Navigating the secretory pathway

Conference on exocytosis membrane structure and dynamics

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The EURESCO/EMBO conference 'Exocytosis Membrane Structure and Dynamics' was held in the shadow of the medieval Templar Castle of Tomar, Portugal, April 20–25, 2002. Graham Warren (New Haven, CT) and Bernhard Dobberstein (Heidelberg, Germany) successfully brought together researchers for 4 days of exciting talks and active poster sessions, encouraging the participants to describe their latest and unpublished results towards unravelling the mechanisms of protein transport through the secretory pathway.

material is sequentially transported between various intracellular compartments while the identity of each is retained is a fundamental issue in cell biology (reviewed by Mellman and Warren, 2000). This report highlights the main issues and trends addressed during a meeting that covered this entire journey, from a protein's delivery into the endoplasmic reticulum (ER), through transport and sorting in the Golgi complex, and ultimately to the arrival at the cell surface (Figure 1).

Preparation for the journey ahead: getting into the ER

Newly synthesized proteins embark on their voyage through the secretory pathway at the ER as a result of the interaction between their emerging signal peptide and the signal recognition particle (SRP) complex. The structural requirements for this event and the concomitant targeting of the ribosome to an ER translocation pore (translocon) remain only partially resolved. I. Sinning (Heidelberg, Germany) and R. Beckmann (Berlin, Germany) launched the meeting by discussing the ways that the main molecules involved (the SRP, the ribosome, the SRP receptor and the Sec61 pore complex) drive this entry, based on recently published structures and cryo-electron microscopy (EM) reconstructions. The SRP is composed of six protein subunits and a 300-nucleotide RNA (7SL RNA), and, although the structures of only isolated components of this complex have been solved, the data already reveal interesting features of protein–RNA recognition, ribonucleoprotein particle assembly and SRP function. Protein–RNA interactions between the SRP19 protein and helix 6 of the 7SL RNA occur without direct protein–base contacts but involve a complex network of highly ordered water molecules. In particular, SRP19 recognizes a GGAG tetraloop and a widened major groove in the RNA. Following successful recognition of the signal peptide, the entire SRP–ribosome complex can then dock with the SRP receptor, which is situated on the ER membrane, bringing the ribosome into close proximity to the Sec61 translocon. Results presented by Sinning and Beckmann

Introduction

The Portuguese explorer Vasco da Gama used to spend time on the magnificent castle walls of Tomar thinking about the ocean and the route that would lead him to his ultimate goal: India. The preparations for this difficult journey had already been made in Tomar by the influential Order of Christ and its governor Prince Henry the Navigator. This location was therefore appropriate for the recent EURESCO/EMBO conference 'Exocytosis Membrane Structure and Dynamics', which brought together researchers whose interests encompassed another great journey: the transport of proteins through the entire length of the eukaryotic secretory pathway. This pathway ensures that newly synthesized proteins are correctly folded, modified and delivered to their designated membrane-bounded organelle. Any successful navigation of the secretory pathway requires the correct interpretation of cargo sorting signals in a precise and timely fashion by large families of transport machinery proteins, including enzymes, coat complexes and GTPases. The complete understanding of how

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Fig. 1. Schematic representation of the secretory pathway, highlighting some of the components discussed during the conference. Newly synthesized proteins enter the pathway at the ER and are subjected to repeated sorting and transport between membrane organelles until they arrive at their designated destination.

indicate that this interaction occurs via distinct rRNA elements and proteins of the large ribosomal subunit that form four connections with the Sec61 complex. A gap of between 10 and 20 Å left on the cytoplasmic side could be used for the accumulation of the growing protein during translation. The exact role and structure of Sec61 during this process was a major point of discussion. Debate centred around whether the translocon is sealed except when 'in use', and, if so, what 'unlocks' it on either side of the membrane—potentially the ribosome on the cytoplasmic side during translation and an unidentified complex on the other during protein retro-translocation.

Following the delivery of nascent proteins into the ER, the signal peptide is cleaved and remains anchored in the lipid bilayer. The disposal of this peptide was addressed by

B. Martoglio (Zurich, Switzerland), who has recently isolated an aspartate protease-like enzyme with some structural similarities to presenilins. This signal peptide peptidase (SPP) clearly exhibits the ability to internally cleave signal peptides and therefore appears to fulfil a vital and obvious intracellular function. However, the equivalent enzyme does not exist in lower eukaryotes, including yeast, despite the long use of this organism as a paradigm for the study of the secretory pathway. Martoglio therefore raised the intriguing possibility that, in higher eukaryotes, the signal sequence itself is more sophisticated than formerly believed, possibly having secondary roles in the cell. Indeed, he presented recently published data implicating SPP in the generation of antigenic peptides from the signal sequences of MHC class I molecules themselves. Moreover, he also demonstrated a role

for SPP in the processing of the hepatitis C virus core protein. This pathogen appears to be able to exploit this protease for its protein processing, a key requirement in its maturation. This was one of many examples demonstrating how defects in, or subversion of, components of the secretory pathway results in disease.

