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A REVIEW ON PROTEIN PRODUCTION AND SECRETION IN EUKARYOYTES

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Review

ABSTRACT

Protein secretion is an important process in any living organism, and this is mediated by numerous steps and several cellular components. This review provides an overview of protein production from nascent polypeptide synthesis stage to secretion of these proteins into extracellular space by vesicular sorting. This review also provides insights on the factors involved in ER and Golgi in this pathway and their role. It also covers responsibility of translocons and chaperones in stress response pathways such as UPR and ERAD. Beyond all these a series of quality control checks are performed by cellular machinery to ensure the quality of protein delivered. So essentially this review covers all the pathways involved from initiation of protein expression to secretion.

Keywords: *E. coli: Escherichia coli*; protein translocation, glycosylation, protein folding, vesicular sorting

INTRODUCTION

One of the crucial functions in any cell is synthesis, folding and transfer of proteins from the cytosol into different compartments of the cells as per their functions. These proteins play a key role in physiological processes such as pathogenicity, nutrition, adaptation, adhesion, and survival **(Green *et al*., 2016)**. Protein secretion plays an integral role in the survival of cells as most of the pathogens use this mechanism for existence. The toxins secreted by these pathogens have the ability to enter the host cells and induce colonization through toxin secretion **(Tseng *et al*., 2009)**. Translated mRNA is targeted into ER through signal recognition particle (a protein – RNA complex). In an SRP dependent mechanism signal sequence in the newly translated polypeptide is recognised by the SRP and translocated into ER lumen. Here the signal sequence is cleaved by signal peptidase and secreted into the extracellular space through secretory pathway. Proteins are properly folded in the ER and by a coordinated approach, it is secreted into the extracellular space.

Bacterial Secretory System:

In a bacterial secretion system, both gram positive and gram-negative bacteria follow different protein secretion mechanisms. Six major classes of secretion system are known and well characterized. These show a considerable amount of diversity within the organism **(Torto-Alalibo *et al*., 2009; Lindeberg *et al*., 2009)**. In general, gram-negative bacteria transports some proteins across the inner and outer membranes via Type I, Type III, IV, or VI pathways in a single step and some proteins through Sec/Tat pathway to the periplasmic space and then to the outer membrane through Type II or Type V. However, gram positive bacteria vary a bit, and they follow Sec/Tat pathway for translocation of proteins across single membrane. This mechanism varies slightly in pathogens **(Papanikou *et al*., 2007; Muller M, 2005; Albers *et al*., 2006)**. Gram negative bacteria such as *E. coli* is of great interest to biotechnology-based industries. This organism is well characterized, can be easily manipulated, and has GRAS (Generally Regarded as Safe) status. Many heterologous proteins have been expressed in this organism and is known to produce in quantities as high as 50% of TCP (Total Cell Protein). But the major downside of this system is lack of efficient secretory mechanism and inability of these cells to execute one of the important PTM's i.e., formation of disulphide bonds. A substantial amount of protein is produced but as inclusion bodies, which eventually reduces the recovery of the proteins thereby increasing the cost of production **(Kliener – Grote *et al*., 2018; Mergulhão & Monteiro, 2007)**. One of the alternate bacterial hosts, which is of interest to researchers now is *Bacillus subtilis*. It is a gram-positive bacteria, which is known to secrete excessive amounts of endogenous proteins such as amylases and proteinases. Since gram positive bacteria lacks outer cell membrane, secretion process is simplified compared to gram negative cells **(Neef *et al*., 2021)**.

Yeast and Fugal secretory system:

Secretory pathways in these organisms are a little complex. A network of proteins and organelles are involved in exporting proteins, lipids, and other molecules from inside the cell to external environment. It begins with the assembly of the secretory vesicle in the endoplasmic reticulum (ER) where proteins are synthesized and packaged into vesicles. These vesicles are transported to Golgi apparatus where it is further modified and sorted into specific pathways based on the signal sequence. These vesicles finally make its way to plasma membrane and get secreted into the external environment through membrane pores **(Saloheimo & Pakula, 2012; Pullmann *et al*., 2020)**. Fungal cells are known to secrete a lot of enzymes to absorb nutrients from the surrounding for survival. This phenotype of the organism is widely exploited by researchers for secretion of proteins in high amounts. Unlike bacterial systems (*E. coli, Bacillus*), yeast system (*P. pastoris, S. cerevisiae, H. polymorpha & K. lactis*), fungal expression systems are complex and laborious to genetically manipulate due to the heterogenicity as these are multinucleated **(Conesa *et al*., 2001; Zhang & An, 2014; Turner, 1990)**.
Efficient secretion of any recombinant protein into the extracellular space completely depends on host and protein compatibility. For a host to secrete these proteins in high levels, it needs to undergo a few genetic level modifications to enhance the strength of cellular machinery. A complex protein with high molecular weight and disulphide bonds might require longer time for processing due to PTM's before secretion compared to peptides. Recent times, researchers are focusing on utilizing the fungal strains as they have the potential to secrete fully processed proteins in high amounts which will be of industrial relevance.

