The Principle of Membrane Fusion in the Cell

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By 1970 the already-classic work of George Palade (Nobel Prize, 1974; Figure 1) had made it evident that secreted proteins are carried from the endoplasmic reticulum (ER) to the cell surface in specialized containers, or transport vesicles, that bud from one membrane and fuse with the next, transiting the Golgi stack *en route* (Figure 2). We now know that such intracellular protein transport is a universal process in all eukaryotes. Many kinds of vesicles traverse the cell, laden with many kinds of cargo for delivery. The result is a choreographed program of secretory, biosynthetic and endocytic protein traffic that serves the cell's internal physiologic needs, propagates its internal organization and allows it to communicate with the outside world and to receive nutrients and signals from it.

All vesicle transfer processes can be thought of as having two basic steps: budding (when the vesicle pinches off from a 'donor' membrane) and fusion (when the membrane of the vesicle merges with the 'acceptor' membrane of the intended target). The membrane fusion process has special importance for both intracellular and extracellular physiology (Figure 3). Fusion of vesicles within the cell must be done with exquisite specificity to prevent one organelle from taking on another's functional properties. Fusion with the cell surface (plasma) membrane (exocytosis) results in the release of the vesicle's contents, almost

George E. Palade (1912-2008)
Yale University, Nobel Prize (1974)

FIGURE 1. The generally-agreed founder of modern cell biology, George E. Palade. He was a recipient of the 1974 Nobel Prize in Physiology or Medicine, when he was at Yale University, where he founded what is now the Department of Cell Biology, which I chaired when I received the Nobel Prize.

always consisting of highly active substances, and therefore must be exquisitely regulated. Exocytosis is used by almost every cell and tissue in the body. The dizzying array of signaling molecules secreted by exocytosis affords a veritable tour of physiology and, frequently, related diseases: neurotransmitters and their ion channel receptors, endocrine hormones like insulin, transporters for

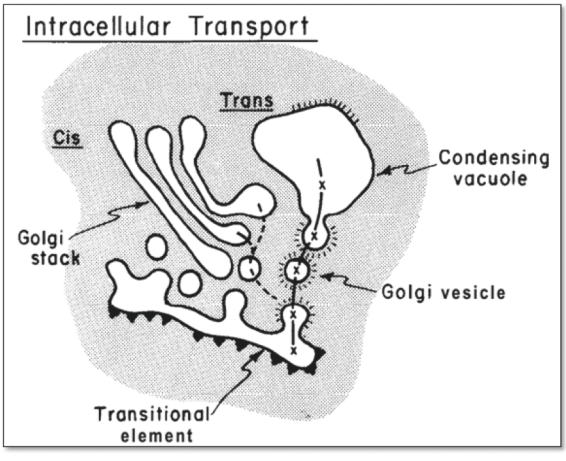

FIGURE 2. The concept of intracellular transport as laid out by Palade in his Nobel Lecture (1974). Fundamental unanswered questions included how the proposed transport vesicles form and how they can fuse specifically with their target membranes to deliver the right cargo to the right place at the right time. This figure is reproduced from the published lecture (Palade, 1975).

glucose and other nutrients, systemic mediators such as histamine and adrenaline, growth factors, and many others.

SETTING THE STAGE

From the earliest time I can remember I wanted to become a scientist, especially a physicist. I am not sure where this came from. Certainly in part from a family that deeply valued education, and especially science and medicine. My mother Gloria, with her enormous focus and drive, would in today's world have been a high-powered executive. She ran the home and my Dad's pediatric practice and

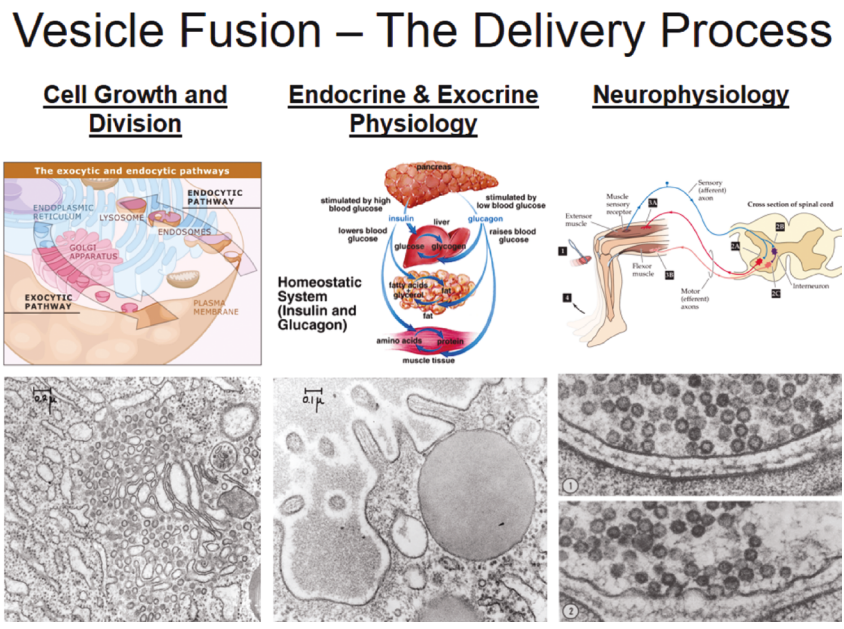

FIGURE 3. Membrane fusion is the fundamental process that allows specific cargo delivery, Fusion of vesicles carrying diverse cargo underlies a great variety of fundamental processes in cell and organismal physiology, ranging from the distribution of specific sets of proteins to designated compartments in the cytoplasm including signaling receptors at the plasma membrane (cell growth and division; top left panel), secretion of hormones and other signaling molecules (endocrine and exocrine physiology; top middle panel), and synaptic transmission (top right panel), a special case of inter-cellular communication. Electron micrographs (bottom panels) illustrate (left to right) the transport vesicles linking the ER to the Golgi in the early secretory pathway (exocrine pancreatic acinar cell), larger secretory storage vesicles containing insulin before and after fusion (endocrine pancreatic beta cell), and synaptic vesicles (containing neurotransmitters) before (above) and (below) after release is triggered by change in the membrane potential (neuromuscular junction).

by example taught me how to organize and manage. My father Martin was an intellectually oriented small-town doctor who had wanted to do medical research as a young man, but had graduated in the Great Depression then been caught up in events of World War II. He was always keen to involve me in the things he did. At perhaps the age of ten, I remember accompanying him on nocturnal house calls, sometimes to the hospital; assisting him in measuring QT intervals in his patients' electrocardiograms; and helping him perform blood analyses in the lab behind his office.

But I believe that my focus on science came at least as much from the ecosystem I grew up in. In the 1950s and 1960s science and technology were viscerally understood by Americans to be mainstays of economic and political power following the victory of World War II. This era began with the polio vaccine eradicating a dread disease and with atomic energy (for better and for worse). It ended with the transistor, the computer, and the first men on the Moon. The best-known of the scientists and practitioners were public heroes: Salk, Einstein, Oppenheimer, and the first astronauts (Figure 4).

FIGURE 4. The period 1950–1965, when I grew up, was in many respects the height of the era of physics. The era of biology had already begun but was not fully underway. Top left, Jonas Salk succeeded in producing the first widely available vaccine to prevent polio. Bottom left, the physicists Albert Einstein and J. Robert Oppenheimer at the dawn of nuclear energy. Right, the Mercury astronaut Alan Shepard preparing for launch.

In such an ecosystem, and with my supportive family, and with an early talent for mathematics, is it surprising that I was building electronics and launching rockets while still in elementary school (Figure 5)? Rockets were a big thing for me. I taught myself basic trigonometry in 7th grade so I could triangulate the height of the rockets, and then calculus a bit later so I could better understand the physics involved. As I studied physics and mathematics formally in high school and beyond, I devoured the subject and read far outside the curriculum at my secondary school (Pomfret School), so much so that I was graduated after my junior year. Entering Yale College in 1967, I was absolutely committed to theoretical physics.

While that isn't how it ended up, physics taught me how to rigorously analyze the components of a problem by first imagining the form a solution would take. This can be a useful approach when engulfed in the fog that envelopes the uncharted waters of biology. A last minute and nearly instantaneous conversion to biology (following my father's suggestion/insistence that I try some biology instead of all physics) occurred during my junior year at Yale. Even at the very first lecture in the general biology course (by the charismatic and brilliant biophysicist Frederic Richards), I was amazed that—in contrast to the highly structured field of physics—the research frontier in molecular biology seemed instantly accessible, and yet could be equally rigorous and structure-based.

FIGURE 5. The author, second from the right, preparing to launch a model rocket, age 12. I was entranced by mathematics, physics, and technology.

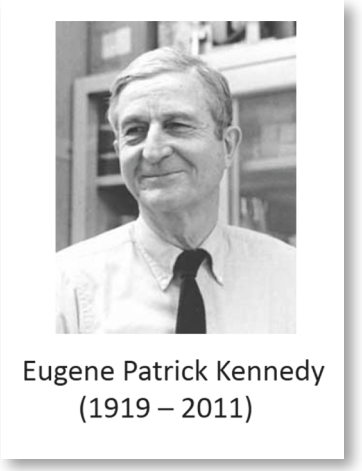

FIGURE 6. My PhD thesis adviser, Gene Kennedy, a master of membrane biochemistry.

A series of events then led me to Donald Engelman, then a new assistant professor of biophysics at Yale, and so my imprinting in experimental science was in the biophysics of membranes. Yale allowed me to drop all formal course work (and yet still graduate; they do not permit that anymore . . .) to pursue full-time research, for which I will always be grateful. That year, I learned from Engelman how to dissect each morsel of data to get the most from it, and I became a scientist. Next, I entered Harvard Medical School (in 1971) with the idea of learning biology broadly (rather than practicing). I succeeded in the former, leaving the MD program more or less after the basic sciences (but with enough clinical exposure to gain a lifetime of respect for clinical medicine).

It was as a first year medical student in histology that I first learned about the secretory pathway, at a time when George Palade's discoveries were still fresh and remarkable. What an astonishing process—how could cells make vesicles from membranes? How could each vesicle know where to go? How could it fuse? It was particularly amazing because at the time it was not even possible to begin to imagine the form a molecular solution might take. This captured my imagination, but not enough was known to productively take up the problem then.

My PhD thesis (as part of Harvard's MD-PhD program) with Eugene Kennedy (Figure 6) at Harvard Medical School, a master of membrane biochemistry, established how the lipid bilayer is formed by asymmetric biosynthesis. Kennedy, a brilliant intellectual and an original thinker, taught me how to formulate a complex problem in biochemical terms.

THE KEY ELEMENTS FOR A BREAKTHROUGH

Looking back, it is easy to see that many elements combined in fortunate ways to enable me to make the discoveries recognized by the Nobel Prize. I took up the sorting problem in the late 1970s with a broad background outside the field that proved helpful in itself and that left me sufficiently naive to be uninhibited. Other key factors included the training and perspective I was equipped with as a young scientist (outlined already); a working environment in a department that encouraged risk-taking; stable federal research funding over a decade that enabled innovation; an astute choice of problem (at the right time, still difficult but not impossible and yet impactful); a unique and productive way of approaching the problem (in my case through the simplifying mind-set of physical chemistry, as I will describe); the right (brilliant) students at the right times; and of course hard work and persistence to develop a method that works in the wake of many painful failures.

