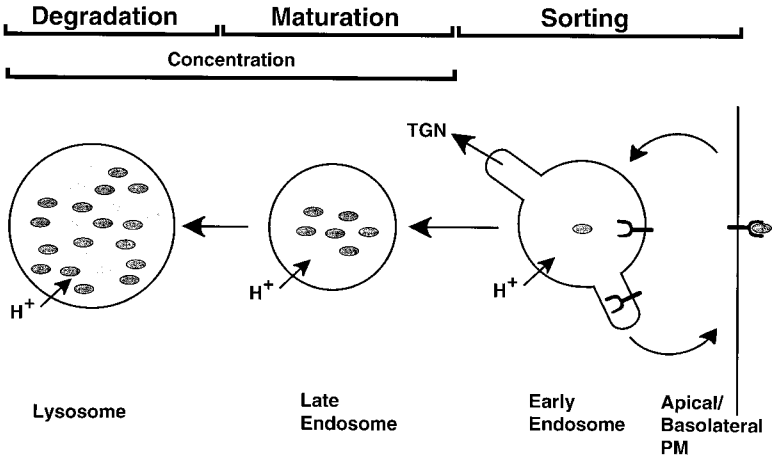

Figure 5. Pathways of Endocytic Traffic

Ligands delivered by receptor-mediated endocytosis to the endosomes are released upon exposure to acidic pH. The receptors are recycled back to the plasma membrane (PM) whereas the ligand is concentrated through maturation of the compartments and eventually degraded by the hydrolytic enzymes in the lysosome.

Endocytic receptors can avoid this fate because they can be recycled to the plasma membrane.

Endosomes were first identified as an anastomosing population of hydrolase-poor tubules and vacuoles through which internalized ligands passed on their way to lysosomes. It rapidly became clear, however, that they were not merely simple conduits but rather central sorting stations controlling the traffic of ligands, receptors, and fluid due in part to the fact that they maintain a slightly acidic internal pH (Mellman et al., 1986). As summarized in Figure 5, receptor–ligand complexes are delivered to "early endosomes" where the low pH facilitates their rapid dissociation. The vacated receptors then accumulate in the endosomes' tubular extensions, which bud off and initiate recycling back to the plasma membrane or, in some cases, to the TGN. In contrast, the ligands that are free in the endosomes' lumen accumulate in the more vacuolar elements of the endosome complex, which results in their being concentrated and exposed to increasingly inhospitable environments as the early endosome transforms or "matures" into late endosomes and then lysosomes.

Endosome maturation reflects the progressive removal of recyclable components and the introduction of newly synthesized lysosomal hydrolases, by fusion of TGN-derived clathrin-coated vesicles. Importantly, these enzymes are deposited into the endocytic pathway in much the same fashion as endocytosed ligands. Because lysosomal hydrolases all contain a specific mannose-6-phosphate recognition marker, they bind in a low pH–sensitive fashion to mannose-6-phosphate receptors in the TGN, which then localize at clathrin-coated buds that ferry enzyme–receptor complexes to endosomes, where they dissociate. The hydrolases are retained within the endocytic system and are concentrated in lysosomes while the receptors are recycled back to the TGN (Kornfeld and Mellman, 1989).

Endosomes accomplish the molecular sorting of incoming or Golgi-derived receptors and ligands by using two simple yet fundamental principles. First, the acidic internal pH creates the critical asymmetry between the originating and destination compartments to allow for the vectorial delivery of receptor-bound ligands. Second, the differences in the surface-to-volume characteristics of tubules and vacuoles allow for the simple Euclidean sorting of membrane and contents. Clearly, there are specific sorting mechanisms imposed on top of this framework. The existence of supplemental mechanisms is clear in the case of polarized cells (such as epithelial cells or neurons) that maintain two or more distinct plasma membrane domains. Here, endosomes decode the sorting signals that allow selective inclusion in vesicles destined for the appropriate domain (Mellman, 1996), another clear example of cargo selection. Endosomes also send off transport vesicles containing membrane proteins back to the TGN based on a different signal recognition system. Finally, endosomes can select receptors, usually those involved in signal transduction, that are targeted for downregulation. Such receptors are sequestered into vesicles that invaginate into the endosome lumen, a process that may be controlled by the production of phospholipid signaling molecules (Haigler et al., 1979; Felder et al., 1990; Wurmser and Emr, 1998). Nevertheless, the logic of endosome sorting is both simple and—more importantly—applicable in a fundamental fashion to the secretory pathway as well.

