Lasker Basic Medical Research Award

Dissecting the membrane trafficking system

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SEC mutants and the secretory apparatus

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The research area of membrane traffic in eukaryotic cells represents one of the most vibrant—some would say competitive—topics in modern cell biology. This was not always so. In 1974, I attended my first meeting of the American Society for Cell Biology, where George Palade, just anointed as a Nobel laureate, gave a special lecture on his pioneering analysis of the secretory pathway. Palade and the Rockefeller school, with such notable colleagues and students as Jim Jamieson, David Sabatini and Gunter Blobel, dominated this new discipline. Morphological and cell-fractionation studies had uncovered a labyrinthine network of intracellular membranes interrelated by the process of vesicular traffic[1]. And yet, to an outsider like me, who had trained as a biochemist in Arthur Kornberg's laboratory, the field seemed descriptive and devoid of molecular mechanistic insight. How are these vesicles created; how do they track to and fuse with a proper target; and how do organelles communicating through a vesicular intermediary maintain their characteristic identity? Palade had eloquently posed such questions, but solutions and methods of approach were not at hand. Nonetheless, one could see an opportunity in the offing. I was attracted to the view that membranes were essentially macromolecules, whose assembly could be dissected by the techniques that had proven so powerful in studying nucleic acid and protein biosynthesis in bacteria: genetics, molecular biology and biochemistry.

My first exposure to membranes came when I was a graduate student in the early 1970s. The field of DNA replication was in transition, and Kornberg believed the future lay in membrane-associated DNA synthesis. To prepare for this, he traveled on sabbatical to various labs, including Palade's. However, within a year, Doug Brutlag and a new postdoctoral fellow in the lab, Bill Wickner, developed a phage DNA replication reaction sustained by a cytosolic protein fraction. I then joined the purification adventure, taking full advantage of Kornberg's legendary skills in enzymology. As a sideline, Bill's previous experience in Eugene Kennedy's lab kindled my interest in membrane assembly. Bill and I spent countless hours plotting strategy in this emerging area of research. I am indebted to Bill for this inspiration, and even more so for the fact that he introduced me to Nancy Walls, whom I married in 1973.

The work of Jon Singer influenced me because of the way he thought about membranes: as ensembles of proteins and lipids diffusing in a two-dimensional fluid. Singer had refined the morphological techniques that allowed membrane constituents to be localized, and I joined his lab as a postdoctoral fellow to learn this approach. However, having been spoiled by the tools available for analysis of bacterial DNA replication, I found the experimental limitations of mammalian cells to be unsatisfying. In contrast, another emerging theme of cell biology, cell division, was yielding to the power of genetics as applied by Lee Hartwell in his brilliant dissection of the yeast cell division cycle. I determined to switch to the study of membrane assembly in yeast in my new position on the biochemistry faculty at Berkeley.

The isolation of *sec* mutants

Unfortunately, I knew little of genetics, and after a three-week period of training at a yeast genetics course in the summer of 1976, I had managed to dissect only four tetrads. To my great good fortune, Charles Field, my first technician, was a most knowledgeable yeast geneticist and he became an invaluable resource for the genetic work we were about to pursue. His background would have meant nothing were it not for the even greater fortune I had in attracting Peter Novick as one of my first graduate students (Fig. 1).

In 1976, yeast cells were not considered a particularly attractive source material for the investigation of secretion. Indeed, when George Palade visited Berkeley for a special lecture in 1978, he was surprised to learn that yeast cells secrete glycoproteins. Thin-section electron microscopy and cytochemical staining showed a cluster of small vesicles under the tip of the bud early in the division cycle, and it was assumed that these vesicles derived from a typical eukaryotic secretory pathway devoted to the export of cell wall enzymes[2]. Novick and I speculated that the membrane of the vesicles contained plasma membrane proteins *en route* to the cell surface.

Our crucial assumption was that secretion mutants would be lethal, and we developed a screening procedure to identify temperature-sensitive (ts) mutants that accumulate secretory enzymes intracellularly. A survey of ts isolates from a mutagenized strain yielded two mutants, *sec1* and *sec2*, that blocked secretion and cell surface assembly. During Palade's visit to Berkeley, he encouraged Novick to examine the mutants by thin-section electron microscopy. One of the most exciting moments in my scientific career came when Novick called me down to the electron microscopy room to see profiles of vesicle-filled *sec1* mutant cells[3] (Fig. 2). We now know that *SEC1* encodes a key regulator of membrane fusion, controlling the interaction of SNAP (soluble NSF (*N*-ethylmaleimide-sensitive factor–sensitive fusion protein) attachment protein) receptor proteins in all eukaryotic cells, including at the synapse in the nerve terminal.