First, an ideal working environment: I was very fortunate to receive an offer to join the biochemistry department at Stanford while still a medical student, an opportunity I could not pass up because it provided the rare chance to start my own research in the remarkable environment created by Arthur Kornberg, one of the great biochemists of the 20th century (Figure 7). Happily Stanford was prepared to wait a year or so for me to do a postdoctoral fellowship, so I left Harvard Medical School (with a PhD) in 1976 for MIT and Harvey Lodish, with Stanford much in mind. Lodish taught me how to work with complex cell-free

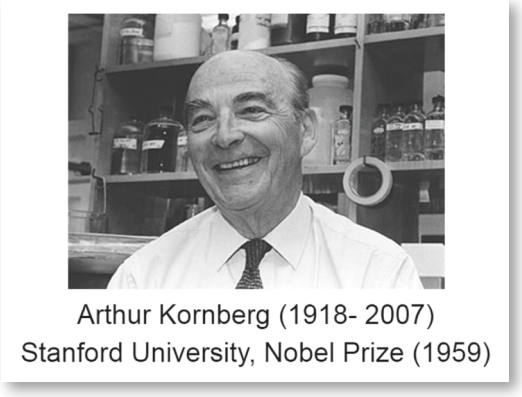

FIGURE 7. Arthur Kornberg, a master of enzymology, founded the Department of Biochemistry at Stanford University and was still its *de facto* leader when I joined as an assistant professor in 1978. In the next door laboratory, he taught me by example how a great scientist equipped with Buchner's world view attacks a complex biological process.

systems (translocation across membranes coupled to protein synthesis) and (frankly) how a large laboratory can be run boldly and energetically.

With the move to Stanford in the summer of 1978 a new era of my development as a scientist began. From Kornberg I learned two critical things at a critical time: how to formulate the strategy for a successful biochemical dissection of a complex system; and a deep faith that no matter how complex the problem, biochemistry would (eventually) succeed and would indeed provide the only sure route to the underlying molecular mechanisms. Kornberg's preaching on this subject was convincing because it stood on very solid ground. After all, cell-free reconstitution had been the central experimental approach of all biochemistry since its founding with the discovery of alcoholic fermentation in yeast extracts by Eduard Buchner (Nobel Prize, 1907) at the end of the nineteenth century, who founded what became modern biochemistry (Figure 8). And by the late 1970s, the core principles of ATP synthesis, DNA replication, RNA transcription, protein synthesis and even the genetic code were all relatively fresh 'trophies' of the reconstitution approach, which effectively strips away the subtleties of physiologic regulation to show the robust core machinery beneath.

The choice of the right problem at the right time: focusing deeply on the central rather than a peripheral aspect of a problem that is impactful and ripe for the right approach. As noted above, I was smitten by the "sorting problem" (as it was then called—how newly made proteins were distributed from ribosomes to their specific destinations in the cell) even as a medical student in the early

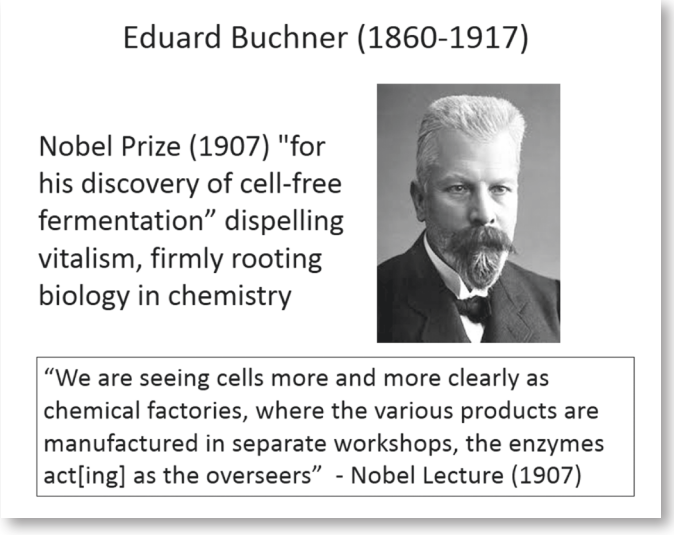

FIGURE 8. Eduard Buchner can be regarded as the founder of modern biochemistry.

1970s, but it was not ripe for attack until the late 1970s. The situation changed dramatically because of several key findings that together allowed the sorting problem to be posed in much more precise terms. Günter Blobel (Nobel Prize, 1999) had by then discovered that these proteins carry built-in signals that direct them into the ER and by implication that proteins quite generally have signal sequences that specify their location in the cell, now a basic principle. Michael Brown and Joseph Goldstein (Nobel Prize, 1985), in the course of elucidating basic mechanisms of cholesterol homeostasis, had just provided the first clear demonstration that selective transport between compartments is mediated by vesicles involving receptors specific for these signals in the transported cargo (particles of low density lipoprotein, LDL). They found that "coated vesicles" (first discovered by the great morphologist Keith Porter in 1964 (Roth and Porter, 1964), and first purified by Barbara Pearse in 1976 (Pearse, 1976) thus discovering the first protein (clathrin) coating vesicles budding from the plasma membrane. Clathrin-coated vesicles carry out the endocytosis of plasma lipoproteins, allowing their cholesterol to be released in lysosomes for re-use by cells. These vesicles garner lipoproteins from the medium by means of a receptor (LDL receptor) localized to the coated regions of membrane involved in budding. As a result of all of this, it became evident that solving the sorting problem essentially required understanding how each type of vesicle targets to and fuses with the correct target membrane in the cell.

A unique way of approaching the problem: Applying the mindset of a physicist to the complexities and mysteries of cell biology afforded me such a perspective and the approach to productively tackle the problem. Physicists seek universal laws to explain all related processes on a common basis, and achieve this by formulating the simplest hypothesis to explain the facts. The prevailing opinion among cell biologists was without doubt that the anatomical arrangement of the endomembrane systems in the cell—for example the fact that the transitional ER (from which vesicles bud to carry secretory products to the Golgi) is placed near the Golgi—is vital to ensure the delivery of cargo. As seen in Figure 9 (left side) in a classic micrograph from Palade, the ER seems almost to "force-feed" vesicles to the entry (cis side) of the Golgi stack, consistent with the idea that anatomy dictates specificity. But the simplest idea is rather the opposite—that intrinsic chemical specificity enables specific cargo delivery, and that the observed anatomy arises as the consequence of chemical specificity in operation. (Figure 9, right side).

The more common and more complex idea, that anatomy dictates specificity would mean that accurate vesicle traffic could never take place in the absence of pre-existing cellular organization. This strong prejudice—deeply rooted in

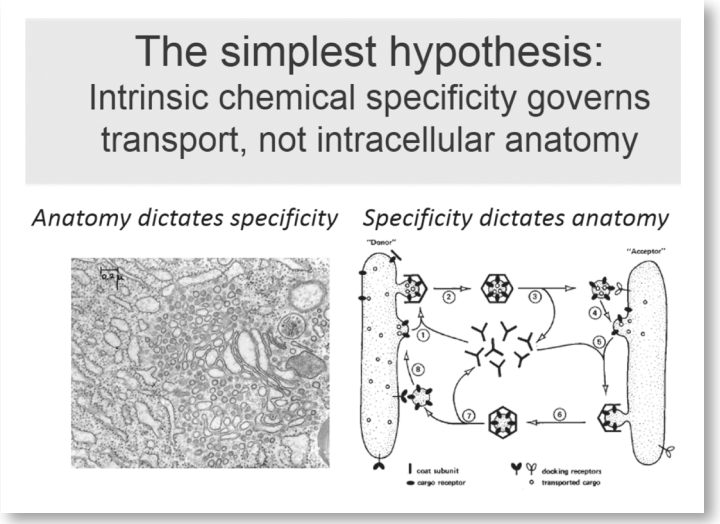

FIGURE 9. My thinking in 1978 on which the cell-free reconstitution approach was attempted. The model at bottom left (published in the 1982 Cold Spring Harbor Symposia on Quantitative Biology, Volume XLVI) was drawn long before any of the protein machinery was known. The logical organization of the pathway was correct, even though the responsible (then hypothetical) proteins were not yet identified.

cell biology from its origin as a branch of microscopic anatomy—no doubt accounted for much of the skepticism with which our reconstitution experiment was to be received for many years.

On the other hand, the great virtue of the simpler idea is the remarkable prediction it makes: that accurate vesicle traffic can in principle take place accurately in cell-free extracts, which would open the door to Kornberg-style enzymology. Once reconstituted, cell-free transport could be used as an assay to permit the underlying enzyme proteins to be discovered and purified according to their functional requirements.

Finally, the right partner to attack the problem, who was my first postdoctoral fellow, Erik Fries (Figure 10, right). Erik, a young Swedish scientist who had just arrived at Stanford from the new European Molecular Biology Lab in Heidelberg, Germany. Erik had worked with Ari Helenius and Kai Simons, providing key insights that led to their uncovering the mechanism of viral entry into cells. As a result, Erik brought with him a deep understanding of both cell biology and of physical biochemistry, and his own rigorous and quantitative style. He also had a rare combination of being adventurous and at the same time persistent, which enabled him to sign on to what most everyone thought would be a hopeless effort.

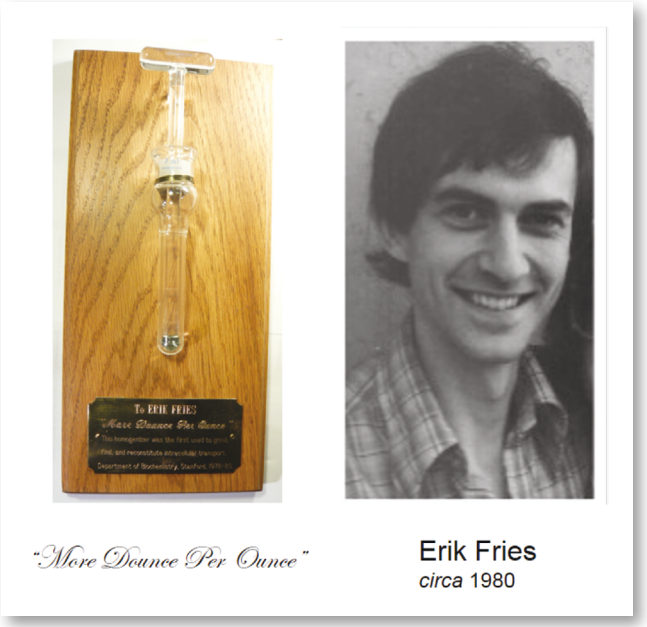

FIGURE 10. At left, the Dounce homogenizer used by my first post-doctoral fellow Erik Fries and me in 1979 to successfully reconstitute "intracellular" transport in a cell-free extract, now in the Nobel Museum. Initially it was mounted as a going-away gift to Erik (pictured at right during this era). Erik is now a professor at Uppsala University, and it was my pleasure to introduce him at my Nobel Lecture.