1. Protein Translocation

The transport of proteins across the cellular membranes i.e., ER during the translation is known as protein translocation. Precursor protein is transported into and across the ER membrane is a highly conserved process and can be divided into 3 major steps:

1. Targeting the newly synthesized nascent precursor proteins towards the ER membrane.
2. Insertion of these newly synthesized precursor proteins into the ER lumen through the translocation channels.
3. Release of this protein into the ER lumen **(Linxweiler *et al*., 2017; Palade, 1975; Borgese & Fasana, 2011)**.

Proteins are translocated into the ER from cytosol by co-translational translocation; in this process of transfer of protein into the ER lumen occurs during the protein synthesis or post translational translocation. Transfer process occurs after polypeptide has been completely synthesized **(Martoglio & Dobberstein, 1998)**. Pre proteins are identified and translocated into the ER membrane by SRP (Signal Recognition Particle) dependent or independent pathway. Molecular chaperones present in the cellular environment bind to these proteins to retain their solubility

during the transition. Proteins which are bound to chaperones translocate into the ER lumen through translocons. Protein folding happens in the ER lumen with the help of chaperones **(Weihofen *et al*., 2002)**.

2. Signal Sequences

Proteins are directed to the destined locations through signal sequences (SS). Highly expressed native protein's signal sequences are preferred for expression of heterologous proteins **(Cullen *et al*., 1987)**. For ex: Glucoamylase from *A. niger, A. nidulans* and *A. awamori*, CbhI from *T. reseei*, amylase from *R. oryzae* **(Madhavan *et al*., 2017)**. Signal sequences are small sequences with ~ 15 – 30 amino acids at the N – terminus of proteins. SS contains 'n' region of 1 – 5 positively charged amino acids followed by 'h' region which contains a hydrophobic stretch of 7 – 15 amino acids and 'c' region with uncharged amino acids as shown in **Figure 1**. As the polypeptide synthesis begins, the hydrophobicity of the core region in SS determines whether these proteins need to be post translationally translocated or co translationally translocated. In yeast if the relative hydrophobicity is high, SS takes post translational translocation path. Well-known example for the SS which takes post translational translocation path is mat alpha signal sequence from *S. cerevisiae*. To further enhance the efficiency of the secretion via these signal sequences, insertion of dibasic amino acids sequence i.e., KR or RR also known as Kex2 cleavage site has known to increase the secretion. This serine membrane bound protease cleaves at KR/RR separating the SS from protein thereby increasing the efficiency of secretion. Overexpressing Kex2 protease has shown improved titres during recombinant protein production **(Koseki *et al*., 2017; Sakekar *et al*., 2021)**.

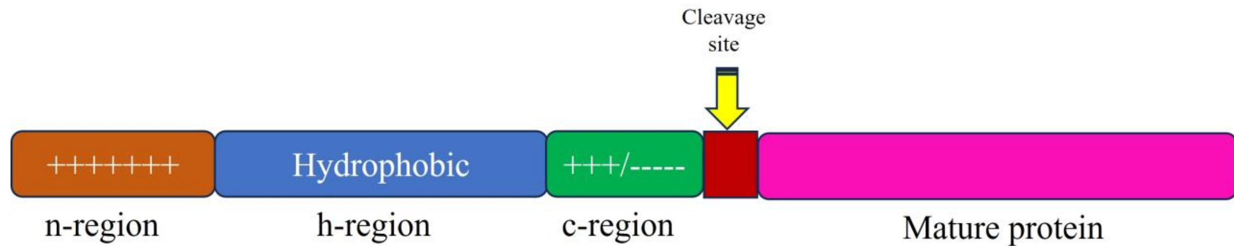


Figure 1 Schematic representation of a Signal Sequence. A general structure of a signal sequence consists of i: positively charged n – region which facilitates the translocation through ER membrane; hydrophobic core h – region, which forms alpha helix and uncharged c – region which ends with signal peptide cleavage site.

The co - translational translocation pathway is identified to be SRP dependent. Signal recognition particle (SRP) recognizes the N – terminus of the nascent polypeptide emerging out of ribosomes by identifying the signal sequence or (TMD) Trans Membrane Domain. SRP is a 11s ribonucleoprotein particle comprised of a single 7s RNA and 6 proteins. SRP anchors to the nascent chain and forms a complex with nascent protein still attached to the ribosome. This complex is recognised by SRP receptor present on the ER membrane **(Chartron *et al*., 2016; Jan *et al*., 2014)**. SRP receptor to which SRP - Nascent protein - Ribosome complex attached sends signals to Sec61 translocation channel and gates are opened to allow the entry of the protein getting synthesized across the membrane **(Jung & Kim, 2021)**.

In SRP independent pathway also known as post - translational translocation pathway, the protein is synthesized in the cytosol. To prevent aggregation, cytosolic Hsp70 chaperones get bound to these proteins and escort them to Sec62 / Sec63 translocation gateway. Bip/Kar2 present in the ER lumen interacts with Sec63 and ensures this is an unidirectional movement as shown in **Figure 2 (Harada *et al*., 2011; Lyman & Schekman, 1995; Brodsky *et al*., 1995; Fang & Green, 1994)**.

