13 Moving Proteins into Membranes and Organelles

Review the Concepts

1. In eukaryotes, protein translocation across the endoplasmic reticulum (ER) membrane is most commonly cotranslational; it can also occur post-translationally, particularly in yeast.

1a. The energy source for cotranslational translocation comes from the translation process itself – in other words, the nascent chain is pushed through the translocon channel. Please note, however, that as translation is completed a portion of the newly synthesized protein still resides within the translocon. This portion is drawn into the ER lumen rather than being pushed.

1b. In post-translational translocation, the newly synthesized polypeptide chain is drawn through the translocon by an energy input from ATP hydrolysis by BiP. BiP is luminal protein of the ER and is a member of the Hsc70 family of molecular chaperones. BiP-ATP activates by binding to the Sec63 complex that in turn binds to the Sec61 translocon complex. Activated BiP is enzymatically active and cleaves ATP to ADP plus P_i. It is BiP-ADP that binds to the entering, unfolded nascent chain. Sequential binding of BiP-ADP to the nascent chain serves to block any sliding of the chain back and forth in the translocon and to ratchet the nascent chain through the translocon.

1c. Translocation across the bacterial cytoplasmic membrane also occurs post-translationally. SecA, a cytosolic protein, acts to drive the incompletely folded, newly synthesized polypeptide through the translocon (SecY complex). SecA associates with the tranlocon and newly synthesized polypeptide. SecA uses ATP hydrolysis to push the polypeptide through the translocon.

1d. Translocation into the mitochondrial matrix occurs through a bipartite Tom/Tim complex in which Tom is the outer membrane translocon and Tim is the inner membrane translocon. Three energy inputs are required. First, ATP hydrolysis by a cytosolic Hsc70 chaperone keeps the newly synthesized mitochondrial precursor protein unfolded in the cytosol. Second, ATP hydrolysis by multiple ATP-driven matrix Hsc70 chaperones may serve to pull the translocating protein into the matrix. Matrix Hsc70s interact with Tim44 and hence may be analogous to the BiP/Sec63 interaction at the ER membrane. Third, energy input from the H^+ electrochemical gradient or proton-motive force is required. The inside-negative membrane electric potential may serve to electrophorese the amphipathic matrix-targeting sequence towards the matrix.

2. SRP (signal recognition particle) acts as a cycling cytosolic factor for the translocation of ER targeted proteins. It binds to both the signal sequence and SRP receptor, a heterodimer associated with the ER membrane. In doing this, SRP initiates ribosome binding to ER membranes and positions the nascent chain proximal to the translocon. Both SRP and the SRP receptor are GTPases. The unfolded nascent chain then translocates. Cytosolic Hsc70 functions as a cytosolic factor required for protein translocation into mitochondria. It acts as a molecular chaperone to keep the post-translationally targeted mitochondrial precursor protein in an open, extended conformation. At least two different cytosolic proteins are required for translocation of peroxisomal matrix proteins. These are Pex5, the soluble receptor protein for matrix proteins containing a C-terminal PTS1 targeting sequence, and Pex7, the soluble receptor protein for matrix proteins containing a N-terminal PTS2 targeting sequence. A different receptor, Pex19, is required for peroxisomal membrane proteins.

3. Many membrane proteins are embedded in the membrane by virtue of transmembrane α-helical segment(s). Such segments can be referred to as topogenic sequences. These segments share general principles or properties. They tend to be about 20 amino acids long, a length sufficient to span the membrane, and hydrophobic, an appropriate property for a sequence embedded in the hydrophobic lipid bilayer. Application of these principles through computer algorithms is predictive. In brief, amino acid sequences of polypeptides may be scanned for hydrophobic segments of about 20 amino acids long. Each amino acid may be assigned a hydrophobic index value based on relative solubility in hydropbobic media versus water and these values then can be summed by a computer for all 20 amino acid segments of a protein. Segments exceeding a threshold value are expected to be topogenic transmembrane segments. Similar reasoning with, in practice, less predictive value can be applied on the basis of properties of signal sequences for targeting to ER, mitochondria, chloroplasts, peroxisomes, etc. In general, such an algorithm must examine the full sequence of a precursor protein. In general, the computer identification of topogenic sequences can be predictive. To illustrate, let us consider an ER-targeted, multipass membrane protein with an N-terminal, cleavable signal sequence. Based on principles, such an ER-targeting, N-terminal, cleavable signal sequence can be computer-identified by sequence scanning. Subsequent internal signal anchor and stop-transfer anchor segments similarly can be identified. Such sequences alternate within a multipass membrane protein. Because of this, the overall arrangement of the protein can predicted as described in detail in Chapter 13 of the text.