CELL-FREE RECONSTITUTION OF VESICLE TRANSPORT

Our goal was therefore to detect transport of a protein between membrane-bound compartments in a cell-free extract. As it was not yet possible to express cloned genes in animal cells, we studied the transport of a membrane glycoprotein (G protein) that is copiously expressed during infection by vesicular stomatitis virus (VSV), then a very popular system that I had learned in Harvey Lodish's laboratory. The processing of G protein's oligosaccharide chains during passage through the Golgi also provided a necessary biochemical handle to follow potential transport in homogenates. The detailed pathway by which Asn-linked oligosaccharide chains are matured by processing had then only recently been uncovered by Stuart Kornfeld and others.

As shown in Figure 11, oligosaccharide processing entails the initial addition of a precursor oligosaccharide to the protein in the ER, followed by the sequential removal of certain glucose and mannose residues, and then the addition of the "terminal" sugars *N*-acetyl-glucosamine (GlcNAc), galactose, and

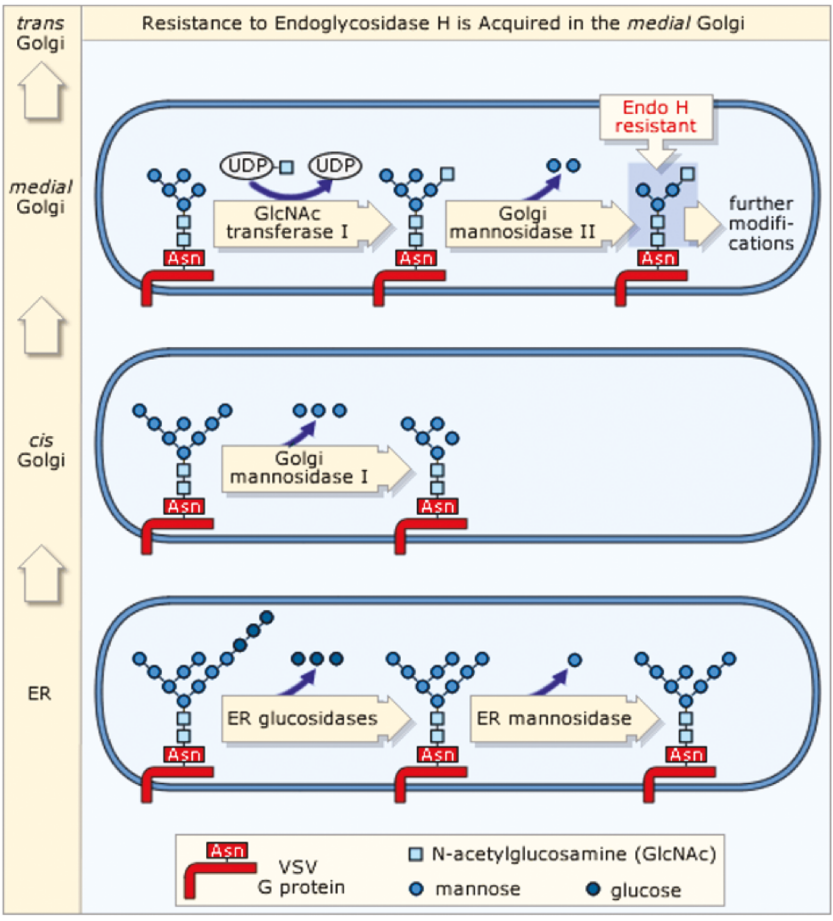

FIGURE 11. The pathway of processing in the Golgi of the Asn-linked oligosaccharide chains of ER-derived glycoprotein, elucidated in 1976–1978 most notably by Stuart Kornfeld. Figure 1. Processing of N-linked oligosaccharides in the secretory pathway. N-linked complexes become resistant to cleavage by Endoglycosidase H following addition of N-acetylglucosamine by GlcNAc transferase I and subsequent release of mannose residues by Golgi mannosidase II in the medial Golgi.

sialic acid at successive locations within the Golgi stack. Phillips Robbins had just worked out a neat shortcut for following saccharide processing using SDS protein gels that exploits an unusual microbial endoglycosidase (Endo H) that cleaves the precursor and immature saccharide chains characteristic of the ER and early Golgi, but which cannot cleave processed chains containing GlcNAc or other terminal sugars added later in the Golgi. Since the saccharide chain (except for the single inner GlcNAc that is directly linked to Asn) is removed, the

overall molecular weight of the glycoprotein is noticeably reduced and its band shifts on an SDS gel (to the G_S position). When only part of the population of G protein has entered and been processed in the Golgi, two bands are observed: the parent band (G_R, resistant to Endo H) and the shifted band (G_S, sensitive to EndoH). Earlier methods for analysis of saccharide chains, which involved multiple steps of fragmentation and chromatography that required days, were prohibitive for the routine enzyme assay we imagined that reconstitution of transport might become.

Erik began by radiolabeling VSV-infected hamster cells in tissue culture with ^{35}S-methionine for what we knew would be enough time (about 5 minutes) to allow newly synthesized G protein to enter the ER while hardly entering the Golgi. Then, we disrupted the cells (with the very Dounce homogenizer pictured in Figure 10, left), incubated the homogenate with ATP, and determined whether any of the Endo H-sensitive G protein present at the outset of the cell-free incubation in the ER had become Endo H-resistant (which would indicate transport from the ER to the Golgi).

Little, if any, G_R was produced. When we extended the labeling time, a small signal (G_R produced in the homogenate) did appear but it was quickly dwarfed by the ever-increasing amount of G_R present in the homogenate at the outset of cell-free incubation due to increasing amounts of G protein entering the Golgi in the cell. No amount of tinkering with the cell-free conditions improved this picture. Worse yet, we could not even be sure that our small signal represented transport taking place in the homogenate. The extra G_R produced *in vitro* could merely have resulted from completion of processing on G protein that had already reached the Golgi before cell disruption.

Facing this quandary led to the breakthrough. I realized that a mutant hamster cell line (clone 15B) defective in a specific glycosylation step in the Golgi could be harnessed in a variation of the above experiment, both to eliminate the background of Endo H-resistant G protein at the outset of the incubation and to ensure that any glycosylation during the incubation could only result from transport. That mutant, clone 15B, had been isolated by Stuart Kornfeld on the basis of its resistance to an ordinarily toxic plant lectin. It lacks the enzyme *N*-Acetyl-Glucosamine Transferase I (NAGT-I; also known as GlcNAc transferase), normally found in the central cisternae of the Golgi stack (Figure 11). As a result of their enzyme deficiency, 15B cells cannot process G protein to Endo H-resistance although they transport the partially processed G protein normally to the cell surface. G protein therefore remains Endo H-sensitive in the ER, Golgi, and in the plasma membrane of 15B cells. So, when homogenates of ^{35}S-methionine-labeled, VSV-infected 15B cells are incubated, the G protein

in their membranes will always remain Endo H-sensitive, even if it were to undergo further transport.

The critical variation (Figure 12) was simply to incubate two homogenates together—one (the "donor") produced from *VSV-infected* 15B mutant cells, and the other (the "acceptor") produced from *uninfected* wild-type cells. Now it is possible for Endo H-sensitive G protein (originating in donor 15B membranes) to be processed by NAGT-I (present in acceptor wild-type cell membranes) and thereby be made Endo H-resistant. For example, if vesicles carrying the G_S protein were to bud off from donor ER membranes and fuse with the Golgi membranes from the acceptor homogenate, then the transported G_S would be converted to G_R. A *bona fide* signal in this revised cell-free reaction explicitly requires that proximity relationships in the cell are not essential for transport, since transport would take place between organelles derived from separate cells. So any signal would suggest that inherent chemical specificity is the key to sorting, not intracellular anatomy.

With this new "complementation assay" design, we could indeed find *in vivo* labeling conditions that allowed cell-free processing at about the same rate and with about the same efficiency as transport in the cell (Figure 13b). As in the cell, cell-free "transport-coupled glycosylation" is ATP-dependent and occurs between closed membrane-bound compartments, the latter shown by the resistance of the lumenally-oriented spike portion of G protein to external proteolytic attack (Fries and Rothman, 1980). The first successful *in vivo* labeling

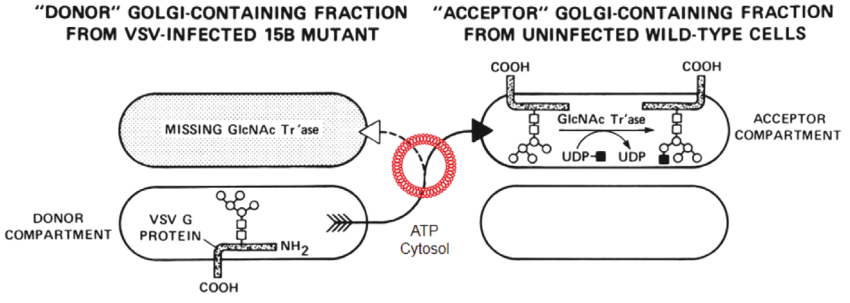

FIGURE 12. The "complementation" method that finally gave successful results. Assay for transport of VSV G protein to the medial Golgi. 15B cells lack GlcNAc transferase I; thus, proteins in these cells never acquire Endo-H resistance, although they are transported through the secretory pathway. To assay for transport, the Golgi-containing fraction from (radiolabeled) VSV-infected 15B cells is incubated with the Golgi-containing fraction from uninfected wild-type cells (plus ATP and cytosol). Acquisition of Endo-H resistance by G protein is a measure of the extent of its transfer from donor compartments to the medial Golgi. The red-walled circle between the two Golgi stacks indicates that transport vesicle that we inferred to bud and fuse in order to account for our results.

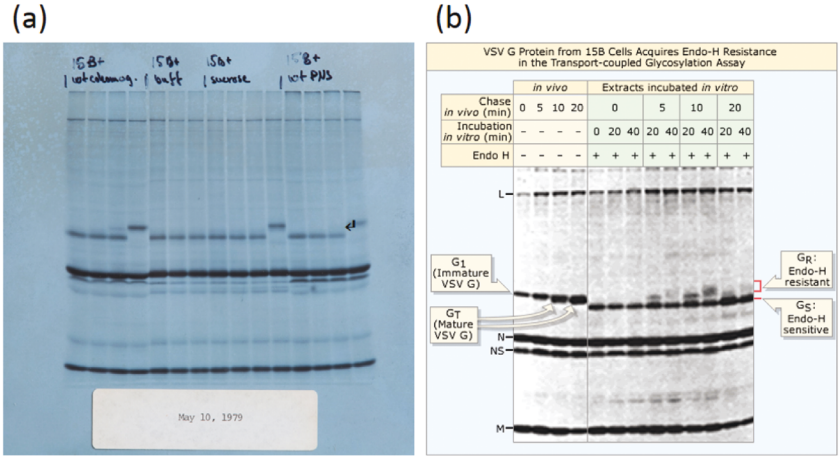

FIGURE 13 A). The first successful reconstitution of cell free transport. From Erik Fries' laboratory notebook of May 20, 1979. The faint band above the main VSV G band in the second lane from the far right (arrow) is due to maturation of the G protein (derived from donor membranes) in the acceptor Golgi compartment. b) Further optimization of incubation conditions improved the yield of the significant Endo H-resistant band for the published work. Figure 4. Left panel: VSV-infected 15B cells were pulse-labeled with ^{35}S-methionine for 5 minutes, then incubated in medium with unlabeled methionine for 0, 5, 10 or 20 minutes, and homogenized. Extracts were applied directly to the gel. Note that in 15B cells the mature G protein has a slightly lower apparent molecular weight than the immature G protein. L, N, NS and M are non-secreted VSV proteins. Right panel: VSV-infected 15B cells were pulse-labeled, and the radioactivity was chased as indicated for the samples in the left panel. Cells were homogenized, and the Golgi-containing fraction was incubated with the Golgi-containing fraction from uninfected, unlabeled wild-type cells for 0, 20 or 40 minutes, then treated with Endo-H. Note that by 5 minutes chase in vivo and 20 minutes incubation in vitro, Endo-H resistant G protein is detected. Adapted from Fries and Rothman (1981).

conditions involved a short "pulse"-label with ^{35}S-methionine followed by a 20-minute period of "chase" with unlabeled methionine in the presence of a proton ionophore "uncoupler" that stops transport by inhibiting ATP synthesis in mitochondria (Figure 13a). That ATP (or other NTP) is required for transport had been found in 1968 by James Jamieson and George Palade, who used a similar protocol to block exit from the ER at its "transitional elements," specialized regions where vesicles appear to bud off from the Golgi. With this background, the simplest working hypothesis was that we had reconstituted transport from transitional elements of the ER (from 15B cells) to the Golgi (from wild-type cells). However, we also recognized that the identity of the donor compartment

was not firmly established as the ER and could be a later compartment (Fries and Rothman, 1980).