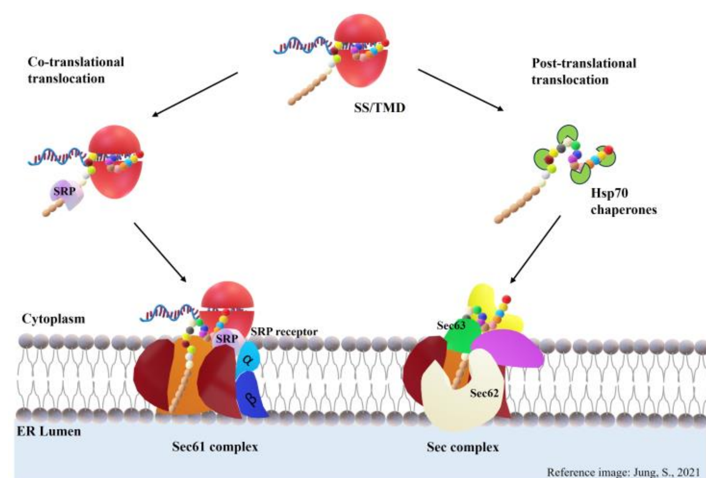


Figure 2 Co - translational and Post - translational Translocation. In co - translational translocation, SRP recognizes Signal sequence and forms a complex with SRP receptor on the ER membrane. Through Sec61 channel nascent polypeptide synthesized by ribosome directly enters into the ER. In the ER, with the help of chaperones, polypeptide is further processed for proper folding. During post - translational translocation, polypeptides is synthesized completely in the cytosol as they are not recognized by SRP. Hsp70 chaperones present in the cytosol binds to these newly synthesized polypeptides (to prevent aggregation) and escort them into ER through SEC channel (Sec62/Sec63) for further folding and maturation.

3. Translocation Channels

Blobel and Dobberstein first hypothesized the design of the membranous channels present on the ER for translocation of preproteins **(Blobel & Dobberstein, 1975; Steel *et al*., 2002)**. For proper folding and cellular signalling, ER lumen must maintain environment and high Ca^{2+} concentrations. To attain these, the translocons needs to be highly regulated. For example, Sec62 induces ER – phagy as a process for recovery of cells from ER stress. Sec61 acts as passive ER calcium leak channel to maintain the ER homeostasis **(Rapoport, 2007)**. As per the studies by X – ray crystallography and Cryo – electron microscopy, it comprises of seven components, Sec61, SbhI and SspI forms trimers, whereas Sec62, Sec63, Sec71 and Sec72 forms tetramer. These complexes form a lateral gate, a central pore and cytosolic funnel. Sec62 and Sec63 are present in two copies forming a hetero tetrameric complex which interacts with Sec61 channel **(Boisrame *et al*., 2006; Brodsky, 1999; Willer *et al*., 2003)**. Binding of signal sequence of precursor protein to SEc62/Sec63 causes a conformational change in the channel allowing translocation of protein regardless of signal sequence hydrophobicity. Sec62 is specific for translocation of precursors with moderately hydrophobic signal sequences and tail anchored membrane proteins **(Borgese and Fasana, 2011).**

Alternatively, chaperones present in the ER lumen such as Bip functions as "molecular ratchets" as they work similar to that of socket wrenches guaranteeing unidirectional movement leading to transport of precursor protein through channel into lumen. As this is an energy dependent activity, Sil1 and GRP170 helps in ATP hydrolysis. During this translocation signal sequence is cleaved at the cleavage site **(Nguyen *et al*., 1991; Sanders & Schekman, 1993; Brodsky & Schekman, 1993; Panzner *et al*., 1995; Matlack *et al*., 1997; Lang *et al*., 2012; Weitzmann *et al*., 2007)**. Apart from transport of precursor proteins, Sec61 is also involved in maintaining cellular Ca^{2+} homeostasis. These channels allow passive efflux of Ca^{2+} to cytosol from ER lumen during essential cellular processes such as apoptosis and cell migration. But they also restrain active import of Ca^{2+} from cytosol into ER through SERCA (Sarcoplasmic / ER Ca^{2+} ATPase) **(Wirth *et al*., 2003; Simon & Blobel, 1991; Lomax *et al*., 2002; Roy & Wonderlin, 2003; Van *et al*., 2004; Wuytack *et al*., 2002; Fumagalli *et al*., 2016)**. Cytosolic Calmodulin and Bip are known to be involved in regulating uncontrolled Ca^{2+} efflux. Many models have been proposed on the mechanism of this action. Another additional function of Sec62 which was identified by **Fumagalli *et al*., 2016,** in eukaryotic cell on relieving the ER stress, was due to extensive accumulation of misfolded proteins or impairment in transport of proteins due to disturbance in the ER environment **(Lang *et al*., 2011; Erdmann *et al*., 2010; Linxweiler *et al*., 2013)**. Depending on the stress level, cell chooses either Sec62 mediated autophagy by formation of autophagosomes to relieve the stress or UPR (Unfolded protein response) pathway **(Crowley *et al*., 1994; Walter & Ron, 2011; Schauble *et al*., 2012)**.