Our next task was to perfect an enrichment technique to isolate more *sec* mutants. Novick found that *sec1* mutant cells become phase refractile and show an increase in buoyant density during a period of 1–3 hours after a shift to the restrictive growth temperature (37 °C). This enrichment proved a gold mine, and Novick and Field succeeded in identifying 23 genes

Fig. 1 Peter Novick (left) and Randy Schekman with son, Joel (right) 1978.

defined by *sec* mutant cells that passed our tests[4]. Phenotypic characterization of the mutants demonstrated there were three classes based on the accumulation of membranes reflecting blocks in traffic from the endoplasmic reticulum (ER), from the Golgi complex or, as with *sec1*, to the cell surface. Subsequent genetic and morphologic inspection of the mutants by Chris Kaiser uncovered a vesicle intermediate in traffic between the ER and Golgi[5]. Simple genetic and biochemical tests demonstrated the broad contour of a pathway in yeast formally analogous to that documented by Palade in the pancreas (Fig. 3) (ref. 6). Tom Stevens found a branch point in the pathway leading to the vacuole, the yeast equivalent of the lysosome[7], and he and Scott Emr, who joined the lab to engineer gene fusions to study protein localization, went on to develop the study of protein traffic to the vacuole into a vibrant discipline in its own right.

In parallel to our effort to define the *SEC* genes and where they act in the secretory pathway, Jim Rothman's lab isolated the first proteins to be clearly associated with the process of transport vesicle targeting and fusion[8]. They showed that mammalian NSF is encoded by *SEC18* (ref. 9), and subsequently our laboratories collaborated to show that α-SNAP is encoded by *SEC17* (ref. 10). The degree of evolutionary conservation is so great that yeast cytosolic proteins will function in place of NSF and α-SNAP in a vesicle transport reaction reconstituted with mammalian Golgi membrane. Chris Kaiser's work indicated that *SEC17* and *SEC18* act at a stage before the one implicated in the Rothman lab vesicle transport assay (ER-to-Golgi versus intra-Golgi). It is now apparent that these proteins act at every stage in which a vesicle docks at a target membrane.

Protein translocation into the secretory pathway

Although the original *sec* mutations defined many stations in the secretory pathway, none caused an arrest in the translocation of secretory proteins from the cytosol into the ER lumen. Ray Deshaies joined the lab as a student, and within a year he had conceived of two very different genetic strategies to define the translocation apparatus. In a more general approach, he reasoned that a secretory signal sequence, if appended to the N terminus of cytoplasmic enzyme, might sequester the enzyme in the ER away from its substrate. Ray documented this behavior for a chimera containing a signal sequence fused to the N terminus of *HIS4C*, the enzyme that converts histidinol to histidine. Mutations in *SEC* genes required for translocation should delay or block the ER localization of the chimeric *HIS4C*, thus permitting cytoplasmic retention of the signal–enzyme chimera and cell growth on histidinol. Because *SEC* genes are essential, however, Ray screened among mutants growing on histidinol plates for those that showed ts growth even on histidine-containing medium. His logic was rewarded with the discovery of several new *SEC* genes, the most important of which, *SEC61*, encodes the main channel-forming subunit of the polypeptide translocase[11–13]. Tom Rapoport's research group cloned the mammalian version of *SEC61* and showed that the core subunits of the translocase are conserved in prokaryotes and eukaryotes. Thus, the genetic approach demonstrated a fundamental conserved mechanism and provided considerable support for the existence of a channel through which secretory and membrane proteins are conveyed into and across membranes.

Blobel's group had shown that secretory proteins are imported into the mammalian ER co-translationally, and no requirement for active unfolding of a cytoplasmic precursor problem was anticipated. However, co-translational import seems not to be a universal feature of membrane assembly because secretion in bacteria, translocation of certain secretory proteins in yeast (such as the precursor of the mating pheromone, α-factor) and protein import into the mitochondrion use post-translational mechanisms. Ray Deshaies theorized that such post-translational processes may require cytoplasmic chaperone proteins to prevent premature folding of translocating precursor proteins. The 70-kD heat-shock proteins seemed logical candidates for such chaperones.

