Cell, Vol. 102, 139–144, July 21, 2000, Copyright ©2000 by Cell Press

Transport across Membranes: A Question of Navigation

Meeting Review

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question (n.)*: a lack of conviction or certainty, a request for data, a situation that presents difficulty, uncertainty, or perplexity.*

Organizing a meeting around a series of questions, rather than around the subdisciplines that already exist within a field, is an unusual strategy with a certain amount of inherent risk about the coherence of the outcome. That was the challenge presented by Rob Jenson and Art Johnson to the participants of the American Society of Microbiology conference on Macromolecular Transport Across Cellular Membranes in Savannah, Georgia. Most protein targeting meetings are subdivided by organelle, which has the obvious advantages of being easier to organize and of greatly simplifying the nomenclature within each session. However, the one membrane, one session format has created membrane-specific "language barriers" and has contributed to the bias within the community that translocation across each membrane is fundamentally unique. A major success of this ASM meeting was the demonstration that protein translocation machineries (translocons) from various membranes share many features and that most of the problems being grappled with are not organelle specific. Given the challenges of the format, the speakers made it surprisingly easy to navigate diverse areas of the field.

Only the Names Have Been Changed . . .

A relatively cogent view of protein targeting emerged in which the basic steps used to target and translocate a protein across a membrane are conserved between organelles. The individual steps that are more or less conserved between many different targeting pathways are outlined in Figure 1. These steps include (1) recognition of a signal sequence on the protein to be targeted, (2) directing the protein to the target membrane, (3) engagement of the translocation machinery, (4) translocation across the membrane, or (5) integration of the polypeptide into the lipid bilayer. The last three steps must be accomplished without compromising the permeability barrier established by the membrane. In any one membrane, the basic steps and some of the specific protein components are shared by multiple pathways. In some cases components are shared even when the system operates in the opposite direction (retrotranslocation) to return damaged, defective, or unwanted substrates to the cytoplasm.

For some time it was thought that the only targeting systems that recognized and bound substrates in the cytoplasm, rather than on the surface of the membrane, were those that directed proteins to the endoplasmic reticulum (by signal recognition particle) or the nucleus (by Ran, Karyopherins etc.). However, over the last few years evidence has accumulated for cytoplasmic receptors for translocation of integral membrane proteins in *E. coli*, and of both soluble and integral membrane proteins into peroxisomes. Only for chloroplasts and mitochondria do recognition of a signal sequence and targeting it to the outer membrane appear to be mediated by membrane proteins already associated with the import machinery. Therefore, for translocation across the outer membrane of mitochondria and chloroplasts, steps 1–3 in Figure 1 are accomplished at the translocon. However, these organelles have multiple membranes and reiterate the steps outlined in Figure 1 for subsequent membranes as well. Therefore, comparisons to Figure 1 can be drawn for virtually any subcellular membrane, independent of whether targeting is cotranslational (and therefore involves the targeting of ribosomes along with their nascent polypeptides) or posttranslational.

The extent to which the steps illustrated in Figure 1 are conserved is perhaps best illustrated by considering a process that works in the opposite direction (retrotranslocation), to enable degradation of proteins. Several participants presented data suggesting that retrotranslocation proceeds by similar steps to those for import into the endoplasmic reticulum. Using a chemical crosslinking approach, Karin Römisch demonstrated that protein disulfide isomerase (PDI) interacts with a retrotranslocation substrate in the endoplasmic reticulum that lacks disulfide bonds. Endoplasmic reticulum–derived microsomes prepared from a yeast strain lacking PDI or in which the peptide binding site of PDI is mutated cannot retrotranslocate this substrate. Thus, PDI is a candidate for one of what are likely to be multiple signal receptors, analogous to those in steps 1–2 of the import reaction. Another candidate discussed by Jeff Brodsky is BiP, in which retrotranslocation-specific mutations have been uncovered. A membrane-associated receptor for retrotranslocation substrates is calnexin (equivalent to step 3 in Figure 1); degradation is reduced by ~50% in yeast strains deleted for calnexin.

