Protein Targeting by Cleavable Signal Sequences

• Export signal sequences (degenerate) -- ER membrane targeting (16-30 residues in length)
  -- N-terminal (+) domain
  -- Hydrophobic domain (6-12 residues) -- interacts with ER membrane receptor
  -- Signal peptidase cleavage site

• Mitochondrial import signal sequence (20-30 residues)/tandem signal sequence
  -- Import signals = amphipathic α-helix (3-5 Arg/Lys residues)
  -- Sort outer membrane, inner membrane and intermembrane space proteins (stop transfer, second cleavable signal sequence)
Uptake-Targeting Sequences of Imported Mitochondrial Proteins

- N-terminal matrix targeting sequences (pink) are similar but not identical & are cleaved by a matrix protease
- A second targeting sequence (brown) functions in directing proteins to intermembrane space and outer membrane
- Second targeting sequence also removed from proteins destined for intermembrane space
Proteins Targeted to ER are Cotranslationally Synthesized on ER Membrane-Bound Polysomes

- Signal recognition particle (SRP) recognizes signal sequence when nascent chain ~70 residues long & arrests translation
- SRP-nascent chain complex binds GTP, associates with α-subunit of SRP receptor
- 60S ribosomal subunit binds to ribosome receptor (Sec61), signal sequence binds to translocating chain-associated membrane (TRAM) receptor -- translation block relieved
- Polypeptide chain extruded cotranslationally through transmembrane Sec61 channel -- SRP recycled (GTP hydrolyzed for SRP release)
- Signal sequence cleaved in lumen by signal peptidase
- Hsc70 chaperone protein binds polypeptide chain and facilitates folding (yeast)
- Transmembrane spans of integral ER membrane proteins contain “stop-transfer/membrane-anchor sequences”
Structural Model for Signal Recognition Particle (SRP)

- SRP consist of one 300-nucleotide RNA and six proteins designated by Mol. Wt.
- P54 also functions together with α-subunit of SRP receptor to hydrolyze GTP
Crystal Structure of Ribonucleoprotein Core of Signal Recognition Particle Determined at 1.8 Å Resolution

- (A) Structure for domain IV of *E. coli* 4.5S SRP RNA (dark blue) & Met-rich M domain of SRP54 homolog (cyan), representing universally conserved ribonucleoprotein SRP core. M domain is involved in recognition of signal peptide & SRP RNA. Pink ribbon is superimposed structure for unbound M domain from *Thermus aquaticus*. Universally conserved & highly conserved SRP nucleotides are yellow & green, respectively (Batey et al., Science 287:1232-1239, 2000)

- (B) Molecular surface representation of complex showing signal peptide recognition groove with hydrophobic region in yellow & adjacent RNA phosphates in red
Synthesis of Secretory Proteins on Rough ER

- Synthesis begins on free ribosomes -- N-terminal signal sequence emerges when chain is ~70 AA’s long
- Steps 1 & 2: SRP binds to signal sequence & SRP-nascent chain-ribosome complex binds α subunit of SRP receptor
- Step 3: SRP & its receptor dissociate from nascent chain with hydrolysis of GTP -- gate opens & nascent chain binds to translocon & loops back with N-terminus of signal sequence facing cytosol
- Step 4: elongation of nascent chain and cleavage of signal sequence by luminal signal peptidase
- Step 5: Nascent chain continues to elongate & is extruded into lumen. Chaperone protein Hsc 70 (in yeast) binds growing chain -- powered by ATP hydrolysis, cycles of this process release nascent chain & facilitate its folding
- Step 6: Nascent chain elongates until translocation & release are complete -- ribosome is released, gate shuts & chain folds into final conformation
Insertion of Nascent Secretory Proteins into Translocon is Driven by GTP-GDP Exchange & GTP Hydrolysis

- Both P54 & α subunit of SRP receptor bind & hydrolyze GTP
- Step 1: SRP with associated GDP binds to signal sequence on ribosome-nascent chain complex, triggering GTP-GDP exchange on SRP (Step 2)
- Step 3: SRP- signal sequence-ribosome complex binds to SRP receptor with GTP bound to α-subunit
- Step 4: GTP hydrolysis by SRP and its receptor powers opening of translocon gate & transfer of nascent chain to translocon channel
- Step 5: SRP with associated GDP is released and recycled
Structure of the Translocon Channel