Once in the oxidative environment of the ER lumen, proteins must fold and assemble. At this point, the secretory pathway operates a quality-control mechanism designed to ensure that aberrantly processed proteins are not delivered to their sites of function. Nascent proteins are first glycosylated, after which the terminal glucose moieties are trimmed by glucosidase I and II as the folding protein enters the calnexin/calreticulin cycle. A. Helenius (Zurich, Switzerland) described how this cycle distinguishes between the folded and unfolded states of a glycoprotein. Although the exact mechanism is still to be defined, it is known to involve a glucosyl transferase (GT), which senses the folding status of newly synthesized proteins and specifically tags the oligosaccharide moieties of incompletely folded proteins by adding glucose residues to their N-linked glycans. This allows them to enter the folding cycle again and prevents their export. When folding defects are local, GT reglucosylates glycans only within the defective domain. N-linked glycans are thus used in the ER to effectively record the folding state of newly synthesized proteins.

Proteins that are persistently misfolded are retro-translocated back to the cytoplasm, where they are degraded by the proteasome in a process termed ER-associated degradation (ERAD). Recent reports have indicated that some of these substrates are first transported to the Golgi before being brought back to the ER and directed into the ERAD pathway. Using a synthetic lethal unfolded protein response (UPR) screen in yeast, D. Ng (University Park, PA) has isolated over 800 mutants and classified them according to their effect on the retention and/or retrieval of various degradation substrates (Ng *et al.*, 2000). Interestingly, in one retrieval mutant (*per18*), the corresponding gene was determined to be *HMG1*, which encodes an enzyme involved in the synthesis of a component of lipid rafts, suggesting that a subdomain of the ER membrane is specifically involved in retrieval but not degradation. Moreover, the UPR screen demonstrated that the ERAD pathway is saturable and that, under stress conditions such as ER overflow, the secretory pathway can be regulated to allow an alternative means of degradation using the vacuole. Clearly, linking the ERAD and secretory pathways is vital, and the mutants identified thus far provide an opportunity to characterize this relationship further.

Boarding the right boat: leaving the ER

The sequential transfer of material between membrane compartments of the exocytic pathway involves coated vesicular and tubular carriers. Therefore, proteins with destinations beyond the ER need to be sorted away from ER residents and packaged into transport vesicles to undergo this next step. ER-derived vesicles are formed by the binding of the protein complex COPII to the external side of the membrane, followed by the selection of cargo. Following budding, these vesicles are directed towards the Golgi complex and then uncoat prior to membrane fusion and cargo delivery. Studies in the yeast *Saccharomyces cerevisiae* have led the field in terms of dissecting this process, as exemplified

by work presented by H. Riezman (Basel, Switzerland). His recently published data were unexpected, demonstrating that different cargoes are sorted into distinct populations of ER-derived vesicles (Muniz *et al.*, 2001). This raises the critical issue of how the COPII coat, which is comprised of just five proteins (the Sec13/31 complex, the Sec23/24 complex and the small GTPase Sar1), is able to selectively recognize and sort this plethora of cargo accumulating in the ER. Using a large-scale synthesis, purification and analysis of COPII vesicles from yeast, C. Barlowe (Hanover, NH) has identified a class of molecules termed Ervs (ER vesicle proteins), which now provide some answers to this question. He demonstrated that Ervs are transmembrane proteins that bind components of the COPII coat through their cytoplasmic tails, and, although non-essential, they enhance the efficiency of ER-derived vesicle formation. Recently published work (Belden and Barlowe, 2001; Powers and Barlowe, 2002) has demonstrated that two of these molecules, Erv14p and Erv29p, selectively bind certain transmembrane and soluble cargo, respectively, thereby providing a critical link between cargo and the cytoplasmic COPII coat. He also introduced two further possible sorting molecules, Erv46p and Erv41p, which function as a complex. Mutation analysis of their cytoplasmic tails has revealed the presumptive COPII-binding residues of Erv41p, and, interestingly, swapping the tails of these two proteins renders them defective for ER budding, indicating that their actual orientation or conformation is important. Furthermore, this Erv complex can bind glucosidase II, implying that either Erv46p/41p functions in the retrieval of glucosidase II lost from the ER or (the more fascinating possibility) that ER quality control and ER export are more tightly linked than previously postulated.