4. Improperly folded or oligomerized proteins fail to exit the ER — in other words, exit from the ER is protein quality dependent. A fairly wide array of accessory proteins is involved in the processing of newly synthesized proteins within the ER lumen. These include proteins necessary for N-glycosylation of appropriate proteins, protein-folding chaperones such as BiP, disulfide-bond-processing proteins such as protein disulfide isomerase, enzymes that accelerate rotation about peptidyl-prolyl bonds such as peptidyl-prolyl isomerases, and proteins such as calnexin and calreticulin that bind to glucose and act to prevent aggregation of unfolded or misfolded proteins. Proteins unable to exit the ER are generally degraded. To the surprise of most investigators, this degradation occurs in the cytosol in an ubiquitin-dependent, proteasome-dependent manner. The Sec61 translocon may be involved in transporting these misfolded proteins into the cytosol for degradation. Cytosolic p97 ATPase has also been implicated in retrotranslocation, and may use the energy of ATP hydrolysis to pull misfolded proteins from the ER membrane into the cytosol.

5. The seven-sugar intermediate is synthesized by sugar addition to cytosolic-facing dolichol phosphate. The intermediate is flipped from the cytosolic face of the ER membrane to the luminal face. Further sugar additions then occur within the lumen of the ER. Short forms of the intermediate are on the wrong side of the membrane to add to nascent polypeptides within the ER lumen. Incomplete adductants within the ER lumen are located appropriately to N-glycosylate nascent polypeptide.

6. Several proteins facilitate the modfication or folding of secretory proteins within the ER. These include signal peptidase, BiP, oligosaccharyl transferase, various glycosidases, calnexin and calreticulun, protein disulfide isomerase, peptidyl-prolyl isomerase, and others. Of these, BiP and peptidyl-prolyl isomerase act to facilitate conformation changes. Protein disulfide isomerase facilitates the making/breaking of disulfide bonds to ensure correct protein folding. Calreticulin and calnexin are lectins that bind to glycoproteins during folding. The others all directly support the covalent modification of proteins within the ER lumen.

7. BiP-ADP binds to nascent chains. Replacement of ADP with ATP releases BiP from nascent chains. The result observed is consistent with this mechanism for BiP binding.

8. Each mutation has a different effect.

8a. Tom22 together with Tom20 act as outer mitochondrial membrane receptor proteins for N-terminal matrix targeting sequences. A defective Tom22 receptor protein would result in accumulation of mitochondrial matrix targeted proteins in the cytosol, possibly followed by their turnover within the cytosol.

8b. Tom70 signal receptor is an outer mitochondrial membrane protein recognizing multipass mitochondrial membrane proteins that have internal signal sequences. Mutation in Tom70 will have no immediate effect on mitochondrial matrix protein import as Tom70 does not recognize this class of protein.

8c. Matrix Hsc70 has a role in the folding of matrix proteins. Also, it is one source of energy for powering translocation. Defective matrix Hsc70 should result in clogging the Tom/Tim translocon complex with incompletely translocated proteins.

8d. Retention of the matrix targeting N-terminal signal sequence because of a defective matrix signal peptidase might well result in defective folding of the imported protein. The sequence normally is removed.

9. On the whole, protein import into the mitochondrial matrix and the chloroplast stroma, topologically equivalent locations, is by functionally equivalent mechanisms. Functionally analogous proteins mediate each process. However, the proteins are not homlogous, indicating a separate evolutionary origin of mitochondria and chloroplasts. Energetically, unlike the situation for mitochondria, there is no need for a membrane electrochemical gradient for import into chloroplasts. Presumably stromal Hsc70 pulls proteins into the stroma.

10. This is basically a molecular ruler question. How many amino acids must span the Tom/Tim complex to expose the matrix-targeting sequence to the matrix-processing protease? DHFR in the presence of the drug methotrexate is locked into a folded state. A chimeric mitochondrial protein with folded DHFR fails to translocate fully into the mitochondria matrix. Instead, it is stuck in the Tom/Tim complex. The number of amino acids between the matrix targeting sequence and the folded DHFR sequence could be varied to provide a molecular ruler. Any unfolded N-terminal DHFR sequence must be included within the ruler. With respect to channel length, an overestimate will result from this approach as the matrix targeting sequence must be spaced out from Tom/Tim to be accessible for cleavage.

11. Mitochondrial targeting sites include matrix, inner membrane, intermembrane space, and outer membrane. Chloroplasts targeting sites include stroma, inner membrane, intermembrane space, outer membrane, and thylakoid membrane. Despite some commonalities, significant differences exist between the mechanisms by which mitochondrial ADP/ATP antiporter and cytochrome b_2 are targeted in a site-specific manner. ADP/ATP antiporter has internal signal sequences recognized by Tom70 receptor and by Tim22 complex. Cytochrome b_2 has a N-terminal matrix targeting sequence that is followed by an intermembrane space-targeting sequence. Both use Tom40. However, the Tim components differ. Both are released from the Tim complex into the inner mitochondrial membrane. The ADP/ATP antiporter stays there. Cytochrome b_2 is released into the inner membrane space by proteolytic cleavage.