Indeed, the latter proved to be the case. We subsequently found (Fries and Rothman, 1981) that transport could be reconstituted without ATP depletion simply by extending the *in vivo* chase period by enough time (5–10 minutes) to allow the ^{35}S-labeled G protein to reach the 15B cell Golgi before homogenization. This implied that the donor is the Golgi—not the ER or its transitional elements. Since the acceptor is also a Golgi membrane, it followed that transport between two Golgi stacks, one from the 15B cells and the other from the wild-type cells, had been reconstituted.

This was very surprising because it was then textbook knowledge that the Golgi cisternae flow across the stack from its "immature" or "forming" (now termed "cis") face to its "mature" (now termed "trans") face. This view had been based on anatomical observations rather than functional evidence. (Now, more than three decades later, it is increasingly clear that transport across the Golgi stack in animals indeed mainly the result of vesicle transport, though some still adhere to cisternal flow models). The straightforward interpretation of our data was that transfer between Golgi compartments can be mediated by vesicles, and that we had reconstituted this process. Unfortunately, this put us in the unenviable position of having reconstituted a process not then known to exist, a dual burden that slowed acceptance of the significance of these results for many years until we (Wilson et al., 1989) ultimately found that one of Randy Schekman's yeast secretion genes (Novick et al., 1980) was the protein required for vesicle fusion in cell-free extracts, NSF.

We did, however, have some powerful criteria to suggest that specific membrane fusion was important for the assay signal. As seen in Figure 13b, Endo H-resistance was observed in samples that had been labeled with a 5-minute pulse followed by a 5-minute chase *in vivo* before homogenization and incubation with acceptor membranes. At this time point, much of the labeled VSV G is known to be present in the Golgi. However, no Endo H resistant G protein is produced *in vitro* when cells were disrupted right after the 5-minute pulse (no chase); at this time point, the labeled VSV G is still in the ER. And importantly, with longer times of chase, as G protein is progressively depleted from the donor Golgi as it transfers to the plasma membrane before cell disruption, and cell-free transport is correspondingly attenuated. Re-examination of our first experiments confirmed that even though energy production was poisoned, this did not occur instantly, and transport had continued during the several minutes required for the cell to use all of its existing ATP. This period was ample to permit much of the G protein to enter the Golgi, thereby reconciling the two

experiments. (It now turns out that transport requires only about 10 mM ATP whereas cells normally maintain ATP in the low millimolar range, so transport can continue until the cell has used up most of its ATP.)

The strong dependence of the efficiency of cell-free transport on the presence of VSV G protein in Golgi vs. other cellular membranes convinced us that this was a *bona fide* reconstitution, and not the result of non-specific membrane fusion. More detailed analysis (Balch et al., 1984a; Balch, Glick, and Rothman, 1984b; Braell et al., 1984) soon confirmed this interpretation and provided key improvements. Adding UDP-[^{3}H]GlcNAc (the donor of GlcNAc for glycosylation by NAGT-I) marks each transported G protein with a fixed quantity of radioactivity as it arrives in the acceptor Golgi. Transport is then simply measured by the amount of [^{3}H]-G protein produced. This improvement, made together with then-postdoctoral fellow William Balch (Balch et al., 1984a), massively improved the signal-to-background and the dynamic range of the assay, and made

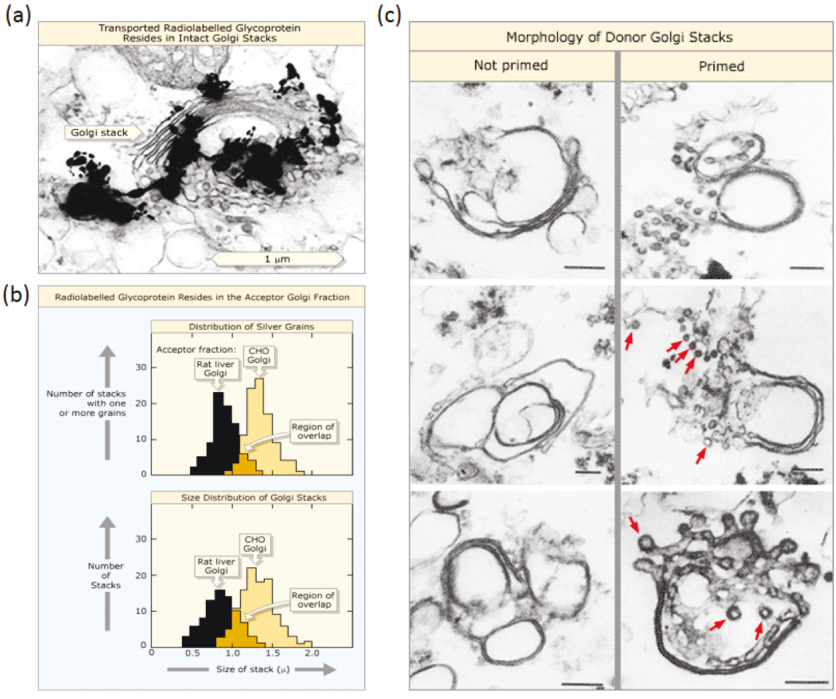

FIGURE 14. (a) Electron microscope autoradiograph showing that protein containing ^{3}H-GlcNAc is associated with Golgi stacks. The Golgi-containing fraction from VSV-infected 15B cells was incubated with the Golgi-containing fraction from wild-type cells that had taken up UDP-^{3}H-GlcNAc, and the mixture prepared for electron microscope autoradiography. Adapted from Braell et al. (1984).

it possible to accurately measure initial rates. This, in turn, made the assay a practical springboard for enzymology to guide the purification and discovery of required components.

With ^{3}H to indicate the transported G protein, we could now use autoradiography of electron microscope sections to localize the acceptor site where the labeled protein resides (Figure 14a). This analysis, performed by postdoctoral fellow William Braell (Braell et al., 1984), confirmed that the glycosylated G protein resides in morphologically-intact Golgi stacks derived from the acceptor homogenate. Thus, the donor- and acceptor-derived Golgi stacks remain as two distinct and unaltered populations, and the processed G protein resides exclusively in the acceptor-derived Golgi population (Figure 14b, forcefully implying that G protein is transferred between them by vesicles. We then discovered by electron microscopy that 70–90 nm-diameter vesicles containing G protein form at the donor Golgi stacks (Figure 14c) (Balch, Glick, and Rothman, 1984b). Later work, with Lelio Orci of the University of Geneva, directly demonstrated that these vesicles (now termed COPI-coated vesicles) contain VSV G protein and are captured by the acceptor stacks and thereby equilibrate between the two populations (Orci et al., 1989).

THE BASIC PRINCIPLE OF VESICLE BUDDING

Having reconstituted for the first time the process of vesicle budding, we could now apply the methods of enzymology to identify the responsible proteins and learn their mechanism of action. This in turn required the ability to visualize budding intermediates to enable the site of action of responsible proteins to be established.

By good fortune, having just read our three back-to-back papers in *Cell* (December, 1984) describing biochemical intermediates and the first images of vesicles formed in cell-free extracts, the great electron microscopist Lelio Orci (Figure 15) reached out to me in a phone call over the Christmas holidays. Thus began what turned out to be a long-term and exceptionally productive collaboration which made it possible to use electron microscope immunocytochemistry to more precisely delineate the nature and composition of the intermediates in transport. Soon, together with a graduate student, Benjamin Glick, we confirmed that the budding transport vesicles contained VSV G protein in their membranes and found that they had a coat on their cytoplasmic surface distinct from the then-known clathrin coat (Orci et al., 1986).

The essential step in establishing the budding mechanism stemmed from my finding (during a brief sabbatical in William Balch's lab, then at Yale) that

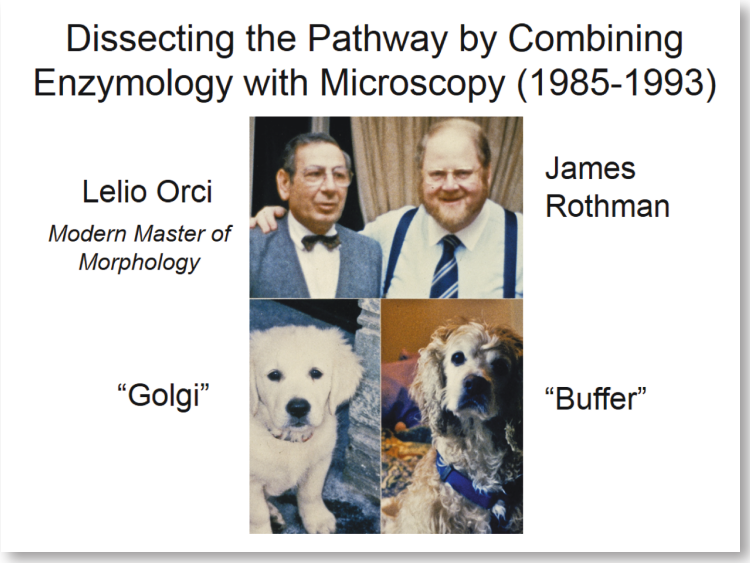

FIGURE 15. My long-term collaborator and friend Lelio Orci, the modern master of morphology. Together, between 1985 and 1993 we combined morphology and enzymology using the cell-free system to dissect a cycle of vesicle transport and in the process discovered COPI-coated vesicles and the GTPase switch mechanism that governs transport vesicle budding and uncoating for fusion. Below us our our treasured dogs whimsically named in the midst of this period to connote the interface between biochemistry (our dog "Buffer") and morphology (his dog "Golgi").

transport was inhibited by a non-hydrolyzable analog of GTP, GTPγS (Figure 16). Lelio and I and a postdoctoral fellow, Paul Melancon, then found (Melancon et al., 1987) that GTPγS blocks uncoating, so that the 'COP-coated' transport vesicles (as we termed them, now re-named COPI to later make way for Randy Schekman's COPII vesicles (Barlowe et al., 1994)) massively accumulate, which enabled their purification (Figure 17) by Vivek Malhotra and Tito Serafini (Malhotra et al., 1989). From the isolated vesicles came two central findings: the seven subunit 'coatomer' (Waters et al., 1991) that assembles to constitute the coat; and the discovery that the GTPase ADP Ribosylation Factor (ARF) is present along with coatomer in stoichiometric amounts, explaining the previously mysterious effect of GTPγS. The latter observation also pointed the way to the basic principle underlying the budding mechanism (Serafini et al, 1991; Tanigawa et al., 1993; Ostermann et al., 1993): GTP-bound ARF recruits the coatomer to the Golgi (triggering coat assembly and vesicle budding), and releases it back to the cytosol after it hydrolyzes the GTP (uncoating).