The theme of using yeast to study ER-to-Golgi transport was continued by B. Glick (Chicago, IL). His model system is the yeast *Pichia pastoris*, which, unlike *S. cerevisiae* that has individual Golgi cisternae scattered throughout the cytoplasm, arranges its Golgi in entire stacks, more closely resembling the situation in mammalian cells. In *P. pastoris*, cargo leaves the ER in COPII vesicles from a small number of transitional ER (tER) elements (marked by Sec12p) that lie in close proximity to these Golgi stacks (marked by Sec7p) (Figure 2, inset). Recent work from Glick's laboratory has focused on how *P. pastoris* Sec12p is able to localize to these specific sites. He reported that expression of the *P. pastoris* Sec12p protein in *S. cerevisiae* is insufficient to create these localized tER structures in this system, indicating that other binding partners are also required. This specific localization information and/or partner binding site is most likely situated in the luminal domain of Sec12p, since this domain is significantly extended in the *P. pastoris* protein. His work is now at an exciting stage, since a mutagenesis screen has identified Sec12p-delocalized mutants, and these are currently being analyzed. This approach has been made possible by the construction of a GFP-tagged component of the COPII coat, Sec13p. Time-lapse movies of this construct elegantly demonstrated that, in *P. pastoris*, these tER sites can form *de novo*, move within the plane of the membrane and fuse with existing sites.

The visual analysis of GFP-tagged proteins has also been fruitful for studying ER-to-Golgi transport in mammalian cells. R. Pepperkok (Heidelberg, Germany) has used multi-colour time-lapse imaging to demonstrate that, similar to yeast, different

Fig. 2. Physical arrangement of ER exit sites and the Golgi complex in mammalian and yeast cells. A mammalian NRK cell stained for ER exit sites (the COPII component Sec13, green) and the early Golgi complex (giantin, red). Inset is a *P. pastoris* yeast cell expressing GFP-tagged markers for tER (Sec13p, green) and the early Golgi complex (Sec7p, red). Note the huge physical distance of ER-to-Golgi transport in mammalian cells compared to yeast cells. Scale bars, 5 μ m. Images courtesy of Ben Glick.

cargoes (procollagen and the viral glycoprotein VSV-G) are sorted into distinct ER-derived carriers. Furthermore, there appears to be an intimate relationship between the many ER exit sites and the microtubule network. Indeed, in the presence of the microtubule depolymerizing drug nocodazole, those microtubules in contact with COPII-coated ER exit sites appear to be preferentially stabilized. Given the relatively large physical distance over which cargo must be transported in mammalian cells compared to yeast cells (Figure 2, note scale bars), the importance of motors, the cytoskeleton and regulators for efficient transport is perhaps not surprising. However, since many of the players involved in this process remain unknown, an ongoing strategy of Pepperkok's group is to screen novel GFP-tagged cDNA products and to further characterize candidates localizing to the cytoskeleton and compartments of the secretory pathway. A. De Matteis (Santa Maria Imbaro, Italy) presented her recent work using correlative light immuno-EM (CLEM) to address how cargo reaches the Golgi complex in mammalian cells. This emerging technique is becoming increasingly popular as an effective method to reconcile the limitations of light-microscopy resolution with the detailed, but often hard-to-interpret, ultrastructural analysis of membrane compartments by EM. Although it is widely accepted that, in higher eukaryotes, transport between the ER and Golgi occurs via an intermediate compartment (IC), the nature of this organelle is not well understood. De Matteis' approach has shown that the IC is a highly dynamic and complex set of membrane structures, often situated peripherally in the cell, and contains a pool of the Golgi matrix protein GM130 but not other Golgi markers such as giantin or mannosidase II. Photobleaching experiments indicate that these pools can communicate with each other in a microtubule-dependent manner and that they serve as sorting stations, which, after receiving cargo from the ER, can homotypically fuse and

then transfer these proteins on to the Golgi via tubular carriers (Marra *et al.*, 2001).