Ron Kopito presented data suggesting that ubiquitination, acting as a Brownian ratchet, may not provide the driving force for retrotranslocation (analogous to step 4 above) as has been proposed previously. His data suggest that a functional ubiquitination pathway is essential for retrotranslocation, but that ubiquitination of the substrate polypeptide is not required. An intriguing possibility suggested was that the 19S cap of the proteasome, a protein complex reported to exhibit chaperone-like activity (Braun et al., 1999), may provide the driving force for retrotranslocation. According to this attractive hypothesis, unfolding would be coupled to retrotranslocation. This proposal is consistent with the observation that transit across the endoplasmic reticulum membrane is the rate-limiting step in retrotranslocation. However, Jeff Brodsky showed that when degradation of an integral membrane protein is prevented in yeast containing mutations in the 20s "core" of the proteasome, the substrate concentrates at distinct sites in the endoplasmic reticulum. Possible interpretations of this result include that degradation is imperative for extraction,

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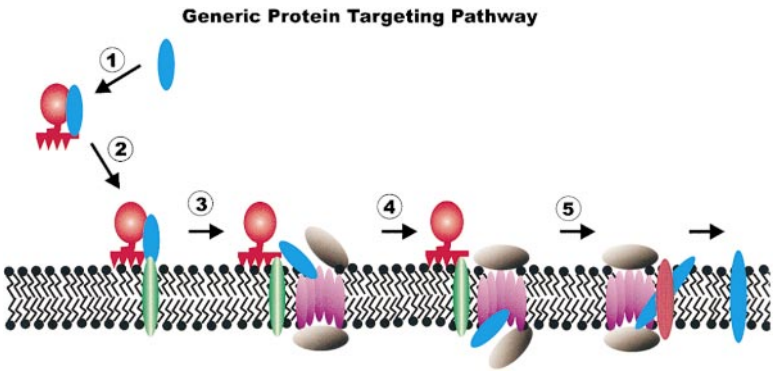

Figure 1. The Steps Involved in a Hypothetical Generic Protein Targeting and Translocation Pathway

(1) A substrate protein (blue) is recognized by a signal sequence binding protein (red) after or during synthesis in the cytoplasm. If targeting occurs cotranslationally, then the substrate is recognized as a nascent polypeptide-ribosome complex. For simplicity ribosomes are not shown.

(2) The substrate protein is targeted to the correct membrane via an interaction with a specific receptor on the target membrane (green). Lipid binding may also contribute to targeting. The signal sequence is examined in a proofreading step concurrent with targeting to the membrane and/or during transfer to the translocon (purple). In mitochondria and chloroplast, outer membranes steps 1–3 are combined at the translocon in the outer membrane. However, soluble targeting proteins contribute to translocation across the inner membrane.

(3) The mechanism of transfer of the substrate protein from the signal sequence binding protein to the translocon is unknown for most membranes. However, in all cases membrane impermeability is not compromised by transfer of the signal sequence to the translocon or by translocation across the membrane. The mechanisms that maintain membrane impermeability are either completely unknown or hotly debated (gray).

(4) The mechanism by which proteins are transported through the translocon remains an area of controversy for most membranes.

(5) Integral membrane proteins may be targeted to the membrane by the same proteins as fully translocated proteins or they may be targeted by a series of receptors functioning in parallel. However, it appears that in many membranes the same translocon pore protein(s) is used for both types of proteins. In contrast, the inner membrane of the mitochondria maintains separate translocons for integral membrane and translocated proteins. Additional recognition steps and proteins (brown) have been implicated in moving the protein out of the aqueous pore formed by the translocon and into the lipid bilayer.

and/or that retrotranslocation substrates coalesce at distinct sites in the endoplasmic reticulum.

Karin Römisch reported that in yeast, some endoplasmic reticulum translocon (Sec61) mutants are defective in translocation of any substrate in any direction, others are defective only for misfolded protein or peptide retrotranslocation. Export of glycopeptides from the endoplasmic reticulum lumen to the cytoplasm was shown to be dependent on Sec63. Since Sec63 is a component of the posttranslational import machinery, glycopeptides may use this translocon to exit the endoplasmic reticulum. Together, these results suggest that the translocons involved in co- and posttranslational translocation of proteins share subunits with the translocons involved in retrotranslocation. It remains to be determined if precisely the same translocons are used for both import and export directions of translocation, or if retrotranslocation is mediated by distinct translocons or translocons are reengineered "on the fly" to perform either forward translocation or retrotranslocation. One indication that misfolded proteins are retrotranslocated via distinct translocons comes from low level expression of an unstable Sec61 mutant. At the permissive temperature, this mutant is expressed at about 40% of wild-type levels, retrotranslocation of misfolded proteins is abolished, but translocation of proteins into the endoplasmic reticulum and glycopeptide export to the cytoplasm continue. Thus, misfolded proteins may be retrotranslocated to the cytoplasm by translocation through the roughly 1/3 of the Sec61 molecules on the membrane that are neither ribosome- nor Sec63 complex–associated.