Incubation	[^{3}H] GlcNAc Incorporated into VSV-G Protein	
Complete	3500 cpm	
- ATP	50	
- Cytosol	75	
- Golgi membranes	95	
+ GTPγS (10 μM)	420	Transport Vesicles Accumulate
NEM-membranes	225	

FIGURE 16. The requirements for cell-free transport of VSV G protein between Golgi stacks, using the simplified assay developed with Bill Balch (Balch et al., 1984). In later work, with Lelio Orci, we found two specific inhibitors of transport (GTPγS and NEM) which accumulate transport vesicle intermediates at distinct stages of maturation.

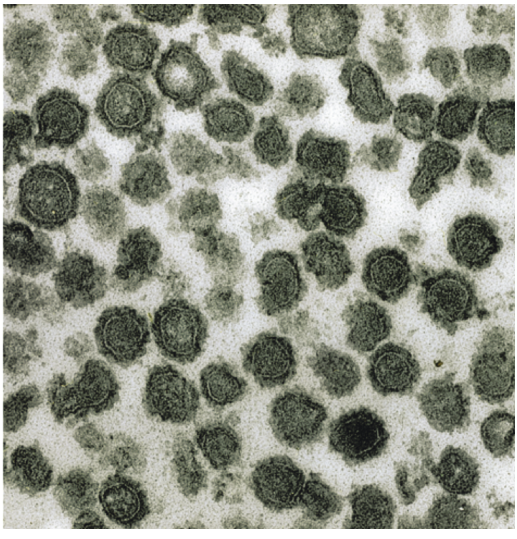

FIGURE 17. Inhibition of transport by GTPγS accumulates intermediate ~ 70 nm diameter transport vesicles encased in a protein coat (originally termed COP-coated vesicles and now termed COPI) containing the cargo VSV G protein. A pure fraction of COPI-coated vesicles produced in cell-free incubations of Golgi membranes (Malhotra et al, 1989) made possible by the blockage of uncoating by non-hydrolyzable analogues of GTP. This key development allowed the discovery of the coat protein subunits (coatomer) and the role of the GTPase switch of ARF protein in triggering sequential assembly and disassembly of the coat (Figure 18).

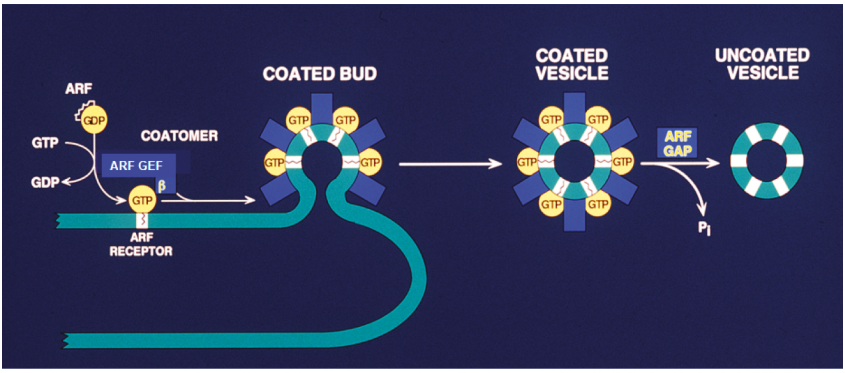

FIGURE 18. The GTP-switch mechanism for sequential budding and uncoating of transport vesicles for membrane fusion. Rothman, Orci and co-workers 1991–1993. See text for details.

By 1993, the validity of this simple and intuitive mechanism was confirmed using pure proteins (Orci et al., 1993). ARF is charged with GTP at the Golgi surface, 'switching on' budding by recruiting coatomer from the cytosol. Coatomer, now locally concentrated and oriented on the membrane surface, self-assembles by polymerization into the coat, including ARF [GTP]. The growing coat acts as a mechanical device to sculpt the applied membrane into the shape of a vesicle whose size is determined by the inner diameter of the coat. The coat now forms an exoskeleton that must be shed to enable the enclosed vesicle to fuse, which occurs when ARF hydrolyzes GTP (Figure 18).

The same principle extends to clathrin-coated vesicles (Stamnes et al., 1993) and to COPII-coated vesicles budding from ER, as found by Orci and Schekman (Barlowe et al., 1994) The particular ARF GTPase family member used and the species of coatomer varies, allowing diversity in physiologic regulation (by GTP exchange/hydrolysis) and in cargo selection (by binding subunits of the coat). But in all cases cycles of GTP binding and subsequent hydrolysis promote unidirectional (vectorial) cycles of vesicle budding and uncoating for membrane fusion.

THE CYTOSOLIC PROTEINS ENERGIZING MEMBRANE FUSION

The identification of the first proteins needed for membrane fusion also stemmed directly from the cell-free reconstitution of protein transport. This part of the story begins in 1987 with the finding by my graduate student Benjamin Glick

(Glick et al., 1987) that cell-free transport is blocked (Figure 16) by low concentrations of the sulfhydryl alkylating reagent N-ethylmaleimide (NEM). Felix Wieland (then on a sabbatical from Regensburg) and a postdoctoral fellow, Mark Block, then purified the N-ethylmaleimide-sensitive factor (NSF) from cytosol of CHO cells based on its ability to restore transport following NEM inactivation (Block et al., 1988). Electron microscopy and other tests revealed that NSF is required for fusion since vesicles accumulate after NEM inhibition (Figure 19; Malhotra et al., 1988). We soon appreciated that NSF is an ATPase and that NSF and ATP hydrolysis are required for vesicle fusion at many compartments in the cell, and that it is extremely well conserved in evolution. As noted above, our identification of NSF as the animal equivalent of Schekman's Sec18 yeast gene was pivotal because it cemented the physiologic relevance of the mechanistic results from the cell-free system and foreshadowed the universality of the fusion mechanism (Wilson et al., 1989).

Because NSF is a soluble cytoplasmic protein, it must bind to membranes to function in the fusion process. How this happens was clarified with the identification of *S*oluble *N*SF *A*ttachment *P*rotein (SNAP) which was purified according to its ability to bind NSF to Golgi membranes (as diagrammed in Figure 20) by my graduate student Douglas Clary (Clary et al., 1990).

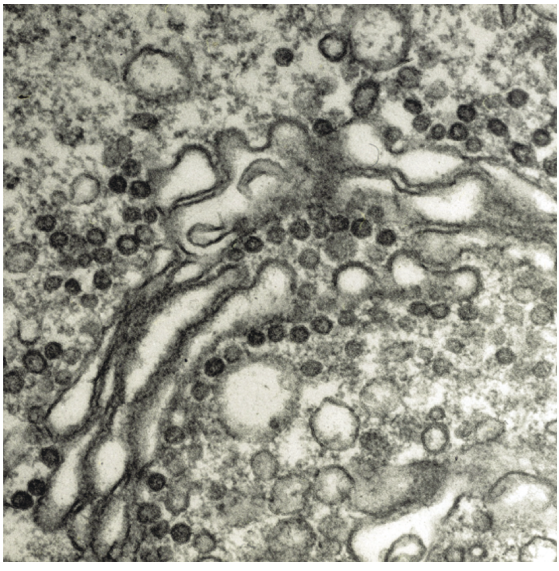

FIGURE 19. NEM inhibition accumulates ~ 70 nm diameter uncoated vesicles containing VSV G protein that fail to fuse. From Malhotra et al. (1988).

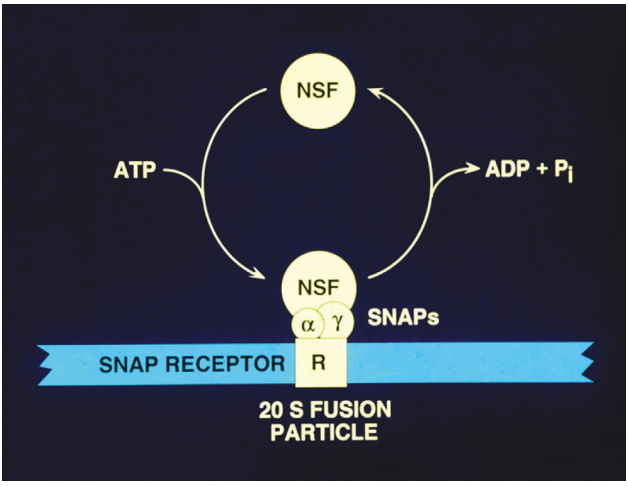

FIGURE 20. Cycle of 20S particle assembly and disassembly. NSF, SNAPs and SNAREs form hetero-oligomeric complexes, then termed 20S particles. ATP hydrolysis by NSF dissociates the 20S particles, regenerating the individual components for another round of membrane fusion.

THE DISCOVERY OF THE SNARE COMPLEX

How, then, does SNAP—which is also a cytosolic protein—bind to membranes? SNAP binds to one or more saturable, high affinity "*SNA*P *RE*ceptor*s*" ("which we termed SNAREs") on Golgi membranes before binding to the ATP-bound form of NSF. This complex of NSF, SNAP and SNAREs sediments as a 20S particle after extraction from membranes with mild detergents. When NSF hydrolyzes the ATP, it releases itself from the complex (Wilson et al., 1992).

It seemed likely that SNAREs would be directly inserted into membranes because the SNAP receptors retain their ability to bind SNAP, even after extraction of membranes with strong alkali, a harsh treatment that removes all but integral membrane proteins (Weidman et al., 1989). That put purification of this membrane protein(s) at the very top of our agenda because of the expectation that lipid bilayer fusion would require membrane-anchored proteins. The SNARE proteins thus became the prime candidates for the fusion proteins.