12. Catalase is responsible for breaking down H_2O_2 to H_2O. Catalase, like most other peroxisome-localized enzymes, contains a peroxisome-targeting sequence (PTS1) consisting of three amino acids, serine-lysine-leucine, at its C-terminus. This PTS1 is recognized and binds in the cytosol to the Pex5 receptor. The catalase-Pex5 heterodimer moves to the peroxisome membrane, where it interacts with the Pex14 receptor located on the membrane. In this position, the complex interacts with three membrane proteins, Pex2, Pex10 and Pex12, that facilitate the translocation of catalase into the peroxisome.

13. Separate mechanisms are used to import peroxisomal matrix and membrane proteins. Hence mutations can selectively affect one or the other. Either can result in the loss of functional

peroxisomes. One approach to determining whether the mutant is primarily defective in insertion/assembly of peroxisomal membrane proteins or matrix proteins is to use antibodies to ask by microscopy if either class of proteins localize to "peroxisomal" structures, e.g., peroxisome ghosts. An alternate approach is cell fractionation, in which the assay determines whether the appropriate proteins are present in a membrane organelle fraction.

14. *Nuclear-localization-signal* (NLS) is the name given to a sequence of amino acids present and required for cargo proteins to be selectively imported into the nucleus. Three proteins required for nuclear import are Ran, importin α and importin β. Ran is a monomeric G protein that binds to GDP in the cytosol. In this conformation, Ran·GDP diffuses through the nuclear pore complex (NPC) into the nucleus. Now in the nucleoplasm, Ran·GDP associates with a guanine nucleotide exchange factor (Ran-GEF), causing Ran to release GDP and replacing it with GTP. In the cytosol, the two importins form a heterodimeric nuclear-import receptor, where importin α binds the NLS on the cargo protein and importin β interacts with nucleoporins of the NPC, facilitating the movement through the pore. When this complex reaches the nucleoplasm it encounters Ran·GTP, which causes the importin to decrease its affinity for the cargo protein. The importin-Ran·GTP complex travels through the NPC back to the cytosol where the GTP is hydrolyzed. This hydrolysis causes the importins to dissociate from Ran allowing them, together with Ran·GDP, to prepare for another round of import.

15. Ran–guanine nucleotide exchange–factor (Ran-GEF) must be present in the nucleus and Ran-GAP must be in the cytoplasm for unidirectional transport of cargo proteins across the nuclear pore complex. When the Ran is bound to GTP it has high affinity for cargo proteins. During nuclear export, Ran-GTP picks up cargo proteins in the nucleus and carries them to the cytoplasm. To be able to release the cargo on the cytoplasmic side, GTP must be hydrolyzed to GDP, and this process is stimulated by Ran-GAP. Once translocated back into the nucleus, Ran needs to be in the GTP bound state to pick up more cargo. Ran-GEF in the nucleus stimulates the exchange of GDP to GTP, and this process of export can start again.

Analyze the Data

a. Messenger RNA lengths vary by steps of 20 codons or 20 amino acids each in corresponding synthesized product. When translated in the absence or presence of microsomes, only mRNAs of 130 and 150 codons produce a product that displays any difference in size with the addition of microsomes. The 130-codon mRNA gives a product that is either full length—showing no difference in size when compared to the minus microsome product—or a smaller product that is the presumed result of signal peptidase cleavage. This suggests variable or incomplete accessibility of the product to signal peptidase. In contrast, the next-step-size-longer mRNA codes for product that is fully sensitive to signal peptidase cleavage when synthesized in the presence of microsomes. The key datum is the smaller size (faster mobility) of the product plus microsomes versus the product minus microsomes. Hence the conclusion is that the prolactin chain must be somewhere between 130 and 150 amino acids in length for the signal sequence to be fully accessible for cleavage.

b. The polypeptide must be mostly α-helical. A 100-amino-acid polypeptide as an α-helix spans 150 Å, the length of the ribosome channel. Thirty amino acids span about 50 Å, the membrane thickness. In sum, a total length of about 160 amino acids (130-amino-acid spacer) is required to space the signal peptide out by 150 Å, the necessary minimum length. Only a 60-amino-acid spacer is required to give a minimally accessible signal peptide if the polypeptide were in extended conformation.

c. The fact that there is no prolactin cleavage if microsomal membranes are added after prolactin translation is complete indicates that prolactin must translocate cotranslationally.

d. A series of parallel reactions were done and a microsomal membrane fraction was prepared by centrifugation. No prolactin labeled polypeptide is seen in the membrane fraction for mRNA shorter than 90 codons. Hence, it is only at this nascent chain length that any engagement of ribosomes with SRP and hence binding to membrane occurs. If the mRNA is 110 codons or longer, the membrane fraction contains all labeled product found in the total reaction as indicated by the equal intensity of the gel bands. So, roughly a total chain length of 100 amino acids is required to expose the 30 amino acid signal peptide for SRP binding. The two bands seen with the 130-codon reaction are due to partial cleavage of the product by signal peptidase. The single band seen with the 150-codon reaction reflects full cleavage of the signal peptide by signal peptidase.