A Membrane by Any Other Name . . .

Perhaps one of the most important insights from the meeting was that a surprising number of translocation pathways appear to operate in parallel to guide protein traffic across any individual membrane. Most translocation-competent membranes appear to have a translocon for proteins that cross the membrane entirely, a partially overlapping pathway for integral membrane proteins, and a specialized transporter for folded proteins. Thus, signal peptides that appear to be relatively similar sequences, with apparently low information content, not only direct proteins to a specific destination but to one of several pathways for that destination. In addition to these three pathways, some membranes also include a machinery that works in the opposite direction for retrotranslocation (described above). Most membranes have separate processing components for proteins with unusual targeting sequences such as those for peroxisomal thiolase, the mitochondrial protein Tim11, or the SRP receptors of eukaryotes and *E. coli*. The only organelle that seems to have dispensed with parallel translocation machineries is the nucleus, where a tight seal is not required, and the enormous complexity of nuclear pores permits regulated transport of a tremendous variety of substrates in both directions.

There are at least three different signal sequences that target proteins to peroxisomes, two of which have been identified and designated as PTS-1 and PTS-2. The targeting proteins for these signal sequences (equivalent to step 1 in Figure 1) are Pex5p and Pex7p, respectively. A third system apparently mediates a parallel import pathway for transmembrane proteins. Both Steve Gould and William Snyder have shown that the soluble primarily cytoplasmic protein Pex19p binds to many integral peroxisome membrane proteins and, therefore, discussed possible roles analogous to steps 1–3 in Figure 1 for Pex19p in targeting of integral membrane proteins. However, the precise function of this protein, especially relative to Pex14p (a candidate for step 2, Figure 1), remains controversial. Steve Gould demonstrated that several peroxisome membrane proteins are imported into nuclei when a nuclear localization signal is added to Pex19p. However, William Snyder reported data from the Subramani lab suggesting that Pex19p does not interact with the transmembrane regions of peroxisomal membrane proteins and that in

vivo it appears to bind primarily to proteins on the peroxisome membrane rather than in the cytoplasm (Snyder et al., 2000). Taken together these results suggest that Pex19p may function both to target or organize peroxisomal membrane proteins within the membrane and to target them to peroxisomes from the cytoplasm. An obvious question is whether or not Pex19p works alone, has a single function (such as a chaperone) as part of multiple complexes or is a multifunctional protein.

There is now good evidence that as in peroxisomes, fully translocated (secreted) and transmembrane proteins are handled by parallel targeting pathways at the inner membrane in *E. coli* and at the mitochondrial inner membrane. In *E. coli*, the targeting signals on these proteins are first recognized as different in the cytoplasm. Using a substrate that can be unambiguously assessed for targeting pathway, Harris Bernstein reported that the targeting signal alone is sufficient to determine whether a targeted protein uses either the SRP-dependent or -independent targeting pathway in *E. coli*. Nevertheless, the precise features of the targeting signals that sort secreted proteins from integral membrane proteins remain elusive. He also presented very provocative data suggesting that the reason that the SRP pathway is essential in prokaryotes, even though membrane proteins can still be targeted to membranes in its absence (with ~50% wild-type efficiency), is that the SRP pathway prevents the toxic effect of the accumulation of inner membrane proteins in the cytoplasm.

Georg Koch reported studies in *E. coli* using a mutant SecY (the translocon pore protein) that does not function in the translocation of secreted proteins to demonstrate that SecY also facilitates integration of inner membrane proteins (step 5 in Figure 1). Thus, in *E. coli* the initial steps are parallel for protein secretion and integration into the membrane (steps 1–3 in Figure 1) and then they converge at the translocon. The use of SecY mutants that function in only one of the two pathways will be extremely useful in differentiating which proteins regulate specifically either translocation or integration as well as for the identification of proteins that facilitate both translocation and integration.