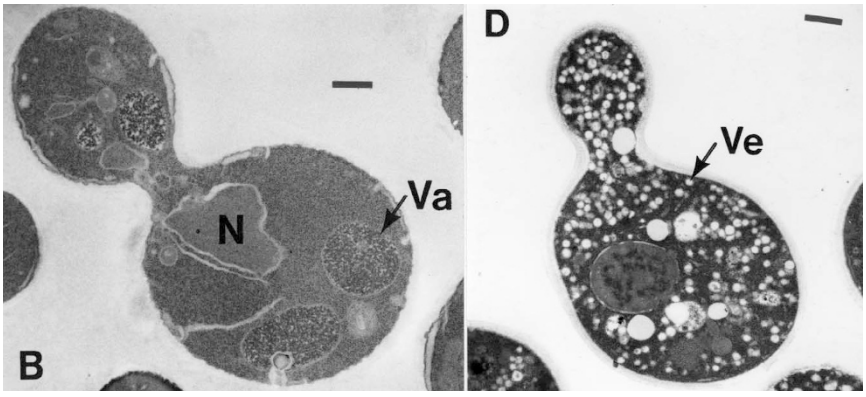

Fig. 2 Thin-section electron micrographs of SEC1 mutant cells grown at the permissive temperature (left) and restrictive temperature (right). Reproduced from ref. 3.

Conveniently, Elizabeth Craig and her colleagues had just created a yeast strain deficient in all but one copy of a heat-shock protein (Hsp)-70 gene. Using this strain, Deshaies found that the post-translational translocation of yeast α-factor precursor and of one of the nuclear-encoded subunits of the mitochondrial ATPase required Hsp70 (ref. 14). We now appreciate that chaperone-assisted import and folding of secretory and organelle proteins applies to the constituents of almost every membrane in the cell.

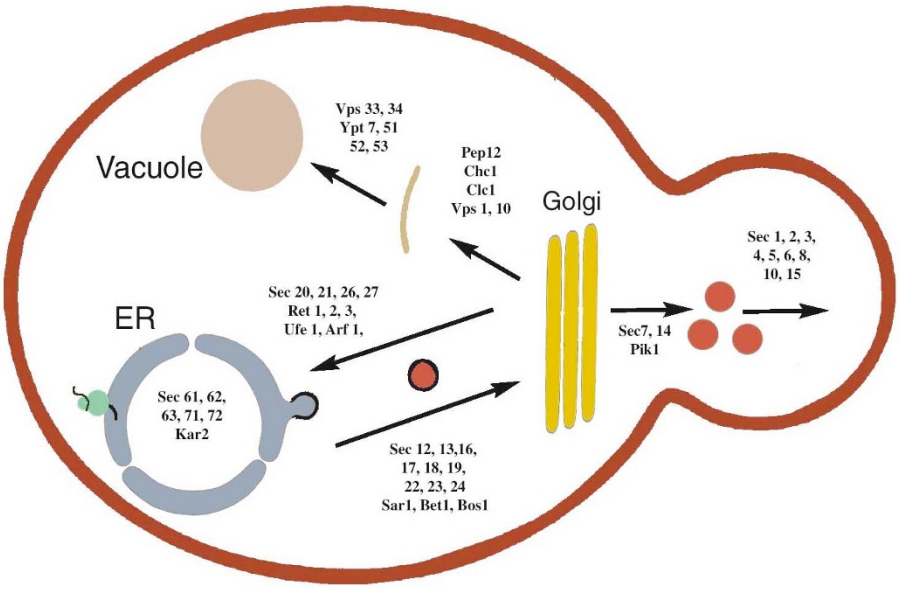

Fig. 3 Yeast secretory pathway.

Biochemical reconstitution of Sec protein function

By the mid-1980s, many of the *SEC* genes had been cloned and sequenced, but little functional insight developed from an analysis of the gene products. One salient exception came from Peter Novick, now in his own laboratory at Yale, who found that *SEC4* encodes a Ras-like GTPase, the first of many such GTPases (called Rab proteins) that populate the secretory pathway[15].

Almost from the outset, my thoughts focused on exploiting the *sec* mutants to guide efforts to reconstitute transport *in vitro*. In the face of tremendous progress on this front in the Rothman lab, our own efforts in yeast did not bear fruit for several years. In 1986, David Baker joined the lab as a student with a bold idea to measure essentially the first half of the secretory pathway in a lysate of wild-type yeast cells. Baker proposed to introduce radioactive α-factor precursor, synthesized *in vitro*, into the ER by post-translational translocation, where it would serve as a tracer of traffic to the Golgi apparatus. The basic translocation assay had been developed by Jonathan Rothblatt and David Meyer at EMBL and Bill Hansen and Peter Walter at UCSF, where they used a lysate, prepared by vigorous homogenization of yeast spheroplasts, as a source of ER membranes[16,17]. In a lysate prepared by this means, the product of translocation, core glycosylated α-factor precursor, showed no evidence of the glycan modifications (outer chain) known to occur in the yeast Golgi complex.