Traffic and Sorting in the Secretory Pathway

Although secretion is not simply endocytosis in reverse, the secretory pathway uses the same basic logic and mechanisms as does the endocytic pathway. To best illustrate how this all works, it is useful to follow the itineraries taken by the three basic types of secretory cargo and recount their relationships to the resident proteins they must meet along the way.

Protein Folding Produces Secretory Proteins Competent for Transport. Although it is a "foundation" of modern cell biology that all secretory and membrane proteins begin their lives by insertion into the ER, only over the past decade has the intimate relationship between translocation and protein folding become clear. This relationship was first and is perhaps best illustrated by protein import into mitochondria, despite the fact that mitochondrial import does not really fall under the rubric of membrane traffic. Most mitochondrial proteins are synthesized in the cytoplasm and are posttranslationally imported through specialized channels that span the outer and inner mitochondrial membranes. Translocation is accompanied by the unfolding of the imported protein but depends on its refolding in the mitochondrial matrix, a process catalyzed by a heat shock protein (Sigler et al., 1998; Ellis and Hartl, 1999).

In the ER, translocation is similarly driven by a different

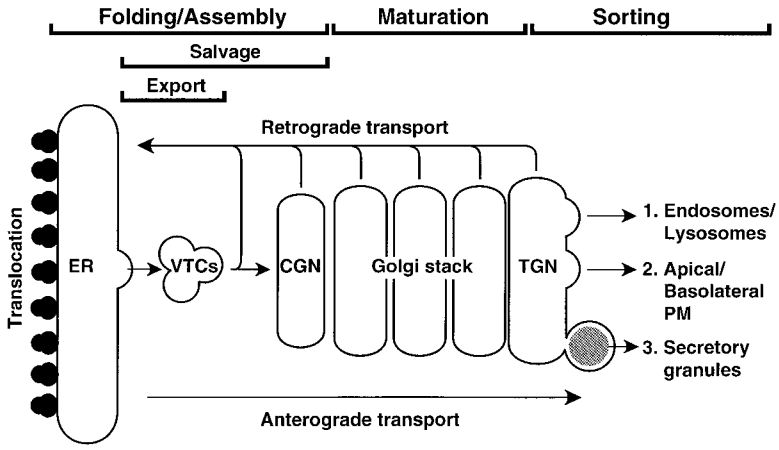

Figure 6. Pathways of Exocytotic Secretory Traffic

Cargo is translocated into the ER and folded before exit via vesicles that fuse to form an intermediate compartment between the ER and the Golgi, referred to here as VTCs. Delivery to the *cis* side of the Golgi complex (CGN) is followed by passage across the stack where extensive posttranslational modifications occur. Cargo is sorted at the *trans* side of the Golgi (*trans*-Golgi network or TGN), packaged into different vesicles, and delivered to the endosomal pathway, the plasma membrane (different domains), or forming secretory granules. Retrograde transport recycles components of the transport machinery and salvages ER residents.

heat shock protein, the Hsp70 family member BiP. However, complete folding depends on a panoply of other folding factors, ranging from proteins that facilitate the exchange of disulfide bonds to those that transfer and process oligosaccharides. These proteins comprise the "quality control system" within the ER that marks unfolded proteins with extra glucose residues at the termini of N-linked sugar chains. This system ensures that unfolded or misfolded proteins remain tightly bound to lectins (calnexin, calreticulin) within the ER restricting their export to the Golgi complex (Ellgaard et al., 1999). Thus, for both membrane and secretory proteins, transport through the secretory pathway cannot begin until folding is completed.