Pier Scotti reported the characterization of one such protein, YidC, that appears to function with the SecY complex to shunt integral membrane proteins into the lipid bilayer (Scotti et al., 2000). It remains to be determined whether the early steps specific to integration of transmembrane proteins select translocons with a preexisiting association with YidC or if YidC is recruited to the translocon during integration of an integral membrane protein. Significantly, there is a mitochondrial homolog for YidC, called Oxa1p, that was previously implicated in the insertion of transmembrane proteins into the inner membrane.

Based on comparisons with these systems that have multiple parallel targeting pathways, it is surprising that there is only one characterized import system for the outer mitochondrial membrane. However, there is some evidence that cytochrome c may be a substrate for an as yet undefined additional pathway. Even more surprising is the absence of candidate cytoplasmic signal recognition proteins (the red protein in Figure 1) for targeting mitochondrial proteins to the translocation machinery in the outer membrane (the TOM complex). In contrast, there appear to be a plethora of proteins within the intermembrane space that are involved in recognition of signals for transport by the translocons of the inner membrane. These proteins direct proteins imported via the TOM complex to either the TIM22 or TIM23 complexes for integration or translocation, respectively.

Rob Jensen and Carla Koehler each reported fractionation and genetic data suggesting that complexes of Tim8/Tim13 and Tim9/Tim10 in the intermembrane space function in parallel, and in some cases in concert, in the translocation of different substrates across the intermembrane space and the inner mitochondrial membrane (steps 1-3 in Figure 1). Using Tim23 as a substrate for the inner membrane TIM22 complex, Rob Jensen identified a pair of transmembrane sequences and loop in Tim23 that function as the signal for translocation via the TOM and TIM22 complexes. Photoactive crosslinking data suggest that Tim9/Tim10 bind to this region of Tim23 while Tim9/Tim10 bind to the amino terminus of Tim23. Walter Neupert presented evidence that Tim23 is itself involved in transport of proteins across the intermembrane space (equivalent to step 3 in Figure 1). Surprisingly, Tim23 appears to span the outer mitochondrial membrane as proteolysis experiments suggest that approximately 20 amino acids at the amino terminus of Tim23 are exposed to the cytoplasm (Donzeau et al., 2000). Tim23 does not appear to be stably associated with the TOM complex nor is there an apparent hydrophobic region in Tim23 that would be a candidate transmembrane sequence. Thus, the mechanics of the unusual topology of Tim23 are not completely clear. Finally, Carla Koehler also reported that translocation of Tim11 and Tim17 are not impaired by mutations that abolish transport of other substrates by either TIM22 or TIM23 complexes. Thus, evidence continues to accumulate for yet another parallel pathway.

Danny Schnell reported the identification of two new chloroplast import proteins in work done in collaboration with Felix Kessler. The new proteins, TOC132 and TOC120, are related to the transit-signal (signal sequence) binding protein TOC159 (Chen and Schnell, 1999). Each of these proteins spans the outer membrane of the chloroplast and appears to function in the recognition of signal peptides (steps 2–3 of Figure 1). Divergent acidic regions (A) at the amino terminus of each protein mediate binding specificity while transfer of peptide to the translocon is likely mediated by the highly homologous M and G domains. The identification of three closely related proteins that all function in the recognition of import signals strongly suggests that transit signals in chloroplasts are recognizably different at the molecular level even though the differences are not immediately apparent from the amino acid sequence.

Plants bearing mutations in TOC159 are viable when grown on sucrose. Therefore, it was possible to demonstrate that loss of TOC159 function blocks import of proteins required for photosynthesis, yet nonphotosynthetic functions of chloroplasts are not impaired. This is a very compelling demonstration of multiple parallel pathways for protein import into chloroplasts. That all three proteins can be coimmunoprecipitated suggests that similar to translocation pathways at the inner membrane of *E. coli*, these pathways may converge at a single translocon.

The Usual Suspects . . .

The first step in the accurate targeting of proteins requires the recognition of the signal peptide in the cytoplasm (step 1, Figure 1). The mechanisms by which the

similar but nonidentical signal sequences that are used to target proteins with exquisite specificity are recognized as distinct from apparently similar nontargeting sequences has been one of the most enduring questions in protein targeting.