At this critical juncture in 1991, I was joined by Thomas Söllner, (Figure 21, right), a gifted scientist who had just arrived in New York City at Sloan-Kettering, where I had just then moved to found the Cellular Biochemistry and Biophysics Department. Thomas had just completed foundational work identifying

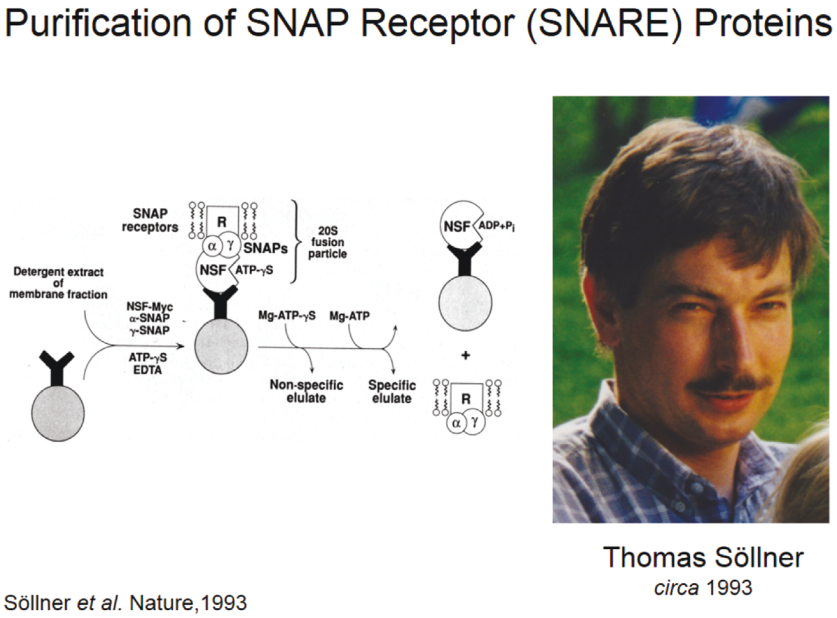

FIGURE 21. Purification of SNARE proteins. At left, recombinant SNAP, and epitope-tagged NSF were assembled into 20S particles together with SNAREs derived from detergent-solubilized membrane fractions in the presence of the non-hydrolizable ATP analogue, ATPγS. The 20S particles were then immobilized on beads via an antibody directed to the epitope-tagged NSF, washed in the presence of MgATPγS ("non-specific eluate") and then disassembled in the presence of MgATP, releasing SNAPs and SNAREs ("specific eluate"). NSF remains bound via the antibody to the beads. At right, Thomas H. Söllner (circa 1993), who joined me at Sloan-Kettering and with whom I discovered the SNARE complex and enjoyed a productive collaboration in the ensuing decade that established the SNARE hypothesis for specific membrane fusion. Thomas is now a professor of Biochemistry at the University of Heidelberg. It was my pleasure that he could join the Nobel lecture, and that I could introduce him on that occasion.

key proteins in the mitochondrial outer membrane needed for protein import, learning biochemistry as a PhD student with Walter Neupert in Munich. This was another very fortunate event, and the beginning of a very productive collaboration that was to last a decade during which we would suggest and then test the tenets of the SNARE Hypothesis.

The meaning of the seemingly futile cycle (Figure 20) of membrane binding and ATPase-driven release of NSF was unclear at the time Söllner joined the Rothman lab. Then, we had imagined that energy from hydrolysis of ATP somehow activated the membrane-anchored SNAREs to power fusion. However, the existence of the binding-release cycle had a huge impact on our strategy for

identifying the SNAREs. The assembly and disassembly of 20S particles, involving binding and release of NSF from SNAP, respectively, could be exploited as sequential affinity purification steps to isolate SNAREs (Figure 21, left). Previous experiments had shown that standard chromatographic methods and a single affinity step were inadequate for isolating SNAREs; the 20S ATPase cycle would add a second level of biological specificity.

SNARE (i.e. SNAP-binding) activity could be found in crude membrane fractions from homogenates of various cell lines and animal tissues, in addition to the purified Golgi membranes in which it was originally detected. It turned out that brain homogenates have the highest specific SNARE activity of the tissues that were tested, and large quantities of SNAREs could be easily obtained. These are, of course, the classic criteria for choosing a source for protein purification, but in our case the choice of brain would soon prove to have been most fortunate for unexpected reasons. Grey matter was homogenized, the total membrane fraction was isolated by centrifugation, and then a "soluble" protein extract was prepared by treating the membrane pellet with a detergent. This detergent extract contained SNARE activity as well as the bulk of integral membrane proteins now "solubilized"; i.e., distributed among micelles of the detergent.

The assembly arm of the NSF cycle was then utilized on a preparative scale as the first of two biologically specific steps, depicted in Figure 20. The idea was that SNAREs would be sequestered from the bulk of membrane protein by incorporation into 20S complexes formed with exogenously added, purified recombinant (bacterially-expressed) NSF and SNAP proteins. This incubation would be done in the presence of ATPγS (a non-hydrolyzable analogue of ATP) and in the absence of free magnesium ion (Mg^{++} is required for hydrolysis of ATP by NSF) to promote 20S particle assembly. The recombinant NSF was expressed with a short peptide epitope from myc to allow the 20S particles to be isolated with a monoclonal antibody (immobilized on beads) directed against this myc tag.

The second biologically-specific step recapitulated the disassembly of 20S particles. As depicted in Figure 21 (left), SNAREs would be released when the beads are incubated with magnesium ion and ATP to allow NSF to hydrolyze ATP. Recombinant myc-tagged NSF would remain bound to the beads by the antibody, but the recombinant SNAP proteins would be released along with the SNAP binding proteins from the brain membranes.

Because vesicle fusion occurs at many membrane compartments, we had suspected that cells would have a large family of SNARE proteins, related in sequence and differing in location. We were therefore surprised when the SNAREs

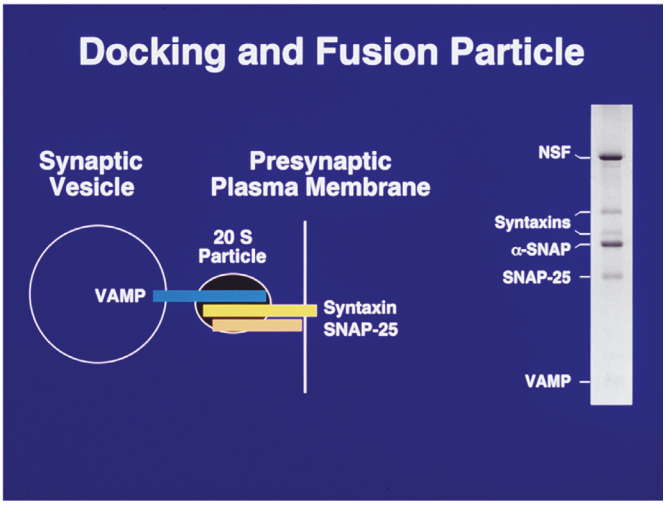

FIGURE 22. The SNARE complex, known at the time of its discovery as the docking and fusion particle. At right, the specific MgATP-eluate was analyzed by polyacrylamide gel electrophoresis. Proteins were revealed by staining with Coomassie blue and then identified by amino acid sequencing and mass spectroscopy. The bands at the top of the gel were also observed in the nonspecific eluate. At left, our interpretation on which a complex of the SNARE proteins links the synaptic vesicle to the plasma membrane to firmly dock the vesicle and initiate fusion.

derived from whole brain yielded a remarkably simple protein pattern (shown in Figure 22, right) consisting of only four proteins, each present in the specific (MgATP) eluate and absent from the non-specific (MgATPγS) eluate (Söllner et al., 1993a).

The identity and purity of these membrane proteins was established by micro-sequencing and by mass spectroscopy of peptides derived from the very small amount of material we had isolated, made possible by the expert protein chemistry of Paul Tempst at Sloan-Kettering. Amazingly enough, all four SNAREs turned out to be proteins found in synapses (Figure 22, right). Although they had all previously been cloned and sequenced, their function was still unknown. Two are isoforms of syntaxin, a plasma membrane protein independently identified by Richard Scheller (Bennett et al., 1992) and Kimio Akagawa (Inoue et al. 1992). The third SNARE protein is SNAP-25, short for synaptosome-associated protein of 25 kDa, cloned by Michael Wilson (Oyler et al., 1989). SNAP-25 mainly resides in the plasma membrane and was originally identified because of its abundance in synapses. Its connection to syntaxin and to membrane fusion was a surprise, as was the coincidental relationship of its acronym to that of the soluble NSF attachment protein, SNAP.

VAMP/Synaptobrevin-2 was the last SNARE protein to emerge. It had been cloned independently by Pietro DeCamilli and Reinhard Jahn, and by Scheller (Baumert et al., 1989; Elferink et al., 1989). In contrast to SNAP-25 and syntaxin, VAMP resides mainly in synaptic vesicles.

The discovery of VAMP in the complex was the lynchpin observation because it immediately suggested how the complex of SNARE proteins (perhaps with NSF and SNAP) could be important for membrane fusions. Since VAMP protrudes from the vesicle membrane into the cytosol, and syntaxin and SNAP-25 likewise protrude from the plasma membrane, a complex involving all three integral membrane proteins could bring the vesicle to the plasma membrane, placing their lipid bilayers within molecular contact range (Figure 22, left).

THE SNARE HYPOTHESIS AND THE BASIC PRINCIPLE OF MEMBRANE FUSION

Instead of limiting ourselves to the special point-of-view of synaptic vesicle exocytosis we chose to interpret the SNARE complex more speculatively from a very broad perspective (Söllner et al., 1993a).

First principles require that vesicles and targets somehow be marked to indicate which vesicles will fuse where. This, in turn, indicates that vesicle and target markers must be matched pairwise. We suggested that the simplest mechanism for matching is self-assembly, in which only matching pairs of "cognate" vesicle ('v') and target ('t') markers bind each other between membranes, thereby forming a 'v-t' complex prerequisite for membrane fusion.

Based on our cognate vesicle and target marker concept, we proposed the "*SNARE hypothesis*" in which the SNAREs are the vesicle and target markers, which we termed v–SNAREs and t-SNAREs (Figure 23). VAMP is the v-SNARE of the synaptic vesicle; syntaxin and SNAP-25 are the subunits of the cognate t-SNARE in the plasma membrane. The SNARE hypothesis provides the framework to generalize our results. We suggested that each type of vesicle in the cell would have its own characteristic v-SNARE, a homologue of VAMP, and that each target membrane in the cell would be marked by a characteristic t-SNARE, having subunits homologous to syntaxin and SNAP-25. In addition, we suggested that "In the simplest view, that is, if there were no other source of specificity, only when complementary v-SNARE and t-SNARE pairs engage would a productive fusion event be initiated" (Söllner et al., 1993a).

Consistent with our simple model, VAMP and syntaxin are membrane-anchored proteins with cytoplasmic domains, and SNAP-25 is anchored to the cytoplasmic side of the plasma membrane via covalently-attached fatty acids. Close to equimolar amounts of VAMP, syntaxin (its two isoforms considered together) and SNAP-25 were recovered in the isolated complexes. Furthermore,

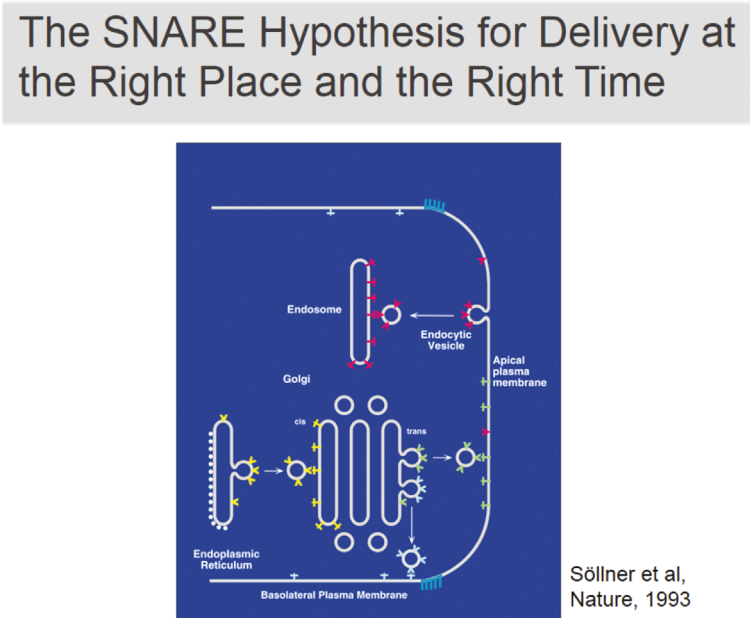

FIGURE 23. The SNARE Hypothesis as initially proposed, explained in the text.

the SNARE proteins were isolated because they bind to and form a 20S particle with NSF and SNAPs, which are known to function in fusion, implying that SNAREs also function in fusion. The SNARE complex progressed from first discovery to final publication in a dizzying sweep lasting only 5 weeks.