ER Export and Recycling. When folding is finished, the newly synthesized proteins exit the ER at specific sites marked by the formation of COPII-coated vesicles (as discussed earlier) (Figure 6). Although it seems reasonable to assume that COPII coats concentrate their cargo in a manner similar to clathrin coats, the COPII system faces a different problem: it must mediate the transport of the entire spectrum of membrane and secretory proteins produced by virtually all cell types rather than a restricted number of specialized plasma membrane receptors. It is not yet clear whether all membrane and secretory proteins have common features that allow them to interact with COPII components or with generic cargo receptors. Nevertheless, there is evidence that at least some secretory and membrane cargo can be concentrated within forming COPII coats, although this is not always the case (Warren and Mellman, 1999). In contrast, the relatively few structural components of the COPII vesicle membrane, such as v-SNAREs like Bet1p and Bos1p, are actively selected for export by interacting with coat components (Springer and Schekman, 1998). Thus, it is possible that for many membrane and secretory proteins, "concentration" in COPII vesicles partly reflects their release from the ER quality control system rather than a direct process of cargo selection. At least some ER residents and misfolded proteins do escape inadvertently in COPII vesicles; these are retrieved by an important salvage pathway that is initiated shortly thereafter.

As COPII vesicles bud, they are almost immediately seen to accumulate as clusters of vesicles and tubules (VTCs) (Tisdale et al., 1997), which begin to lose their COPII coats and move along microtubule tracks toward the *cis*-Golgi complex (Presley et al., 1997). The VTCs are not simple transport carriers but instead behave as the moral equivalents of endosomes. Thus, they sort and concentrate proteins intended for anterograde transport through the Golgi from those that must be returned to the ER. Sorting is initiated by the assembly on VTCs of COPI coats. COPI coats prime the formation of vesicles that provide a critical salvage function (Figure 4), returning resident or structural components as well as inadvertently exported and misfolded proteins to the ER. COPI vesicles perform this function in a remarkably specific fashion. COPI coats interact with the KKXX/RRXX "retrieval signals" common to ER membrane proteins and thereby selectively concentrate these proteins in COPI buds forming in the VTCs and after VTC fusion with the Golgi. Escaped luminal ER components are retrieved in much the same way. Resident proteins, such as the folding chaperones BiP and calreticulin, terminate in a characteristic KDEL tetrapeptide sequence, which binds to a retrieval receptor, known as the KDEL receptor (Pelham, 1996). In addition to ensuring the retrieval of ER components, formation of COPI vesicles effects the concentration of secretory cargo, a process that continues during subsequent steps (Martinez-Menarguez et al., 1999).

Transport through the Golgi Complex. VTCs deliver secretory and membrane proteins to the *cis* face of the Golgi complex. From there, they begin a trip through 2–3 distinct Golgi compartments during which secretory cargo "matures": glycoproteins have their oligosaccharides trimmed and rebuilt, modifications such as sulfation and phosphorylation may occur, and proteolytic cleavages can be introduced. In animal and plant cells, transit through the Golgi complex involves sequential passage through a characteristic stack of 3–5 cisternae (*cis*, medial, *trans*). Precisely how the transit occurs and why the Golgi complex looks the way it does have been fascinating questions almost since the organelle was rediscovered in the mid-1950's.

There are two popular models for transport through the Golgi stack. The first, or maturation model, envisages COPI vesicles carrying resident Golgi enzymes and remaining ER components back to the previous cisterna.

This process may allow the Golgi, like VTCs, to act in a fashion similar to endosomes involving the continuous sorting of cargo from container. After delivery to the *cis* side of the stack, secretory proteins would move across the stack as the *cis* cisterna matures to become a medial and then *trans* cisterna by selective retrograde recycling of non–cargo membrane components. In this model, new *cis* cisternae would be formed continuously from components delivered by VTCs from the ER (Figure 6).