- An mRNA encoding the first 70 residues of secretory protein prolactin, modified to contain a single Lys, was translated in a cell-free microsome system that contained a lysyl-tRNA with a light-activatable cross-linking group @ the Lys ε-amino, thereby forming lys that could potentially cross-link associated proteins.
- Completed chain became stuck in translocon channel & cross linker was activated by light, resulting in covalent linkage to the Sec61p and the translocating chain-associated membrane (TRAM) proteins lining the channel.
- (Right) Freeze-fracture electron micrograph image of translocon channel formed by reconstitution of ribosome-associated Sec61p into phospholipid bilayer. The channel is viewed in the membrane plane & forms a pentagonal cylinder (5-6 nm high, 8.5 nm diameter with ~2 nm central pore).
During biosynthesis, topogenic sequences act to ensure proper orientation of proteins integral to ER membrane, that is retained as they undergo transport to the cell surface.
Secretory Proteins of ER Lumen Are Transported to Golgi Apparatus by Vesicle Budding & Fusion for Further Glycosylation & Modification

- Soluble proteins resident to ER contain KDEL C-terminal sequence that return them to ER by vesicle re-fusion
- Proteins are transported to cell surface via vesicles which fuse with plasma membrane
- Proteins are tagged with mannose-6-phosphate for recognition by specific receptor in *trans*-Golgi & ultimately targeted to lysosomes
Secretory Pathway of Protein Synthesis & Sorting

- As translation of signal-sequence containing proteins by rough-ER bound polysomes is completed, they either insert into membrane or cross into lumen (some remain resident to ER)
- Others move into transport vesicles that fuse to form new cis-Golgi vesicles
- Red arrows show Golgi cisternae moving from cis to trans face of Golgi stack -- many proteins undergo glycosylation with oligosaccharides
- Some proteins are retained in trans-Golgi cisternae, others move via small vesicle to cell surface or to lysosomes
- In regulated secretion (nerve, pancreatic cells), soluble proteins are stored in secretory vesicle & are released by neural or hormonal signals
- In constitutive secretion, proteins move to surface for continuous release via transport vesicles (some proteins remain integral to plasma membrane)
- Retrograde movement via small transport vesicles retrieves ER proteins from cis-Golgi & cis- or medial-Golgi proteins that migrate to later compartments & returns them to original location
Posttranslational Mitochondrial Import of Proteins with Cleavable N-Terminal Targeting Sequences Requires ATP and pmf

- Import competent conformation maintained by chaperones (mitochondria import stimulating factor (MSF), cytosolic Hsc70) -- energy from ATP hydrolysis maintains conformation
- Interact with respective outer membrane receptors (translocases of outer membrane (Tom)37/Tom70 & Tom20/Tom22 complexes)
- Translocation via import channel protein (Tom40 core) -- requires $\Delta \psi$
- Precursor moves through translocase of inner membrane (Tim17/Tim23, Tim44) interacts with chaperon matrix Hsc70 -- ATP-driven import motor
- Combines with intramitochondrial chaperones for active-state folding -- matrix Hsc70, Hsc60, etc. (ATP required for release)
- Mitochondrial processing peptidase cleaves matrix targeting sequence
- Second targeting sequence usually present in proteins destined for inner membrane or intermembrane space
**Protein Import Into Mitochondrial Matrix**

- **Steps 1a, 2 & 3a**: MSF binds matrix targeting sequence (red). Energy released from ATP hydrolysis keeps precursor unfolded. MSF binds Tom37/Tom70 outer membrane receptor.
- **Steps 1b & 3b**: Other precursor proteins bind to cytosolic Hsc70 & also keep precursor unfolded by energy released from ATP hydrolysis. Delivered directly to Tom20/Tom22 receptor.
- **Step 4**: Receptor-bound precursor proteins near contact sites with inner membrane are released to transport channel (Tom40 & three smaller subunits).
- **Step 5**: Precursor protein is translocated across inner membrane through a transport channel of Tim proteins located at contact sites with outer membrane - Requires pmf. interacts with Hsc70 - ATP-driven import.
- **Step 6**: Matrix protease cleaves matrix targeting sequence.
- **Steps 7**: (a) Chaperone-less folding into mature, active conformation (b) ATP-requiring, Hsc60-assisted folding.
Two Pathways Transport Different Proteins From Cytosol to Mitochondrial Intermembrane Space

- Precursor proteins contain tandem N-terminal targeting sequences & are delivered to Tom receptor
- **Conservative pathway** (cytochrome $c_1$): mimics matrix targeting, with cleavage of matrix targeting sequence (1a). Intermembrane space targeting sequence directs $c_1$ to inner membrane receptor (2a) for translocation to intermembrane space. Second targeting sequence cleaved (3a), heme is added & $c_1$ folds into mature conformation (C-terminal TMS not shown)
- **Nonconservative pathway** (cytochrome $b_2$): matrix targeting sequence also cleaved but intermembrane space targeting sequence is anchored in inner membrane (1b) assuring that rest of $b_2$ remains in intermembrane space. Second targeting sequence cleaved (2b), heme is added & $b_2$ folds into mature conformation
Biological Relevance of Protein Turnover