First major port of call: the Golgi complex

The fusion of membrane carriers and the concomitant delivery of secretory cargo to the first Golgi cisterna poses the problem that both escaped ER residents and the machinery molecules just utilized must be returned to the ER. This retrograde transport step is performed by carriers, whose coat comprises seven 'coatamer' subunits (α , β , β' , γ , δ , ϵ and ζ) and the GTPase ARF1, together termed COPI. Although the COPI coat has been seemingly well characterized, C. Reinhard (Heidelberg, Germany) described two new subunits of the COPI complex: $\gamma 2$ and $\zeta 2$. The $\gamma 2$ -COP subunit is present in all tissues and shares 80% homology with the $\gamma 1$ -COP subunit but has two boxes of significant divergence. In contrast, the new $\zeta 2$ subunit is characterized by a 30-amino-acid N-terminal extension with respect to its $\zeta 1$ counterpart. Immunoprecipitation experiments using specific peptide antibodies have determined that these two subunits exist in the COPI coat either in a $\gamma 1\zeta 2$ or in a $\gamma 2\zeta 1$ arrangement. Furthermore, preliminary results from budding assays indicate that the $\gamma 2$ -COP subunit is enriched in Golgi-derived COPI vesicles, whereas the $\gamma 1$ -COP subunit remains associated with the Golgi complex itself. The discovery of new subpopulations of coatamer may lead the field to exciting new perspectives into how cells manage to efficiently utilize the COPI coat to recycle material backwards while still maintaining the physical integrity of the Golgi complex. T. Nilsson (Heidelberg, Germany) continued this theme by describing how ARF1 regulates COPI assembly on membranes. Using an *in vitro* budding assay containing Golgi membranes, he demonstrated that the sorting of retrograde material into COPI vesicles is dependent upon GTP hydrolysis by ARF1. This GTPase functions in conjunction with the activating protein ARF-GAP1, which promotes the continuous hydrolysis of GTP and the subsequent release of the coatamer from the membrane. Nilsson proposed that, in the presence of prospective cargo, and in particular through the binding of members of the p24 family of cargo receptor molecules, this activity is gradually sequestered, allowing the coatamer to remain on the membrane longer and thereby permitting the formation of a COPI-coated transport vesicle. In support of this, his group has observed a strong correlation between the amount of ARF-GAP1 and p24 in vesicles.

The p24 family was also highlighted by F. Barr (Martinsried, Germany), with respect to Golgi matrix proteins such as the Golgi reassembly stacking proteins (GRASPs). He has recently observed that p24a exists in the same complex as GRASP55 and GRASP65 (Barr *et al.*, 2001), a finding implicating the latter proteins, once considered to simply have a structural role, in a more dynamic scenario. He is further examining the GRASP proteins using various strategies, including downregulation by small interfering RNAs and analysis of their phosphorylation state during the cell cycle. These studies have wide implications for the field, considering the ongoing debate of how stable an entity the Golgi complex actually is. In particular, the significance of Golgi enzyme recycling through the ER remains to be resolved, and indeed how this relates to the integrity of the Golgi complex during the cell cycle. The matrix proteins are undoubt-

edly key regulators of this process. During transport either through the Golgi complex or between distinct organelles, the cell must ensure that vesicles dock and fuse only with the intended membrane. One means whereby this fidelity is achieved is through pairs of cognate targeting molecules termed SNAREs, which are present on the vesicles (vSNAREs) and target membranes (tSNAREs). Correct interaction of these molecules ultimately leads to fusion of the vesicle with the membrane. At present, it is unclear how vSNAREs are incorporated into transport vesicles, a question that was addressed by A. Spang (Tuebingen, Germany). Her work has shown that, in yeast, the ARF-GAPs Glo3p and Gcs1p can bind to ER-to-Golgi vSNAREs and that this is vital for the recruitment of ARF1 and coatamer. It was suggested that ARF-GAP induces a conformational change in the vSNARE proteins (converting them to a protease-resistant form), thus allowing the COPI components to bind. These findings therefore imply a second conformation-altering function for ARF-GAPs, distinct from their previously discussed GTPase activating function. Furthermore, the interaction of vSNAREs with both COPI and COPII coat proteins highlights another level at which specificity in vesicle budding is determined.

Distant horizons: beyond the Golgi

Cargo still in transit at the distal face of the Golgi complex undergoes another round of sorting in the *trans*-Golgi network (TGN). M. Robinson (Cambridge, UK) described the ever-growing number of cytoplasmic coats and their adaptors through which this is achieved (reviewed by Robinson and Bonifacino, 2001). The principal players are the adaptor protein complexes AP-1, AP-3 and AP-4 and the relatively recently identified GGAs (Golgi-localizing, γ -adaptin ear homology domain, ARF-binding proteins), each of which interacts with motifs within the cytoplasmic tails of cargo molecules. Such highly integrated sorting must be tightly regulated; however, to date, only two interactors (γ -synergin and rabaptin-5) of the γ -adaptin subunit of the AP-1 complex have been identified. To redress this anomaly, Robinson's group has been employing glutathione *S*-transferase (GST) fused to either γ -adaptin or the GGAs in pulldown experiments. Two novel binding partners of both γ -adaptin and the GGAs, termed p56 and p200, have been identified from pig brain cytosol. The p56 protein is predicted to contain a coiled-coil domain and binds both of these adaptors *in vitro*. Immunofluorescence analysis using antibodies raised against this protein indicate a greater degree of co-localization with the GGAs. The identification of new regulators in this field is always welcome, and further characterization of these candidates is eagerly awaited.