The other model envisages the cisternae as static structures from which vesicles bud for anterograde and retrograde transport. COPI vesicles might well serve this function as they also contain secretory cargo intended for forward transport (Orci et al., 1997). This situation would clearly require the existence of distinct COPI vesicles dedicated to forward or recycling transport.

Whether transport across the Golgi stack involves vesicles moving in both directions, retrograde vesicles combined only with cisternal maturation, or some combination of the two, there are common principles that can be simply stated. First, transport of secretory cargo across the stack occurs by bulk flow, i.e., it is not dependent on interaction with one or more receptor elements. Second, recycling (retrograde transport) relies on the selective removal of resident components not intended for forward transport via the COPI-dependent KKXX/RRXX recognition system. Third, and least well understood, the system provides for the asymmetric distribution of resident Golgi proteins across the stack. In other words, *cis* cisternae contain glycosylation enzymes that perform initial oligosaccharide processing events, medial cisternae contain enzymes for intermediate steps, and *trans* cisternae contain enzymes for the terminal events. Various mechanisms may contribute to this differential composition, including the capacity for "like" enzymes to self-assemble or to partition into membranes of distinct lipid compositions and thickness (Bretscher and Munro, 1993; Nilsson and Warren, 1994).

Multiple Exit Ports from the Golgi. Although the three basic types of secretory cargo are handled in more or less indistinguishable fashions through the stack, upon delivery to the TGN the situation changes dramatically. Proteins intended for the plasma membrane exit in vesicles or tubules that are formed constitutively (Figure 6, option #2). In polarized cells, however, there are two classes of these vesicles: those targeted to the apical plasma membrane, and those to the basolateral plasma membrane. Much of basolateral transport involves a novel clathrin adaptor protein, which is specific to epithelia and which recognizes a canonical cytoplasmic domain–sorting signal common to basolateral proteins (Folsch et al., 1999). Apical transport may involve carbohydrate–lectin interactions or the propensity to partition into glycolipid-enriched domains or "rafts," which presumably bear the appropriate SNAREs and recognition molecules for targeting to the apical surface (Simons and Ikonen, 1997).

Proteins intended for endosomes or lysosomes accumulate at an apparently distinct set of coated buds containing a ubiquitously expressed clathrin adaptor complex. Perhaps the most important example is provided by lysosomal enzymes bearing the mannose-6-phosphate recognition marker bound to mannose-6-phosphate receptors (see above) (Figure 6, option #1). However, cytoplasmic domain signals also play an important role, particularly in the endosomal targeting of membrane proteins. In animal cells, these signals look highly reminiscent of those used for endocytosis (Bonifacino and Dell'Angelica, 1999); in yeast, an alternative signal is used, which is linked to activation of a phosphatidylinositol 3-kinase (Wurmser and Emr, 1998).

Finally, in professional secretory cells such as the acinar pancreas (see Figure 1), secretory content begins to aggregate or crystallize upon reaching the TGN (due to alterations in pH and/or Ca^{2+} concentration) and are then sequestered in larger membrane buds that give rise to the densely packed secretory granules characteristic of exocrine and endocrine cells (Figure 6, option #3). Formation of granules is an exceptionally efficient way to concentrate secretory product and store it intracellularly prior to regulated release. Secretory granules also mature by a process that involves the selective removal of TGN-derived components, in this case by the formation of clathrin-coated vesicles from the newly formed granules (Orci et al., 1984; Tooze and Tooze, 1986; Arvan and Castle, 1998; Thiele and Huttner, 1998).

Alternative Modes of Transportation.

We have concentrated on the most common and best understood modes of transport that explain the majority of events that occur on the endocytic and secretory pathways. However, a number of alternative or atypical modes are becoming known, some of which bear mentioning as they too have broad and fundamental implications. This is particularly true when considering membrane traffic in complex cell types or in relation to higher order functions such as signal transduction.