The PTS-1 signal sequence used for the import of many peroxisomal matrix proteins is composed of only 3 amino acids (the archetype sequence is Ser-Lys-Leu). Steve Gould reported the results of modeling experiments suggesting that, similar to recognition of nuclear localization sequences by Karyopherins, the peroxisome import receptor Pex5p makes use of conserved Asn residues to bind PTS-1 signals. In his model, PTS-1 signals are bound in an extended conformation. Consistent with these modeling experiments, patients with an Asn-to-Lys mutation in Pex5p fail to import proteins with a PTS-1 but still import proteins via PTS-2 signals. Nevertheless, modeling is clearly not going to be sufficient for understanding recognition of even small signals like PTS-1 as the model presented does not account for the essential lysine at position 2. However, diffraction quality crystals for a portion of Pex5p bound to a PTS-1 have been obtained. Once heavy atom derivatives are found, and the structure is solved, it will greatly facilitate experimental testing of models for PTS-1 binding.

The essential features of the signal peptides that target proteins to the endoplasmic reticulum and to the *E. coli* plasma membrane are well established. However, it is still not entirely clear how these sequences are differentiated from transmembrane domains nor are the molecular interactions involved in recognition completely established (Falcone et al., 1999). The signals and early steps in signal recognition are sufficiently similar in eukaryotes and prokaryotes that the signal peptides are interchangeable. Furthermore, in both systems, the initial interaction with the signal peptide is mediated by homologous GTPase containing proteins. However, to understand at the molecular level how signal peptides are recognized and transferred between signal receptors during targeting and translocation, it will be essential to obtain quantitative measurements for the affinity and dissociation rates for signal peptides with their receptors. Art Johnson presented preliminary equilibrium measurements of dissociation constants for ribosome-bound signal sequences with the eukaryotic signal recognition particle (SRP). These measurements promise to permit clear discrimination between the different models for signal peptide recognition that have been contentious for years.

Like endoplasmic reticulum signal peptides, the N-terminal presequences of mitochondrial proteins share very limited sequence similarity. Consequently, the mechanism by which these sequences are recognized as different from other similar sequences in nontranslocated proteins is unknown. Toshiya Endo analyzed the structure of the presequence receptor Tom20 in the presence of different presequences by NMR spectroscopy (Abe et al., 2000). Different sequences were shown to induce similar chemical shift changes, suggesting that Tom20 contains a single presequence binding site. The NMR structure revealed a bed of three α helices surrounding one side of the presequence peptide that was also in a helical conformation. Like binding of signal peptides involved in export of proteins across the endoplasmic reticulum and *E. coli* plasma membrane, recognition appears to be based primarily on hydrophobic interactions. However, since mitochondrial import sequences are amphipathic, binding by Tom20 appears to involve primarily one side of the amphipathic helix. To test this hypothesis, mitochondrial import peptides were synthesized in which the Arg residues were changed to Gln residues. Conversely other peptides were used in which Leu residues were exchanged for Gln. Analysis of both sets of peptides confirmed that hydrophobicity is the driving force for peptide binding by Tom20. Nevertheless, the positive charges in presequences are known to be essential for import. Therefore, it is likely that the positive charges contribute to a feature recognized at another stage of the import pathway. Taken together these results suggest a theme shared by presequence-mediated import into mitochondria and signal sequence–mediated transport across the endoplasmic reticulum and *E. coli* plasma membranes: sequential low-affinity interactions are used to increase the selectivity of transport. Binding of signal sequences by relatively sequence-independent features such as patches of hydrophobicity may account for how peptides of various amino acid sequences can specify a unique localization.