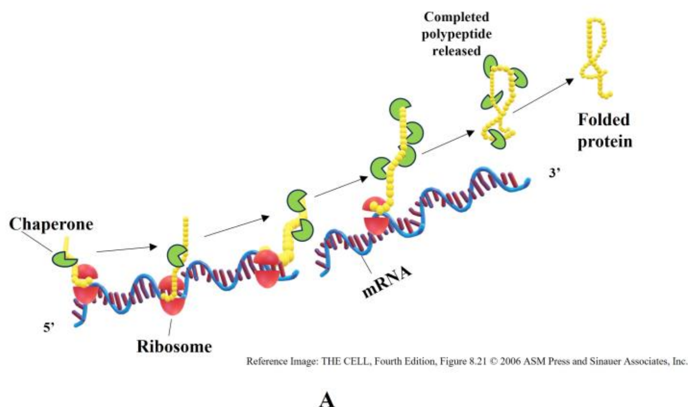
During translocation Sec63 plays different roles in translocation but in all its roles, it is aided by a chaperone known as Kar2. Kar2 closely works with Sec63 in gating translocon pore and in the ratcheting mechanism of pulling the precursor protein during the synthesis into the ER lumen along with Lhs1 and Sil1 **(Brodsky & Mc Craken, 1999; Tyson, 2000)**. Studies have shown that Lhs1 binds to different regions of unfolded polypeptide and regulates reciprocal ATPase activities with Kar2 and also serves as NEF (Nucleotide Exchange Factor). Sil1 is known to bind

to different regions of Kar2 and also promotes Kar2 recruitment by Sec63. Sil1 deletion studies has shown defective ERAD in *S. cerevisiae* (**Biosrame *et al*., 2006; Lakkaraju *et al*., 2012**). There are reports showing increase in the expression of recombinant proteins when Sec63 and SbhI are overexpressed. If YPT7 gene which is involved in vacuolar pathway is disturbed the vesicle fusion with vacuoles is impaired. So, overexpression of SbhI in ΔYPT7 yeast strain has shown two-fold higher expression of recombinant protein. Similarly, when Sec63 is overexpressed along with YDJ1p, heterologous protein secretion was enhanced by 7.6 times. YDJ1p is localized in the cytosol and is known for interaction with Ssa1p which in turn boosts the chaperone activity. This combination helps inducting more chaperones such as Kar2/Bip & Lhs1 bringing a synergistic effect in protein folding and secretion (**Zhang *et al*., 2006; Corsi & Schekman, 1996; Marsalek *et al*., 2019**).

4. Molecular Chaperones

Molecular chaperones are a set of proteins which assists in protein unfolding and proper folding. They bind to the unfolded and newly synthesized polypeptides and controls aggregation or accumulation of synthesized proteins. In ER, it is also involved in quality control mechanism, wherein it triggers UPR pathway. When ER is overburdened with misfolded proteins each organelle depending on their function contain its own chaperones for distinct functions. Some of the chaperones are involved from the synthesis stage and these acts as a catalyst by increasing the rates folding process as shown in **Figure 3** Chaperone mediated protein folding; **A**: In the cytosol/ER **B**: In mitochondria. Proper folding of newly synthesized polypeptide is mediated by chaperones. During / post synthesis, these nascent polypeptides can form aggregates. Chaperones present in cytosol or ER or the ones in the mitochondria interact with them and aid in proper folding. Cytosolic chaperones binds to proteins which are destined to enter mitochondria and release the protein into the mitochondrial membranes.

sHSP's or holdases are ATP independent members and form high molecular weight oligomers. They bind to unfolded protein with high affinity and prevent protein aggregation (**Niforou *et al*., 2014; Benesch *et al*., 2008; Stromer *et al*., 2003**). They are highly expressed during stress conditions. One such example is Hsp12 and Hsp26 found in *S. cerevisiae*. This chaperone gets induced when the cells are exposed to low or high temperatures, osmotic or oxidative stress (**Haslbeck *et al*., 2005; Pacheco *et al*., 2009**). These are low molecular weight proteins which are capable of forming large oligomers preventing aggregation of partially misfolded proteins (**Garrido *et al*., 2012**).
Hsp60 are oligomeric proteins which are known to "protect" proteins during heat denaturation and synthesis. These can be further categorized into Group I [GroEL and GroES] found predominantly in bacteria such as *E. coli* and Group II which is found in eukaryotic cytosol [CCT] and archaeal thermosome.
. Broadly, chaperones are classified into "holdases" (small heat shock proteins) known for modulating proteome stability and "foldases" (Hsp60, Hsp70, Hsp90 family) which are energy dependent chaperones for their functionality (**Delic *et al*., 2013**).