- Regulation of protein levels: e.g., HMGCoA reductase (key regulatory enzyme) -- $t_{1/2} = 2 \text{ h}$
- Prevents abnormal protein accumulation
- Control of growth & development
- Adapting to environmental changes
Proteolysis in Lysosomes

- Non-selective, catalyzed by acid proteases (cathepsins)
- Role of primary lysosomes
  -- Migrate to cell surface & secrete degradative enzymes by exocytosis
  -- Fuse with autophagic vesicles engulfing organelles & give rise to secondary lysosomes
  -- Fuse with phagocytic vacuoles engulfing external material & generating secondary lysosomes
  -- Rupture within cytoplasm, resulting in autolysis (nutritional deprivation)
- Lysosomal storage diseases result when hydrolytic enzymes are missing
Formation of primary and secondary lysosomes and their role in cellular digestive processes. The primary lysosomes that bud from the Golgi can take several pathways. **Path A:** Exocytosis—transport of enzymes to outside of cell. **Paths B and C:** Phagocytosis—formation of phagic lysosomes for digesting organelles (autophagocytosis) or ingested matter (heterophagocytosis). **Path D:** Autolysis—destruction of the cell itself.
Ubiquitin-Mediated Protein Degradation Pathway

- Selective cytosolic, ATP-dependent ubiquitin activation and transfer to amino groups of fated protein
  -- Ubiquitin: highly conserved 8.5-kDa polypeptide -- commits proteins ligated to C-terminal Gly to degradation
  -- Three additional proteins necessary for ubiquitination
    \[ E_1 \] -- *ubiquitin activating enzyme* - ATP-dependent thioester formation at ubiquitin C-terminus (ubiquitin-AMP intermediate)
    \[ E_2 \] -- *ubiquitin carrier protein* (ubiquitin-conjugating enzyme -- protein family) transfers ubiquitin from \( E_1 \) to Cys-thiol on \( E_2 \)
    \[ E_3 \] -- *ubiquitin-protein ligase* (main pathway) transfers ubiquitin via C-terminus to free \( \text{NH}_3 \) group (\( \alpha-\text{NH}_2 \) terminal, \( \varepsilon \)-amino Lys) on selected protein
- Can get formation of multi-ubiquitin chains, marking protein for degradation by proteasome
  -- Tagged protein digested by specific ATP-dependent proteases forming 26S proteasome (20S proteasome core plus 19S caps)
  -- Gives rise to selective degradation products and recycled ubiquitin
Enzymatic Reactions in Ligation of Ubiquitin to Proteins

Garrett & Grisham: Biochemistry, 2/e
Figure 33.33

- Ubiquitin is ultimately attached to selected protein via isopeptide bond formed between ubiquitin C-terminus & free amino group on fated protein.
Signals Marking Proteins for Ubiquitination

- Protein sequences rich in Pro, Asp, Glu, Ser and Thr (PEST) residues
- Destruction boxes of strongly conserved sequences
- N-terminal rule (destabilizing bulky hydrophobic, basic and acidic residues @ N-terminus)
Cartoon of Ubiquitin-Proteasome Degradation Pathway
**Crystal structure of the Thermoplasma acidophilum archaeal 20S proteasome**

- Consist of barrel-shaped 700-kDa structure composed of α and β chains arranged to form four stacked $\alpha_7 \beta_7 \beta_7 \alpha_7$ rings (see (b))
- 20S proteasome barrel is 15 nm in height & 11 nm in diameter & has three-part central cavity which contains proteolytic sites within β subunit
- Access to cavity gated by 1.3 nm opening in outer ring (see (a))
- Rings thought to unfold proteins & transport them into inner cavity  
  (Lowe et al. Science 268:533, 1995)
The Eukaryotic 26S Proteasome

- Obtained by 2-dimensional analysis of electron microscope images of negatively stained Drosophila 26S proteasomes
- A flexible linkage between 19S caps and 20S core was revealed in these analyses (Baumeister et al. Cell 92:357, 1998)
**Some Important Ubiquitin Targeted Regulatory Proteins**

- Cell cycle regulators (associated with cyclin-dependent kinases)
- p53 tumor suppressor (novel oncogenic virus protein mediates E₃ interaction with p53 \(\rightarrow\) cervical cancer)
- Transcription regulator NF-κB (immune and inflammatory response genes)
- Ubiquitination signals ligand-stimulated endocytosis of yeast plasma membrane α-factor (mating pheromone)
  -- Interacts with Ste2p receptor, degraded in lysosomal vacuoles
  -- Other transport proteins suffer same vacuolar fate in yeast (e.g., uracil permease).