At the time of this experiment, membrane fusion seemed complex and confusing because a conceptual framework with which to organize the continuously increasing list of genes and sequences was lacking. A great many genes and proteins of yeast and animal cells, including neurons, were implicated as being somehow involved in the overall process of vesicle transport or fusion or its regulation. It was readily appreciated that many of these genes and proteins belong to evolutionarily-conserved families affecting different transport steps (reviewed by Bennett and Scheller, 1993). A dozen or more proteins were known to reside in the synaptic vesicle alone. But it was guess work as to which proteins could catalyze fusion or provide for its specificity, as distinct from affecting fusion indirectly at the level of cellular regulation, and many proteins had been considered to be candidates for fusion, including at one time or another synaptophysin, synaptoporin and SV2. Interestingly, although VAMP and syntaxin were seen to be important players, they were not highlighted in this context and not suggested to form a complex, and SNAP-25 was not connected to exocytosis.

The primary impact of our paper stemmed from its combination of an unexpected discovery—the SNARE complex—and a broad and clearly-stated concept—the SNARE hypothesis—deduced from it. Its impact was amplified because the discovery of the SNARE complex firmly linked three fields (cell biology (vesicle transport), physiology (endocrine and exocrine secretion), and neurobiology (synaptic transmission)), three disciplines (cell-free biochemistry, yeast genetics, and electrophysiology), and many favorite cells and organisms. As a result our paper changed the focus of cell biology, away from differences in physiology and regulation and on to core machinery and universal mechanisms.

In follow-up work we found that NSF and SNAP function to disrupt the SNARE complex using energy derived by ATP hydrolysis (Söllner et al, 1993b), and it was later shown by William Wickner that SNAP and NSF are not directly involved in bilayer fusion (Mayer et al., 1996). This focused attention on the simplest remaining possibility, that the SNARE complex is all that is needed to mediate fusion. However, NSF and SNAP play a critical role in sustaining ongoing fusion. They separate v-SNAREs from t-SNAREs after fusion (i.e., when they reside in the *same* bilayer), but not during fusion (i.e., when they are paired *between* bilayers) (Weber et al., 2000). This allows NSF and SNAP to recycle SNARE complexes after fusion while sparing fusion in progress.

The SNARE complex is extraordinarily stable, resisting heat denaturation up to 90°C (Hayashi et al., 1994). The rod-like structure of the SNARE complex, with its membrane anchors at one end, implies that it could bring two membranes into close contact and it was suggested that the binding energy from SNARE assembly could drive bilayer fusion (Hanson et al., 1997).

A direct test of the possibility that the SNARE complex is the active principle of fusion could only come from assessing this function in the absence of all other proteins. Reconstituting recombinant exocytic/neuronal SNAREs into liposomes established that the pairing of cognate SNARES between lipid bilayers indeed results in spontaneous membrane fusion (Weber et al., 1998) (Figure 24). Thus, when complementary v-SNARE and t-SNARE pairs engage, a productive fusion event is not only initiated—as we had first imagined—but it is also completed.

The ability to observe fusion by isolated SNAREs opened the door to a very direct test of the central tenet of the SNARE hypothesis, that specificity for membrane fusion is encoded in the physical chemistry of the isolated SNARE proteins (McNew et al., 2000; Parlati et al., 2000; Fukuda et al., 2000; Paumet et. al, 2001; Parlati et al., 2002). A total of 275 combinations of the potential v-SNAREs and t-SNAREs encoded in the genome of yeast, representing ER,

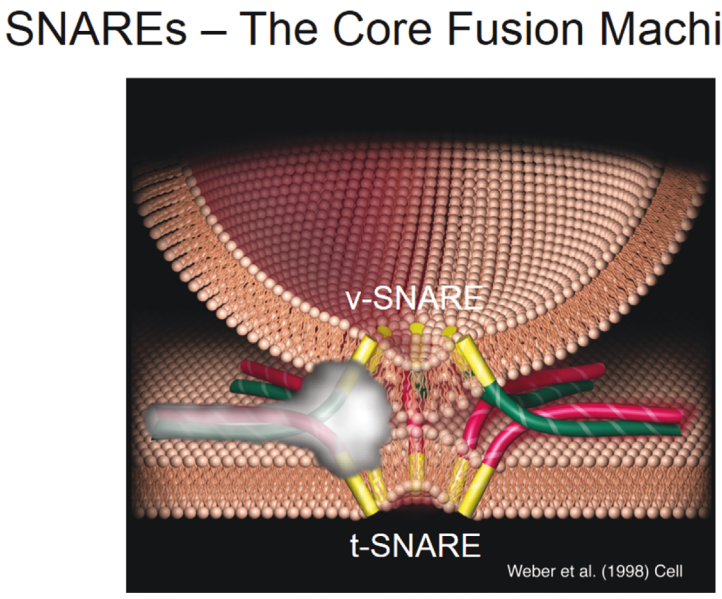

FIGURE 24. v-SNAREs (in green) on a vesicle bind to their cognate t-SNAREs (in red) on the target membrane, forming specific SNAREpins that then fuse the two membranes. For simplicity, the t-SNARE is shown as a single elongated rod, although it is now known to contribute three alpha helices to a four-helix v-t-SNARE bundle. Other proteins regulate the assembly and disassembly of SNAREpins and thus control membrane fusion.

Golgi, plasma membrane, endosomes, and vacuoles (lysosomes), have been tested for fusion. Of these, only 9 combinations (~3%) are fusogenic and all but one (~0.4%) correspond to known transport pathways (Figure 25). Virtually without exception, fusion only takes place with the rare combinations of v- and t-SNAREs that are drawn from compartments connected by vesicle shuttles in the living cell. Put differently, a physical chemist armed only with the DNA sequence of yeast and the SNARE hypothesis could test isolated SNAREs to read out the fusion potential and transport pathways allowed in the cell with at least 99.6% accuracy.

THE MECHANISM OF LIPID BILAYER FUSION BY SNAREPINS

The physical chemical mechanism of fusion was strongly suggested by the X-ray crystal structure of the SNARE complex elucidated by Axel Brunger and Reinhard Jahn later that year (Sutton et al, 1998). It revealed a bundle of four parallel alpha helices that forms a pin-like arrangement forcing the two bilayers together as the SNARE complex "zippers up" to result in fusion (Figure 26). We termed these "SNAREpins." Very recently, in collaboration with Yale colleague

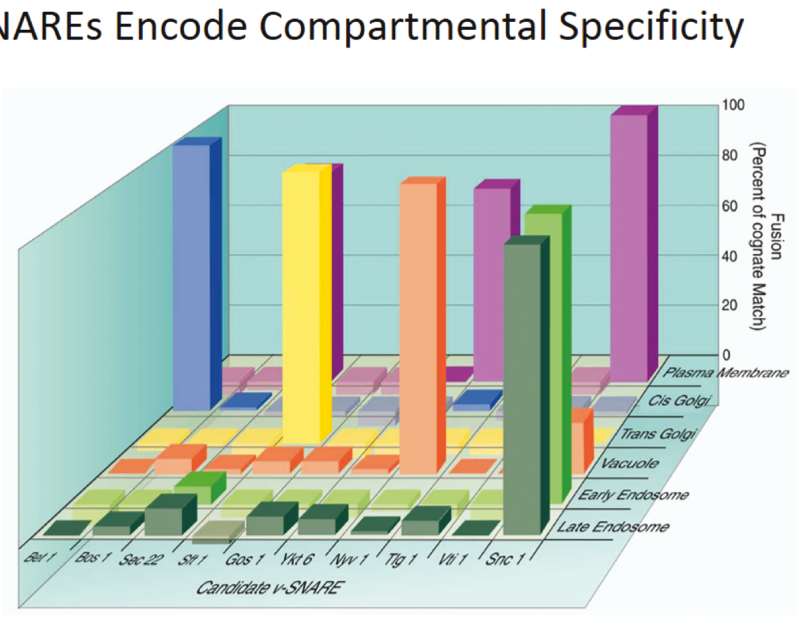

FIGURE 25. Proof that SNAREs encode compartmental specificity was obtained from a large scale experiment (200–2002) in which the complement of SNAREs encoded in the yeast genome was tested for its fusion potential in many combinations in reconstituted lipid vesicles. See text for details.

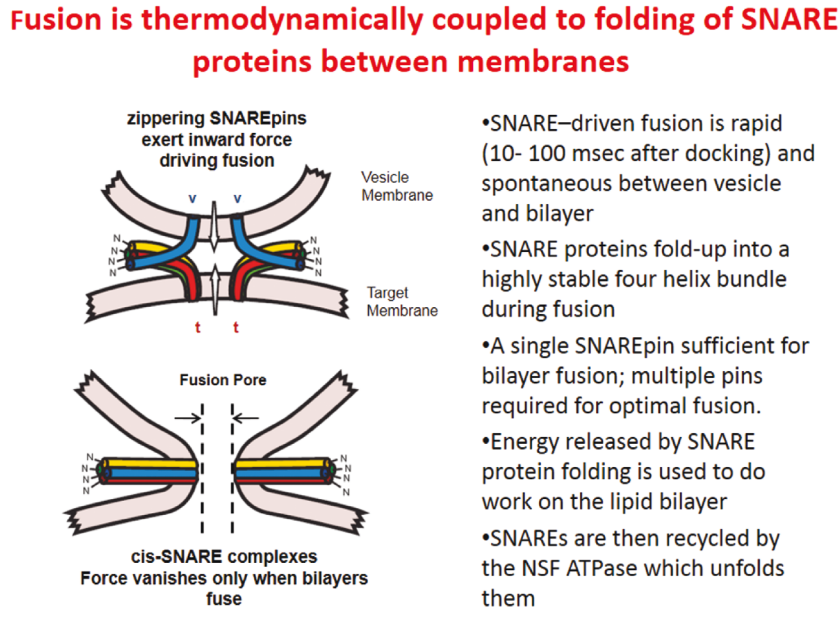

FIGURE 26. Summary of current knowledge of the fusion mechanism. Adapted from Südhof and Rothman (2009).

Yongli Zhang, we have directly measured the force that fuses the bilayers in single molecule experiments (Figure 27) in which a SNARE complex is literally pulled apart and the allowed to zipper back up again—the power stroke of fusion—which it does in two discrete steps (Gao et al., 2012).