Presequences are required to initiate translocation of folded proteins across the *E. coli* plasma membrane, thylakoid membranes, and the inner membrane of mitochondria. In these cases translocation is mediated by the twin arginine translocation (TAT) translocon. The signal sequences responsible for mediating TAT-dependent transport are nearly identical to endoplasmic reticulum signal sequences except that they contain a diarginine motif amino-terminal of the hydrophobic core sequence. Colin Robinson suggested that, at least in thylakoid membranes, a hydrophobic residue two or three residues carboxyl of the two arginines is required for recognition of a TAT pathway signal sequence. Analogous to other targeting pathways the essential features of TAT translocation signals are conserved (*E. coli* and thylakoid presequences are interchangeable), yet the identified features (RRXh, RRXXh, where h is more hydrophobic than alanine) seem to contain insufficient information to be organelle specific. By adding a pair of arginines two residues before a Leu near the beginning of the hydrophobic domain of a conventional secretory signal sequence, it was possible to create a protein that could be exported by either pathway in *E. coli*. Soluble signal receptors (step 1 of Figure 1) have not yet been identified for these pathways, yet it seems very likely that they exist (although they may not be essential in vitro).

Swallowing Hook, Line, and Sinker . . .

In peroxisomes, the Pex5 pathway allows the import of fully folded oligomerized proteins. Klaas Nico Faber used an in vivo system to demonstrate that multisubunit proteins can be assembled in the cytoplasm in a functional form which can still be imported into peroxisomes. Using this approach he attempted to determine the upper size limit for import (previously set at 9 nm by the import of gold particles). The octameric enzyme alcohol oxidase of the yeast *Hansenula polymorpha*, with estimated dimensions of 12.5 nm^3 was not imported. However, it remains to be determined if lack of import is because of an intrinsic size limitation or if folding and oligomerization obscurred the import signal.

Unlike peroxisomes, transport of folded proteins by TAT transporters in chloroplasts, mitochondria, and probably *E. coli* requires a transmembrane potential.

Colin Robinson reported that in *E. coli* this pathway seems to be involved in the export of approximately 20 cofactor binding proteins while some, if not all, thylakoid proteins are imported in a folded form. It may be that the TAT system predominates for proteins destined for thylakoid membranes because the substrates tend to fold in the stromal space. Import reactions using purified proteins revealed that translocation of folded proteins appears to be independent of chaperones. This intriguing observation raises the question of how the transporter accommodates a variety of substrates without compromising the transmembrane potential essential to translocation.

Steve Theg presented an interesting biophysical approach to determining the role of transmembrane potential in import via the thylakoid TAT pathway. By measuring delta pH and protein import at the same time and in the same cuvette, he demonstrated that protons are exchanged for protein import and that the threshold delta pH for translocating a protein was not the same for two different proteins. Whether there will be specific features of a protein that require a higher delta pH for translocation or if the threshold delta pH is related primarily to the size of the protein remains to be determined. Nevertheless, using two different methods to measure the "cost" for importing a single model protein, it was possible to estimate that energy equivalent to approximately 2000–3000 ATP molecules is required to translocate a folded protein across a membrane. This value approximates the "cost" estimated previously for Sec-mediated translocation in *E. coli* and is slightly more ATP than needed to synthesize the protein. Thus, correct localization is clearly a significant energy investment.

The translocation of large molecules is not limited to proteins. Steve Hajduk presented data suggesting that in trypanosomes mitochondria can import RNAs containing 2 tRNAs and a 59 nucleotide intergenic region (Yermovsky-Kammerer and Hajduk, 1999). This intergenic region forms a stem loop that is essential for efficient import into mitochondria. Import was abolished by pretreating mitochondria with protease, by adding uncouplers, or by removing external ATP. Dissection of this pathway is bound to reveal fascinating insights into the mechanisms by which translocation of relatively large folded molecules is accomplished without compromising the transmembrane potential.

GTPases, GTPases, and More GTPases . . .

Identifying the proteins involved in protein targeting and translocation is only the initial step in determining the mechanisms of transport. Evidence was presented by Reid Gilmore that the initial targeting of nascent polypeptides to the endoplasmic reticulum is managed by a unique series of GTPases (steps 1–3 in Figure 1). While strong evidence already existed for roles for two of these, the SRP GTPase and the GTPase in the α subunit of the SRP receptor, the GTPase domain of the β subunit of the SRP receptor has been an enigma (Millman and Andrews, 1997). Reid Gilmore presented data indicating that the GTPase cycle of SRβ has to occur in order to get multiple rounds of translocation. He also demonstrated that SRP receptor heterodimers are required to form SRP-SRP receptor-ribosome nascent chain complexes. Related data presented by David Andrews suggests that nucleotide binding by the β subunit of SRP receptor regulates heterodimerization with the α subunit (Legate et al., 2000). Therefore, it is possible that the α subunit of the SRP receptor cycles on and off the endoplasmic reticulum membrane. Although there is no β subunit in *E. coli*, the homolog of the α subunit, FtsY, has also been proposed to cycle on and off the membrane.