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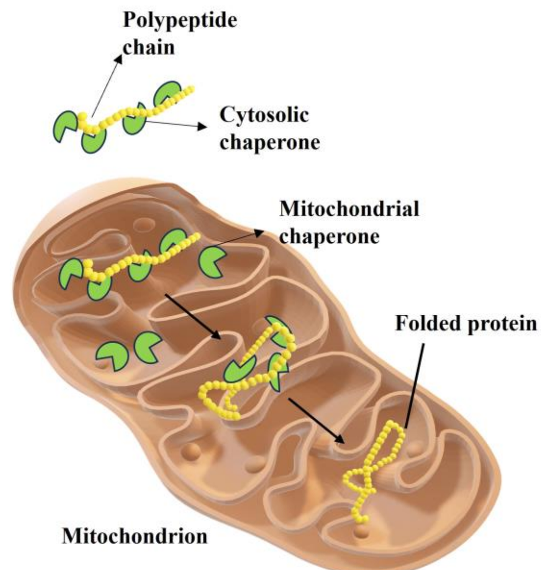

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B

Figure 3 Chaperone mediated protein folding; **A**: In the cytosol/ER **B**: In mitochondria. Proper folding of newly synthesized polypeptide is mediated by chaperones. During / post synthesis, these nascent polypeptides can form aggregates. Chaperones present in cytosol or ER or the ones in the mitochondria interact with them and aid in proper folding. Cytosolic chaperones binds to proteins which are destined to enter mitochondria and release the protein into the mitochondrial membranes.

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GroEL is composed of a two tightly stacked ring structure, similar to that of barrel with an open cavity with hydrophobic surface. GroES which is a single heptameric ring acts like a lid to the open ring thereby transforming it into a closed chamber for coordinated unfolding and folding activity. This is a highly ATP dependent activity and the protein present in the closed chamber is properly folded by a " push – pull" mechanism wherein protein is pushed away from the hydrophobic sites of GroEL and pulled by hydrophilic chamber of GroES for folding. This activity is fuelled by slow ATP hydrolysis and when the lid is opened, only the properly folded protein is released otherwise it is recaptured again by another chaperonin ring as shown in **Figure 4 (Motojima *et al*., 2004; Leitner *et al*., 2012; Munoz *et al*., 2010)**.
Hsp70, the major / abundantly found Hsp's found in all cellular compartments. Hsp70 plays a prime role in retaining the newly synthesized proteins in its primary structure as these are prone to form aggregates. Hsp70 is always supported by another co – chaperone Hsp40. Protein folding is an ATP driven cycle, wherein Hsp40 stimulates ATP hydrolysis. As per the model (**Kampinga *et al*., 2010; Gautschi *et al*., 2001**), Hsp70 is comprised of two domains; Carboxy terminal substrate binding domain which recognizes the hydrophobic amino acids of polypeptide. There is also an amino terminal ATP-ase domain. When ATP is bound helical lid is open and when ATP is hydrolysed lid is closed. During this process the protein substrates are recruited which helps in folding misfolded proteins as shown in **Figure 5 (Niforou *et al*., 2014; Mayer & Bukau, 2005; Rudiger *et al*., 1997; Hartl *et al*., 2011; Rousseau *et al*., 2006)**.

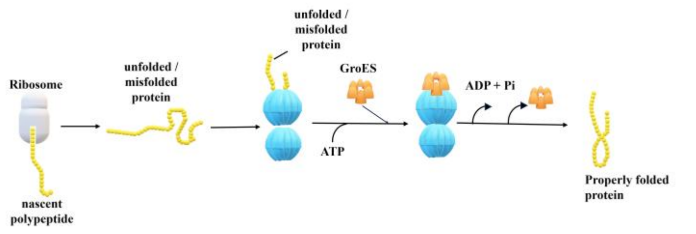

Figure 4 Chaperonin, GroEL and GroES mediated protein folding mechanism. There are two important sets of chaperones functional in *E. coli*. One is DnaK-DnaJ-GrpE and another is GroEL – GroES. GroEL – GroES combination is known to interact with partially folded proteins to assist them in proper folding for yielding a functional protein.

Another Hsp which works closely with Hsp70 is Hsp100, also known as "unfoldases" or "disaggregatases". These helps in recruiting unfolded proteins and present to Hsp70 for folding. After several continual efforts, if the protein is still misfolded, they are presented to proteosomes for degradation **(Kampinga *et al*., 2010; Seyffer *et al*., 2012; Winkler *et al*., 2012; Imamato & Kose, 2012)**.

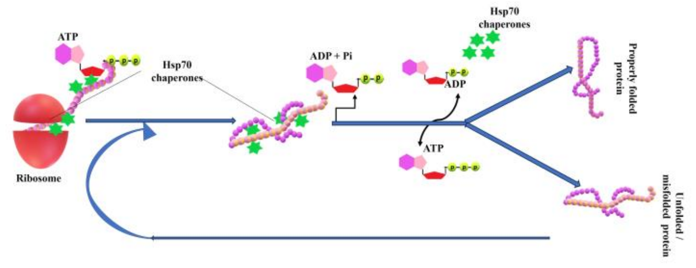

Figure 5 Hsp70 chaperones mediated protein folding mechanism. Hsp (Heat shock proteins) present in the cells, helps native protein in proper folding. They bind to these polypeptides and block them from folding until they are translocated across the membranes of destined organelles. Hsp70 mediated protein folding is an energy intensive process and ATP is hydrolyzed to ADP during any conformational change.