Sorting of material to more specialized subcellular compartments such as lysosomes and secretory granules is also initiated from the TGN. Indeed, G. Griffiths (London, UK), T. Soldati (London, UK) and S. Tooze (London, UK) were amongst those presenting their recent work in deciphering the regulation of transport to these organelles. Tooze described a reconstituted cell-free assay for studying the requirements for the homotypic fusion of immature secretory granules (ISGs) during their maturation (Wendler *et al.*, 2001). These are known to include GTP and ATP, the SNARE machinery (NSF and α SNAP), the tSNARE syntaxin6 and cytosol. Fractionation of cytosol has now revealed

the importance of an additional four proteins: actin, RanTC4 and two ERM proteins (radixin and moesin), the latter two being known to link the actin cytoskeleton with membranes. Particularly appealing is the hypothesis that an as yet unidentified ERM receptor might be able to sequester these activated ERMs to the ISGs, in a process that may be regulated by phosphorylation, and thereby allow actin to be recruited to the complex, bringing the ISGs together.

Parallel approaches to elucidate the molecular mechanisms of membrane fusion were also presented by P. Freemont (London, UK). Combining cryo-EM and X-ray crystallography, he has determined the structure of p97, an ATPase involved in a variety of membrane-fusion events including post-mitotic Golgi reassembly and ER reformation. In particular, p97 is thought to disassemble SNARE complexes formed during the fusion process. His latest reconstructions suggest that p97 and its adaptor p47 assemble as a complex over SNAREs, forming a system of two 'wheels' on top of each other. Upon ATP hydrolysis, these rings are subjected to a profound conformational change that effectively acts like a ratchet to unwind and ultimately dissociate the SNARE complex. Such an unfoldase activity is expected to be universal on all membranes within the secretory pathway, as SNARE complexes need to be disassembled and recycled following every membrane-fusion event.

The fusion of vesicles with the plasma membrane represents the final stage of secretory cargo delivery. F. Hughson (Princeton, NJ) described this process with respect to the molecular interactions between the vesicle vSNARE Snc2p and the plasma membrane tSNARE Sso1p in collaboration with the helper tSNARE Sec9p. Using a mutagenesis approach, Hughson has determined that Sso1p normally exists in a closed conformation, with its N-terminal regulatory domain preventing its C-terminal SNARE motif from binding its assembly partners. Although an engineered constitutively 'open mutant' can functionally substitute for wild-type Sso1p *in vivo*, if additional accessory Sec9p is added, severely deleterious effects on growth are observed. Following vesicle fusion, the cell must therefore tightly regulate the disassembly of potentially toxic SNARE complexes by utilizing a combination of the disassembly machinery (described above), limiting the availability of Sec9p and, whenever possible, maintaining the closed conformation of Sso1p (Munson and Hughson, 2002).

Perspectives for future journeys

The molecular machinery of the exocytic pathway exists to correctly recognize and interpret targeting information within the proteins that enter it. As a result, proteins function in their allocated environment: chaperones remain in the ER to mediate folding, the Golgi maintains a gradient of oligosaccharide trimming enzymes, and receptors accumulate at the cell surface. The goal of this meeting was to assess our understanding of this pathway and consolidate new strategies for its future exploration. Its success was apparent from both the wide variety of experimental approaches that were integrated and the number of new molecules and new screening strategies that were introduced. In this post-genomic age, the rate of identification of such proteins can only increase. However, many key issues remain to be resolved. How does the protein folding machinery so effectively sequester secretory proteins away from ER exit sites? What are

the signals that initiate vesicle budding? If cargo is differentially sorted on leaving the ER, is this segregation maintained throughout the Golgi? How does the Golgi maintain its integrity while balancing forward and backward membrane flow? Does lipid composition regulate vesicle transport? Does the same machinery exist in a wider range of model organisms? Our understanding of the full complexity of this journey is still incomplete: perhaps we have rounded the Cape, but charting the waters to our final destination will undoubtedly bring more surprises.

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