Using a reconstituted retrotranslocation system, Bassam Ali demonstrated that guanylate kinase and GTP hydrolysis are required for export of glycopeptides from the endoplasmic reticulum to the cytoplasm (reverse of step 4 in Figure 1). Thus, GTPases regulate traffic in both directions across the membrane of the endoplasmic reticulum. Taken together with the already well-characterized role of the RAN GTPase in nuclear transport and proposed functions of the transmembrane GTPases involved in signal recognition at chloroplasts (TOC159, TOC 132, and TOC120, described above), it seems likely that GTPases are going to be involved in regulating translocation across most membranes.

Are there other regulatory mechanisms for protein transport? Analogous to the interactions mediated by SRP and SRP receptor, there is an initial docking step (step 2 in Figure 1) at nuclear pores during translocation in both directions across the nuclear membrane. Susan Wente made use of the observation that overexpression of the GLFG region of a class of nuclear pore–associated proteins inhibits mRNA export to identify Gle1p, an essential mRNA export factor in yeast that does not bind to RNA but does bind to NUP42. Gle1p is located on both sides of the nuclear pore complex and has homologs in mammals (Watkins et al., 1998). A screen for genes synthetically lethal with Gle1 identified several proteins including phospholipase C1 and two unknown proteins. Phospholipase C1 cleaves PIP2 releasing IP3 and activating protein kinase C. IP3 is sequentially phosphorylated to higher order polyinositol phosphates, so she examined whether or not the two unknown genes identified in the synthetic lethal screen encode inositol polykinases (IPKs). Knockouts of these genes result in distinct blocks in the metabolic pathway converting IP3 to IP6. Biochemical evidence identified one as an IP5 2-kinase (IPK1) and the other as a dual function IP3/IP4 kinase (IPK2). Mutations in phospholipase C and in the IPK genes result in specific defects in mRNA export. Moreover, IPK1 localizes to nuclear pore complexes suggesting that IP6 facilitates mRNA export by binding to the export machinery at the nuclear pores.

The Future Is Biogenesis . . .

One of the benefits of any meeting is an opportunity to witness the coalescence of techniques that provide unique insights into a variety of processes. For example, not so long ago the most popular method of analyzing protein–protein interactions was the yeast two-hybrid assay. The huge number of false positives obtained with that method led to a resurgence in cross-precipitation techniques. However, current approaches aim toward understanding stoichiometry as well as measuring binding. An interesting extension of standard cross-precipitation techniques was presented by Beth Traxler in which modeling of precipitation reactions was used to estimate stoichiometry (Kennedy and Traxler, 1999).

A clear recent success has been the application of blue native electrophoresis (Schagger and von Jagow, 1991; Schagger et al., 1994). The use of both one- and two-dimensional blue native electrophoresis to map interactions within larger complexes was reported by several participants. In one particularly elegant application

of the technique, Nikolaus Pfanner reported the use of blue native electrophoresis to study the structure and dynamics of the outer mitochondrial membrane TOM complexes. Preparative scale gels were used to isolate 400 kDa TOM complexes that could then be reconstituted into liposomes and fused with black lipid bilayers to analyze the conductance states of the complex. Blue native electrophoresis was also used to characterize TOM assembly intermediates with different compositions and molecular weights. Stepwise assembly of the 400 kDa TOM complex occurred via intermediate complexes of 250 kDa and 100 kDa. The precursor of the channel-forming protein Tom40 first associates with Tom5 to form a 250 kDa complex that is converted to a 100 kDa complex during membrane insertion. The addition of Tom6 and Tom22 to the 100 kDa complex results in formation of the mature 400 kDa complex. Addition of Tom7 then allows cycling between 100 kDa precursor and 400 kDa mature complexes. Cycling between partially assembled and fully assembled complexes provides an interesting twist on the "chicken and the egg" dilemma for the assembly of translocons. For systems such as the peroxisome, where there appear to be many more proteins than identified functions, these techniques promise to be very revealing.