Baker developed a gentle lysis procedure designed to preserve the architecture of the ER–Golgi system and permit the reconstitution of a reaction including traffic of α-factor precursor to the Golgi as measured by the addition of outer-chain glycan epitopes characteristic of mature yeast glycoproteins. Unexpectedly efficient transport, up to 30% of core glycosylated precursor converted to an outer chain–modified form, was detected in a reaction that required cytosol, ATP and magnesium[18]. Within weeks we learned of a comparable effort achieved in the Yale laboratory of my former student Susan Ferro-Novick and her student, Hanele Ruohola (now Ruohola-Baker) (ref. 19). At this point, another student in the lab, Linda Hicke, was working on the cloning of *SEC23*, a gene we had assigned a function in vesicle budding from the ER. She did an essential test showing that a lysate of *sec23* mutant cells demonstrated ts transport of α-factor precursor *in vitro*. This simple test provided compelling evidence that the cell-free reaction measured an authentic transport event.

The reconstituted reaction recapitulates a process that requires the concerted action of at least 30 Sec proteins. Clearly, attempting to fractionate the complete ensemble of these proteins was not feasible. Two important results emerged to simplify our task. Hicke found that wild-type cytosol containing soluble Sec23 repaired the transport defect in a *sec23* mutant lysate. This permitted the detection and purification of one activity at a time by biochemical complementation of the corresponding mutant lysate. Concurrently, another student, Michael Rexach, showed that small vesicles mediate the transport of α-factor precursor and certain SNAP receptor proteins from the ER to the Golgi complex in the cell-free system. Rexach showed that vesicle budding *in vitro* depended on Sec23, but not Sec18, which is required at the fusion step[20]. The simplified budding reaction allowed us to purify three cytosolic proteins necessary and sufficient to reproduce the formation of transport competent vesicles from isolated ER membranes. Yet another in a series of remarkable students, Nina Salama, showed that the pure Sec proteins participate in a protein-sorting event that accompanies the budding reaction; proteins destined for transport are packaged, whereas resident ER proteins are left behind[21].

With pure proteins in hand, it was time to consider the mechanism of vesicle budding. The coat-protein paradigm informed our thoughts on this problem. Clathrin, which was most closely associated with the endocytic process, had also been implicated in secretory protein traffic. Greg Payne, a postdoctoral fellow in the lab, cloned the clathrin heavy chain gene and found that deletion mutant strains, though 'sickly', were very normal in respect to secretion[22]. Just as it became apparent that clathrin was not required for secretion, Lelio Orci and Jim Rothman discovered a coat, now called COPI, and its assembly protomer (coatomer), responsible for vesicle traffic within the Golgi complex[23]. Dori Hosobuchi, another student in my lab, found coatomer in yeast and showed that a gene implicated in traffic between the ER and Golgi, *SEC21*, encodes the γ subunit of coatomer[24]. Although this result was consistent with a function for coatomer in vesicle budding from the ER, the genetic behavior of *SEC21* did not quite fit with other

genes we had assigned this function, and the Sec21 protein fractionated away from the proteins required for vesicle budding from the ER. Cosson and Letourner showed that at least one function of coatomer is to retrieve and recycle proteins from the *cis* Golgi cisterna[25].

What then were the vesicle-budding Sec proteins we had isolated? Charles Barlowe, a postdoctoral fellow in the lab, discovered, in collaboration with Lelio Orci, that anterograde budding from the ER involves a distinct coat, which we called COPII because it superficially resembles but is molecularly distinct from COPI (ref. 26). Meta Kuehn and Sebastian Springer showed that subunits of the coat make direct contact with membrane cargo proteins but not with resident ER proteins[27,28]. It seems likely that most secretory and membrane proteins are sorted directly or indirectly by interaction with a COP coat.

Coats also provide the exclusive driving force for vesicle morphogenesis. Ken Matsuoka and Anne Spang in the lab showed that COPII and I, respectively, can assemble on and bud vesicles from the surface of a liposome formulated with pure phospholipids[29,30].

Prospects

Our understanding of the process of vesicular traffic in eukaryotic cells has moved from a descriptive phase in which the pathways and intermediates were mapped, to a functional stage in which a small number of the machines that drive the pathways have been isolated and to some extent characterized. The mechanism of action and regulation of these machines will occupy the attention of new investigators attracted to the challenges of this field. We are now entering an era that will increasingly be influenced by structural biologists, who have the tools to identify at atomic-resolution detail the inner workings of these machines. The promise of a most satisfying level of understanding is now at hand.

Acknowledgments

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