The half-zippered intermediate provides a natural pause point that can be stabilized by binding to other proteins to permit regulation of membrane fusion after vesicle docking. An important example is provided by the protein complexin (McMahon et al., 1995), discovered by Thomas Südhof, along with the calcium ion sensor synaptotagmin (Geppert et al., 1994)) to be a key regulator of neurotransmitter release in synaptic transmission. In a recent collaboration with Yale colleague Karin Reinisch (Kümmel et al., 2011), we found that complexin stabilizes exactly this half-zippered state (Figure 28), explaining how synaptic vesicles can be ready to release neurotransitters much in < 1 msec, much faster than the time (50–100 msec) required for the overall process of fusion by isolated SNAREs after initial docking by the SNAREpin (Karatekin et al., 2010).

With my current laboratory colleagues in the Department of Cell Biology at Yale (Figure 29) I am primarily hoping to better understand how SNARE proteins are regulated in exocytosis and the still debated details of the dynamics of protein sorting in the Golgi stack.

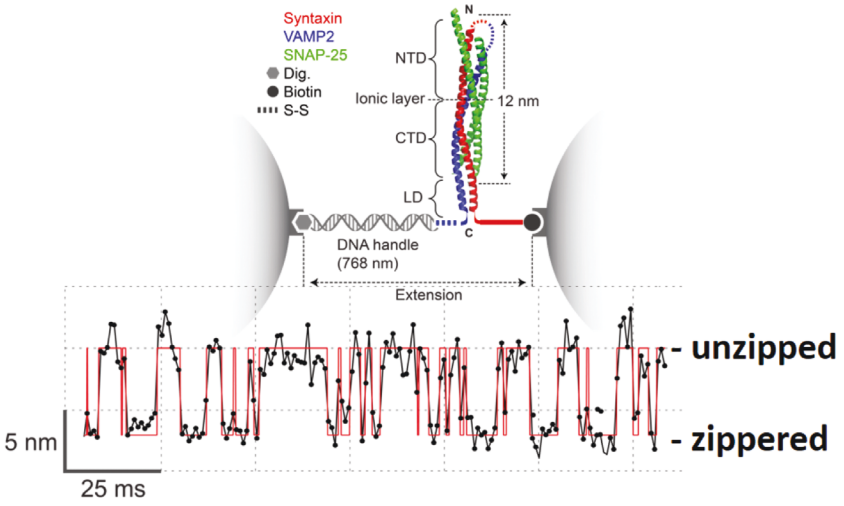

FIGURE 27. The modular structure of the SNARE complex. When pulled apart with optical tweezers, the C-terminal (membrane-proximal) half of the four helix bundle (CTD) unzips and re-zips in an all-or-none fashion, demonstrating a half-zippered intermediate in SNARE-dependent fusion. From Gao et al (2012).

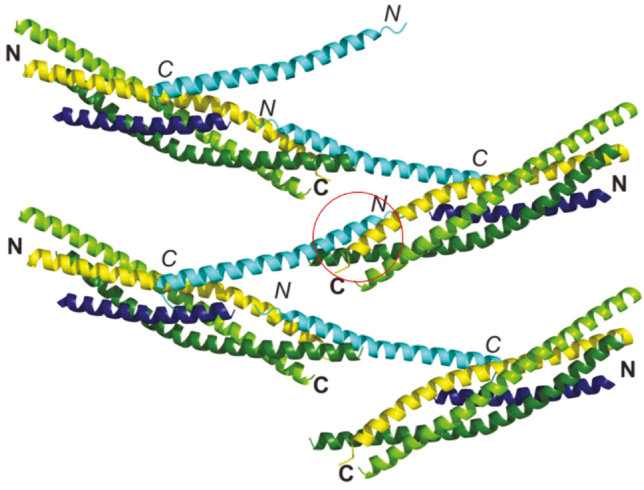

FIGURE 28. Complexin (helices in magenta) trans-clamps half-zippered SNAREpins to synchronize neurotransmitter release to enable rapid synaptic transmission. The accessory helix of complexin reaches across from one SNAREpin to insert into the membrane-proximal portion of another (red circle). Here the accessory helix binds to the t-SNARE (green and yellow helices) in the same place where the v-SNARE (blue) would otherwise bind to complete membrane fusion. Thus, complexin stabilizes the otherwise transient half-zippered intermediate seen with the optical tweezers (Figure 27). From Kümmel et al. (2011).

FIGURE 29. My laboratory and I assembled under the rotunda above the Yale Medical School Historical Library on the morning of the announcement of the Nobel Prize. My wife Professor Joy Hirsch is on my right.

FINAL THOUGHTS

How membranes flow in the cell was a fundamental problem in biology that seemed unapproachable three decades ago. Yet, today we have an understanding of the main features of this vital process at the physical chemical level. This is simply testimony to the power of the reductionist method of science, espoused so insightfully and so early by Eduard Buchner at the very dawn of modern biochemistry more than a century ago (Figure 8).

In writing this lecture, which affords a rare opportunity to look back over decades at one's own contributions, increasingly I see how my work prospered because of the scientific culture (or ecosystem). As Sir Hans Krebs (Nobel Prize, 1953) wrote, "scientists are not so much born as made by those who teach them" (Krebs, 1967). In this excellent article Krebs explains his origins as a scientist in terms of a lineage of great organic chemists and biochemists, each of whom successively trained the next, spanning nearly a century, a chain that is to this very day unbroken, going back to the dye chemist von Baeyer, virtually all Nobel laureates (Figure 30). There are many similar lineages in other fields such as genetics, physiology, microbiology and investigative medicine. Such extraordinary ecosystems have a major temporal component that makes them hard to establish, and correspondingly valuable to the rare societies that possess them,

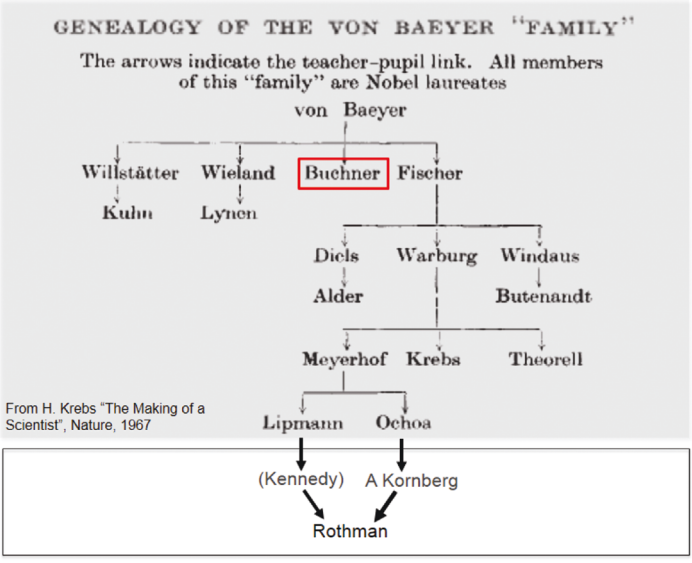

FIGURE 30. A century-long scientific ecosystem—from dye chemistry to enzymology to modern cell biology. The shaded portion is reproduced from H. Krebs "The Making of a Scientist" Nature (1967).

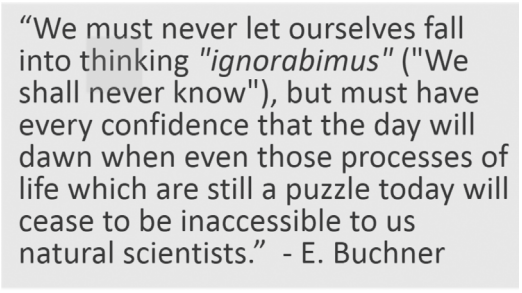

FIGURE 31. Buchner's early faith in reductionism proved to be well-founded, and his words still ring true today.

not only for the knowledge and technologies they generate but also for the economic benefits they provide. Scientific ecosystems can only thrive in societies that sustain science over the very long term.

I am deeply humbled by the insights and accomplishments of these predecessors. As Buchner enunciated in his time (Figure 31), we share the deep conviction, now evidenced by the results of more than a century of discoveries, that there is no process in biology that, at its very core, is not physical-chemical in nature. As the direct consequence, we can expect that, in due course, all of life—even human thought and emotion—will be understood as emergent from physics and we will understand ourselves in health and in disease as complex, organically composed self-determining machines. This is a perspective that may frighten some, but it should not because it offers our species the best hope for the long term.

ACKNOWLEDGMENTS

Though it is often frustrating, Science at the edge combines fun and discovery equally (whimsically illustrated in Figure 32). I wish to thank my personal ecosystem for helping to make my life in science both productive and fun. This includes an enormous debt of gratitude to the many students and postdoctoral fellows who have contributed to this body of work over more than three decades, most of whom have not been specifically mentioned. It also includes the great exemplars and teachers I have apprenticed with as noted in the text; the wonderful environments in which I have been privileged to work, most especially in the earlier years at Stanford and at Sloan-Kettering under Paul Marks' leadership; many close long-term personal and scientific friends who have enriched my life

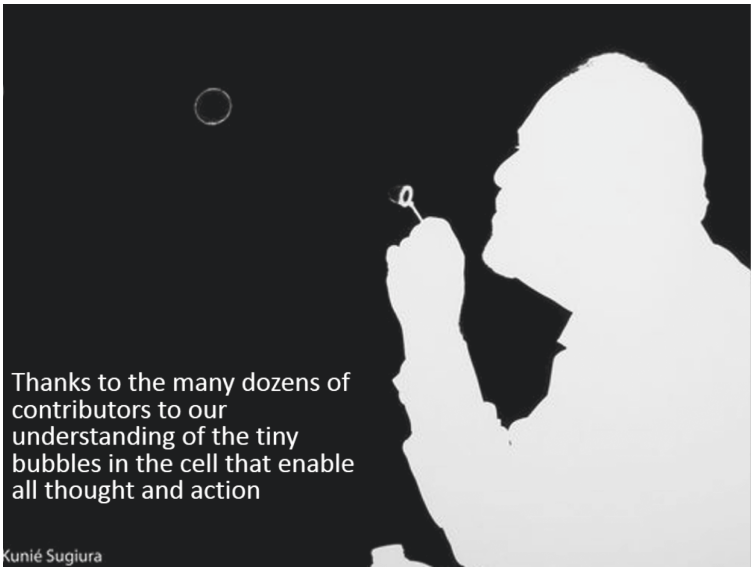

FIGURE 32. Bubbles symbolize vesicles in the cell but also the fun and freedom of good science. So, when I was asked about ten years ago by the Japanese photographer Sugiura to pose for one of her unique photograms while doing something to illustrate my scientific work, I chose to blow bubbles.

and my work; and most especially my wife, soul-mate and intellectual partner, Joy Hirsch; and my children, Lisa and Matthew.

In preparing the written form of this Nobel Lecture I have drawn liberally on my own language previously published in connection with earlier awards, notably the Lasker Award and the Kavli Prize, and also from two publications in the Great Experiments series, a web-based publication from 2001 that is unfortunately no longer available. Finally, a special mention to Willa Bellamy who for 22 years at three academic institutions was my executive assistant, retiring this year.

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Portrait photo of James E. Rothman by photographer Alexander Mahmoud.