Lastly, Hsp90 class of chaperones. These are also ATP dependent and present in non-stressed cells. They are more involved in cell signalling and regulation. Hsp90 also binds to polypeptides and prevent from aggregation **(Saibil, 2013)**. Molecular chaperones present in the ER facilitate proper folding of polypeptides into its tertiary structure. After several attempts if the protein does not attain its right conformation, then the ER triggers either UPR pathway or ERAD pathway to maintain ER homeostasis. Some of the chaperones and their functions are listed in **Table 1**.

Table 1 Chaperones and their functions

Chaperones	Organism	Functions
Chaperonins		
Hsp60	Yeast	With the help of co factors Hsp10 and GroES, helps in Protein folding and preventing aggregation
GroEL	*E. coli*	
CCT	Mammals	
***Hsp70* system**		
DnaK	*E. coli*	Stabilizing unfolded or partially folded proteins and presenting it to GroEL for subsequent folding
Ssa, Ssb	*Saccharomyces cerevisiae*	Stabilizing misfolded proteins, targeting misfolded proteins for degradation
Bip	Mammals	Prevents aggregation, Targeting misfolded proteins for retrograde translocation and eventually degradation
Hsp90 system		Binding to misfolded proteins for preventing aggregation, delivery of misfolded proteins to proteases
Hsp100 system	Prokaryotes and Eukaryotes	Unfolding, solubilization of aggregates

5. UPR (Unfolded protein response) pathway

UPR pathway aims to restore the balance in the ER by reducing the protein synthesis and increasing the protein folding capacity. Foldases, chaperones, protein disulphide isomerases assisted by thioredoxin system are involved in this process. Only properly folded proteins are destined to pass through exit sites or transitional ER sites. In fungal cells these exits are present in hyphal tips **(Almanza *et al*., 2018; Benham, 2018; Kimura *et al*., 2010; Wang *et al*., 2020)**. If there is accumulation of properly folded proteins present in the ER, UPR pathway gets activated **(Hetz &Glimcher, 2009; Dassanayake, 2015)**.

This triggers induction of ER resident foldases, chaperones and other enzymes which are involved in helping the proteins attain their native conformation. However, increased accumulation leads to impairment of ER function thereby causing detrimental effects to cell physiology. This activates the unfolded protein response pathway, UPR pathway helps in increasing the ER protein folding capacity by inducing production of more helper proteins and parallelly regulates the disposal of completely misfolded proteins which cannot be restored by any means **(Sidrauski *et al*., 1996)**.

Ire1-Bip/Kar2 complex helps in regulating UPR pathway. Bip/Kar2 complex dissociates from IreI when they sense ER stress. This change activates RNase, now RNAse helps in removal of intron sequence in Hac1 to mRNA leading to translation of Hac1 protein. Hac1p now travels to the nucleus and in turn activates a lot of UPR target genes. In the meantime, ERAD pathway is activated and the protein which are irreparable are retro translocated back to cytosol, where it is ubiquitinated and released for degradation by proteases **(Krishnan & Askew, 2014; Mustalahati *et al*., 2013)**.

6. ERAD (Endoplasmic Reticulum Associated Degradation) pathway.

One of the important functions of ERAD pathway is to detect and eliminate aggregated, misfolded, or unassembled proteins by retro – translocation of these proteins into cytoplasm with the help of AAA ATPase protein (Cdc48/pr7 complex) **(Hoseki *et al*., Carvalho *et al*., 2006)**. In the cytoplasm these are ubiquitinated and degraded by proteosome. ERAD plays a critical role in retaining the ER homeostasis and reducing the aggregation of toxic misfolded proteins **(Gauss *et al*., 2006)**. Since ERAD is one of the quality control mechanisms built within the cell, several steps and key components are involved in this pathway, and they are:

i. Recognition: Hrd3p and Yos9p proteins present in the ER recognizes the misfolded proteins **(Tanaka, 2009)**.
ii. Retro-translocation: In this step membrane anchored ubiquitin ligases, such as Hrd1p/Der3p helps in retro-translocation of misfolded proteins from ER lumen or membrane into the cytosol **(Hitt & Wolf, 2004)**.
iii. Ubiquitination: The process of tagging misfolded protein present in the cytosol with ubiquitin molecules is facilitated by Ubiquitin ligases present on the ER membrane **(Bhamidipati *et al*., 2005)**.
iv. Proteosomal degradation: Proteosome is a large protein complex made up of several subunits which are responsible for identifying ubiquitinylated, misfolded, or unassembled proteins and hydrolyze them into smaller peptide units. Some of the proteases involved in the degradation are a) Chymotrypsin like activity protease is observed in the β5 subunit of proteosome and is responsible for hydrolyzing the peptide bonds after hydrophobic amino acids. b) Trypsin like activity from β2 subunit is responsible for cleaving peptide bond adjacent to basic amino acids, preferably lysine and arginine. c) Caspase like activity from β1 subunit is known for hydrolyzing peptide bonds after acidic amino acids **(Kloetzel, 2001; Bhamidipati *et al*., 2005)**.