Sorting and Traffic in Polarized Cells. In Figures 5 and 6, reference has already been made to the fact that the endosomes and TGN of epithelial cells are capable of sorting apical and basolateral membrane (and secretory) proteins into distinct transport vesicles. The logic of the system is rather simple: a cytoplasmic domain signal for basolateral targeting is *cis* dominant to a recessive signal for apical targeting (Matter and Mellman, 1994). The basolateral signal is decoded by a clathrin adaptor, and the apical signal by an as yet unidentified lectin. A similar logic, if not necessarily the same mechanism, governs targeting in other polarized cells such as neurons (Dotti and Simons, 1990; Jareb and Banker, 1998).

However, this system is not the end of the story. Interactions of membrane proteins with infrastructural elements influence polarized traffic in dramatic ways. For example, the existence of PDZ-binding domains can determine polarity, indicating that the distribution of PDZ proteins (often found at epithelial junctions and nerve endings) plays an important role in directing sorting, vesicle traffic, or retention of newly inserted membrane proteins (Cohen et al., 1998; Rongo et al., 1998). Indeed, it has long been thought likely that interaction of some membrane proteins with ankyrin and actin results in retention at cell–cell junctions, achieving polarity (Nelson and Veshnock, 1987). One can presume that other cytoskeletal interactions will emerge as having other effects.

Digging for Gems in Rafts. Some forms of polarized targeting correlate with the partitioning of incipient apical proteins in lipid rafts. This partitioning is thought to reflect the lectin interaction mentioned above or possibly the propensity of certain membrane-anchoring domains to segregate into detergent-insoluble glycolipid

domains (DIGs), glycolipid-enriched membranes (GEMs), or more simply, lipid rafts. Much of these data is only correlative, but the extent to which rafts are turning up as associated with a variety of important cell functions is quite remarkable. Signal transduction is perhaps the top of the list. Initial studies suggested that many important signal transduction mediators are localized at distinctive nonclathrin invaginations of the plasma membrane known as caveolae. Subsequently, many of the same molecules were found biochemically to partition within rafts, even in the absence of caveolae. It is conceivable that rafts exist to concentrate trace components that must interact for the purposes of signal transduction. As such, they restrict and/or specify traffic by sequestering selected membrane proteins away from the endocytic pathway or targeting them to a distinct cell surface domain. The latter function may emerge as playing a large role in lymphocytes, in helping these cells to establish functionally distinct signaling domains during antigen recognition.

Foundations for the Future

In the larger scheme of things, millennial transitions are wholly arbitrary demarcations. However, such transitions do exist and cause the unlucky few to summarize the past and predict the future. Summaries and predictions do little more than create opportunities for derision by one's current and future colleagues. Even confident statements of what we currently know must be made with some level of trepidation, however small.

With such disclaimers in mind, it does seem reasonable to state that the principles governing membrane traffic are now quite clear. However, it is also clear that a number of the underlying mechanisms remain controversial. This situation reflects the fact that key molecules have yet to be identified and their functions studied. An even greater limitation to our current understanding, even at the conceptual level, is the difficulty in integrating the functions of these molecules and pathways to understand how organelles form, why organelles and cells form the shapes that they do, how individual cells work together to form complex tissues, and how all of this goes wrong in the context of human disease.

Identification of individual molecules has been greatly facilitated by the complete sequence of the *S. cerevisiae* and *C. elegans* genomes, an advantage that will be enhanced further upon completion of the *Drosophila* and human genomes. There is every reason to expect that the cast of proteins involved in each vesicle-mediated step will be complete in the near future. In a sense, the completion of one or more genomes represents a closing of the frontier, simply because no gene will remain to be discovered. This typification is not infrequently met with defensive derision on the part of devotees of the organism whose frontier has been closed. Yet, just as the closing of the frontier in the American West toward the end of the 19th century marked the beginning rather than the end of a great period of progress, there is every reason to suspect that this will mark the beginning of a new era in the biological sciences.