Finally, the question most frequently debated between the posters was organelle biogenesis. One of the major difficulties with analyzing biogenesis is that the pathways have evolved along with the translocons that direct membrane assembly. It has already been shown that in yeast, both subunits of the SRP receptor must be inherited. Yet only one of these polypeptides uses the translocation machinery to target to the endoplasmic reticulum. Of the 5 polypeptides essential for protein translocation across the endoplasmic reticulum, all but two of them (the β subunit of SRP receptor and the α subunit of the translocation pore, Sec61) are assembled independently of the translocon. In this case it is tempting to speculate that the dependence on the translocon for assembly of these two proteins is a relatively recent development. Therefore, it will not be surprising if the role of maternal inheritance varies considerably from one organelle to another and for the same organelle from one organism to another. For example, in cells that do not require peroxisome hyperproliferation, maternal inheritance may be essential and fission may be sufficient to provide new peroxisomes. In contrast, yeast such as *Hansenula polymorpha* may be unable to synthesize sufficient membrane at the peroxisome quickly enough to accomplish the massive hyperpoliferation of peroxisomes that is induced by growth of this organism on oleate or methanol. These yeast, then, may have quite different routes to the synthesis of new peroxisomes.

The analysis of organelle biogenesis is further complicated in cells in which maternal inheritance is an important contributor to biogenesis. In these cells it may not be reasonable to expect that recovery from even transient elimination of the organelle would approximate normal biogenesis. Thus, the question of the "chicken or the egg" may give us many avenues to explore in the years ahead.

References

Abe, Y., Shodai, T., Muto, T., Mihara, K., Torii, H., Nishikawa, S., Endo, T., and Kohda, D. (2000). Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. Cell *100*, 551–560.

Braun, B.C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.M., Finley, D., and Schmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. *1*, 221–226.

Chen, X., and Schnell, D.J. (1999). Protein import into chloroplasts. Trends Cell Biol. *9*, 222–227.

Donzeau, M., Kaldi, K., Adam, A., Paschen, S., Wanner, G., Guiard, B., Bauer, M.F., Neupert, W., and Brunner, M. (2000). Tim23 links the inner and outer mitochondrial membranes. Cell *101*, 401–412.

Falcone, D., Do, H., Johnson, A.E., and Andrews, D.W. (1999). Negatively charged residues in the IgM stop-transfer effector sequence regulate transmembrane polypeptide integration. J. Biol. Chem. *274*, 33661–33670.

Kennedy, K.A., and Traxler, B. (1999). MalK forms a dimer independent of its assembly into the MalFGK2 ATP-binding cassette transporter of Escherichia coli. J. Biol. Chem. *274*, 6259–6264.

Legate, K.R., Falcone, D., and Andrews, D.W. (2000). Nucleotide dependent binding of the GTPase domain of the signal recognition particle receptor beta-subunit to the alpha-subunit. J. Biol. Chem., in press.

Millman, J.S., and Andrews, D.W. (1997). Switching the model: a concerted mechanism for GTPases in protein targeting. Cell *89*, 673–676.

Schagger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. *199*, 223–231.

Schagger, H., Cramer, W.A., and von Jagow, G. (1994). Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal. Biochem. *217*, 220–230.

Scotti, P.A., Urbanus, M.L., Brunner, J., de Gier, J.W., von Heijne, G., van der, D.C., Driessen, A.J., Oudega, B., and Luirink, J. (2000). YidC, the Escherichia coli homologue of mitochondrial Oxa1p, is a component of the Sec translocase. EMBO J. *19*, 542–549.

Snyder, W.B., Koller, A., Choy, A.J., and Subramani, S. (2000). The peroxin pex19p interacts with multiple, integral membrane proteins at the peroxisomal membrane. J. Cell Biol. *149*, 1171–1178.

Watkins, J.L., Murphy, R., Emtage, J.L., and Wente, S.R. (1998). The human homologue of Saccharomyces cerevisiae Gle1p is required for poly(A)+ RNA export. Proc. Natl. Acad. Sci. USA *95*, 6779–6784.

Yermovsky-Kammerer, A.E., and Hajduk, S.L. (1999). In vitro import of a nuclearly encoded tRNA into the mitochondrion of Trypanosoma brucei. Mol. Cell Biol. *19*, 6253–6259.