The ERAD pathway is well sorted, depending on the position of misfolded lesion, recognition of the substrate, its transportation, polyubiquitination and degradation by the proteosome is structured. (i) ERAD – L pathway if the protein misfolded at ER luminal domain, (ii) ERAD - C for membrane proteins, misfolded at cytosolic domains, (ii) ERAD – M for membrane proteins, misfolded at intramembrane domains. Each pathway involves different machinery and follows certain mechanisms. However, all three pathways comprise of several converging pathways and involves Yos9p/ Kar2p/ Hrd3p complex **(Rock *et al*., 2002; Friedman *et al*., 2002)**.

a. Yos9p – It is lectin protein responsible for recognizing and binding to unfolded / misfolded glycoproteins. It is known to recognize only terminally misfolded proteins.
b. Kar2p – Kar2p is a chaperone and plays a crucial role in protein folding, ER quality control, maintaining ER homeostasis and UPR. In ERAD it forms a complex with Yos9P and Hrd3p for facilitating misfolded protein degradation.
c. Hrd3p – A major component in ERAD pathway. It is a transmembrane protein responsible for recognition and translocation of misfolded proteins from ER lumen to cytosol. Hrd3p is also known to interact with Hrd1p and Der1p **(Kim *et al*., 2005; Nakatsukasa *et al*., 2001; Meusser *et al*., 2005)**.

The three ERAD pathways differ with one another only in recognition step, but they converge during retro-translocation and degradation stage. In ERAD – L, the misfolded luminal protein is identified by Kar2p/Hsp70 chaperones and is maintained in its soluble state. Core membrane complex consisting of Hrd1p (E3 ubiquitin ligase), Hrd3p, Der1p and Usa1p along with Yos9p and Cdc48pATP forms complex, which present the misfolded proteins for retro – translocation,

ubiquitination and proteosome degradation. Whereas, in case of ERAD – M membrane proteins which are misfolded at intramembrane domains are recognised by Hrd1p and Hrd3p. They are also involved in recruiting ATPase complex and move them into cytosol for proteosome degradation (**Tsai *et al*., 2002; Vashist & Ng, 2004**). ERAD – C (cytosolic domain) follows a similar pathway as ERAD – L. Retro-translocation of misfolded proteins is facilitated by complex of proteins such as Hrd1p, Cdc48p ATpase. Cdc48p complex along with its cofactors Ufd1p, Np14p hydrolyses ATP and facilitates translocation of misfolded proteins to cytosol and then proteosome degradation (**Vashist *et al*., 2001; Huyer *et al*., 2004**).

7. Calnexin a quality regulator

Calnexin is a molecular chaperone that plays a major role in quality control of glycoproteins. It is a type I integral membrane protein residing in the ER membrane and has a luminal domain which interacts with the newly synthesized glycoproteins by assisting in the proper folding and maturation of N – linked glycoproteins. Calnexin recognizes and binds to mono glycosylated N – linked glycans or glycoproteins present on misfolded or incompletely folded glycoproteins, thereby preventing their premature release from the ER. Calnexin also interacts with folding enzymes and chaperones, such as PDI and Calreticulin to facilitate proper folding and prevent aggregation of glycoproteins (**Antonny & Schekman, 2001; Martinez Benitez *et al*., 2011**). PDI assist in formation and rearrangement of disulphide bonds in the misfolded proteins whereas calreticulin retain the misfolded glycoprotein in the ER and recruits other chaperones for proper folding as shown in Figure 6. Calreticulin also helps in regulating Ca^{2+} concentration in the ER lumen by acting as calcium binding protein sequester Ca2+ ions. Binding of calnexin is controlled by the presence / absence of terminal glucose. Calnexin / Calreticulin complex transiently bind to terminal glucose residue which is removed by Glucosidase II. Further modification by mannosidase cleavage ($Glc_3Man_9GlcNac_2$) terminates the cycle and directs the protein further into the secretory pathway (**Harty *et al*., 2001; Nakatsukasa *et al*., 2004**). If the glycoprotein is properly folded it exits the ER or prolonged retention of protein in the ER leads to removal of terminal mannose by Mannosidase I (Man9 to Man8 conversion). Man8 protein interacts with EDEM and subsequently pushed out of ER for ERAD (**Sanders *et al*., 1999; Trombetta & Parodi, 1992; Belden & Barlowe, 2001**).

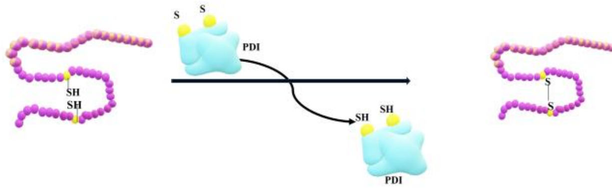

Figure 6 PDI assisted Disulphide bond formation. Protein Disulphide Isomerase catalyzes the disulphide bond formation reaction. It breaks and makes these bonds over and again until the protein achieves its right conformation.