The massive effort and intellectual power that has been unavoidably devoted to activities of marginal conceptual creativity such as sequencing, mapping, cloning, and mutant searches will hopefully now be turned toward the real business at hand: translating the shimmering ocean of information that has been provided by two decades of genomics, genetics, and gene expression analysis into useful cell biological information. This is a labor intensive, highly creative process, as each new gene product or cellular function represents a new problem requiring its own set of assays and procedures. Cell biology has not yet lent itself well to industrialization or high throughput methods. Perhaps it will in the future.

Specifically in the area of membrane traffic, the immediate issues that remain involve the functional study of individual transport steps. Here, available techniques are inadequate. Biochemical assays can measure the production of budded vesicles but not the intermediates that lead to them. The same applies to vesicle docking. Vesicles bound to membranes can be separated from those that do not, but the different stages of docking cannot be deconstructed. This is a particular concern because recent work suggests that there are at least a dozen or more proteins involved in what the assays would suggest is the comparatively simple act of tethering a vesicle to a membrane. They point to a far more sophisticated and perhaps orchestrated process.

Increasing the resolution of the assays will involve improved visualization techniques that allow vesicle trafficking to be followed in real time, eliminating the need to take snapshots of fixed samples that then have to be placed in some sort of order. What is needed is the development of instruments and methods that are familiar to Star Trek fans, namely tools that will allow real time visualization of events occurring in vitro as well as within cells at the molecular level. Current attention is being paid mostly to optical methods combined with the use of fluorescent probes (e.g., green fluorescent protein). As powerful and visually exciting as these may be, their analytical potential will always be limited by the wavelength of light. In principle, atomic force microscopy offers even more hope since the resolution should allow the coating, uncoating, and docking of vesicles to be observed in real time. The ultimate biophysical techniques would be those that have the spatial resolution of EM and the time resolution of fluorescence. Video electron microscopy would represent a quantum leap in technology and understanding that could only be superseded by video X-ray or NMR microscopy. Perhaps this is how Star Trek's "tricorders" work.

Although a mechanistic understanding of vesicle-mediated traffic will be an important goal for the immediate future, perhaps a more important if also more distant goal is understanding the contribution that positional information makes to cell and tissue function. Why do organelles look the way they do? Why are they positioned in characteristic regions in the cytoplasm? Most importantly, how do organelle function, cytoskeletal assembly, and signal transduction determine cell polarity and thus tissue organization and development? Our knowledge of these issues remains fragmentary, in need of more information, better methods, and intellectual integration.

An allied area of research is the biogenesis of intracellular membrane compartments. Are they autonomous structures that can grow and replicate in a manner analogous to the chromosomes? Or are they component parts of a collective with no individual identity of their own? Mitochondria and chloroplasts are clearly autonomous structures, each with their own complement of

genetic material. The autonomy of compartments connected by vesicles is much less clear. The ER is autonomous since a cell that did not inherit a single copy would be unable to synthesize a new one. But what of the Golgi and endocytic membranes? Are they also autonomous, or simply renewable products of the ER?

The underlying aim of molecular cell biology has always been to explain cell function in terms of cell structure. Its roots lie in the common language of cells and its success in the multiplicity of approaches that have been brought to bear at different times in different organisms to solve common problems. If the major challenge of the next period of biomedical science is to translate genomic information into function and thus to the understanding of human disease, cell biology clearly lies at the epicenter. It is ironic that this should be the case, as cell biology has always played Cinderella to its more visible sisters genetics, genomics, and molecular biology. All future progress will be marked in cellular terms, leaving cell biology at the nexus of structure, neurobiology, immunology, physiology, genetics, developmental biology, and biochemistry. Most importantly, advances in medicine will increasingly be dependent on cell biological understanding, a pleasing symmetry as it was an interest in human biology and disease that led Golgi and Metchnikoff to give rise to the field. As the boundaries between disciplines become blurred, molecular cell biology will disappear as a separate discipline and become subsumed by the rest of the biological sciences. This transition, already begun, will be cell biology's inevitable fate, and the mark of its ultimate success.

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