8. Vacuolar transport

Vesicles carrying protein cargo is surrounded by membrane which upon reaching the destined location fuses with the target membrane releasing the components into the organelles is known as vacuolar sorting. This transfer of proteins between the cellular compartments by fusing to the membranes is mediated by vesicles. There are different kinds of protein transport from ER, and all are membrane bound (**Letourner *et al*., 1994**).

a. **Anterograde transport**, also known as forward direction travel, is involved in the transport of protein cargo from ER to Golgi apparatus and is mediated by COPII (Coat protein complex II) vesicles transport (**Castillon *et al*., 2011**).
   i. Sec12 – GEF activates Sar1 (a G protein) by coupling it with GTP. This leads to a conformational change causing a hydrophobic tail to protrude into the ER membrane.
   ii. The hydrophobic tail of Sar1 recruits COPII proteins and vesicles formed at the tER sites of the ER region. During the budding of the vesicles from the ER membrane cargo proteins are captured along with the membrane lipids.
   iii. The TRAPPI (Transport protein particle I) tethering complex guides and binds the COPII vesicles to the target membrane i.e., the Golgi apparatus.
   iv. RabYrt1 (Rab GTPases) helps in anchoring COPII vesicles and facilitate the fusion of vesicle with Golgi membrane. SM (Sec1 / Munc18) proteins such as Sly1p helps in pairing V – SNARE and T – SNARE. SNAREs are Soluble N-ethylmaleimide-sensitive factor activating protein receptors present on the vesicle (V – SNARE) and on the target (T – SNARE) membrane. SNAREs are the proteins which are actually involved in the fusion of vesicle and target membrane allowing the cargo proteins to be delivered into Golgi (**Szul & Sztul, 2011; Hughes & Stephens, 2007; Lee *et al*., 2005; Wendeler *et al*., 2007**).

b. **Retrograde Transport**, also known as going backwards. It involves the transport of protein from Golgi back to ER. This process is like that of anterograde transport but is mediated by COPI vesicle transport (**Behnia & Munro, 2005; Brocker *et al*., 2010**).
   i. Instead of SarI, ARF (ADP – ribosylation factor) is loaded with GTP, causing the hydrophobic tail to protrude and stick to Golgi membrane.
   ii. Hydrophobic tail of ARF recruits COPI (Coat protein Complex I). COPI is responsible for capturing cargo proteins in the Golgi along with membrane lipids. COPI vesicles mediate intra Golgi trafficking from trans – to – cis – Golgi causing structural differences between the two COPI vesicle populations. A heptameric complex composed of subunits COPI, Sec26, SEC27, Ret2, SEC28 and RET3 are involved in COPI vesicle formation.
   iii. The Rab – ARF present in the COPI complex cycles between cytosolic GDP bound inactive state to GTP bound active state on the membrane. Transport from endosome to trans – Golgi and trans – to – cis – Golgi is facilitated by TRAPII.
   iv. Similar to that of anterograde transport, SNARE assembly is mediated by Sly1 which selectively interact with GosI at Cis Golgi and Tlg2 at Trans Golgi (**Gillingham & Munro, 2003; Gupta & Brent, 2002; Kienle *et al*., 2009**).

Concentrations of Ca^{2+} and Mn^{2+} ions inside the ER lumen are very critical as they are involved in vesicular sorting and Glycosylation. The concentrations of these two ions and influx and efflux are tightly regulated by Pmr1 gene. Pmr1 acts as a pump to supply these ions across Golgi and plasma membrane. Any sort of imbalance in the concentration of these ions leads to impaired sorting and hyper glycosylation (**Delic *et al*., 2014; Devasayaham *et al*., 2006**).

c. **Exocytosis:** Transport of proteins from trans - Golgi to the plasma membrane by the exocyst is known as exocytosis. The exocyst is a multimeric complex composed of eight subunits Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. Both COPII and COPI vesicles are involved in this transport and follow similar path (**Yeaman *et al*., 2004).** The exocyst' s are localized on the plasma membrane at defined regions. For ex: In *S. cerevisiae*, it is localized at the tip of growing bud and in fungi it is localized at the hyphal tip (**Hazuka *et al*., 1999; Hsu *et al*., 2004**).

CONCLUSION

We have provided a comprehensive review of production and secretion of proteins in eukaryotes. Different factors and steps are involved in the journey of nascent polypeptide from synthesis to fully folded functional proteins secreting out of the cell. Each protein produced in the cell plays a crucial role in maintaining the physiology of cells. These proteins are responsible for processes involving nutrition, survival and adaptation, adhesion, and pathogenicity. Some of the proteins secreted by pathogens acts as toxins and help in their survival. A network of organelles is involved in the secretory process of which ER and Golgi plays very important role.

With growing interest in the field of recombinant protein production, choosing host based on the protein compatibility is advantageous for increased expression of heterologous proteins. The coordinated efforts of translocons and chaperones help repressed proteins in proper folding and secretion into extracellular space. Based on the characteristic / property of protein, a few genetic level modifications can be considered such as overexpression of translocons or chaperones etc. High molecular weight proteins with multiple disulphide bonds requires a lot of chaperones for efficient folding and secretion. It is also important to carefully design the expression system to ensure that the recombinant protein is expressed at the appropriate level and in the correct conformation. Considering such trivial points would help researchers achieve high expression of heterologous proteins.

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