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Minireview

Insertion and translocation of proteins into and through membranes

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In prokaryotic and eukaryotic organisms, proteins are efficiently sorted to reach their final destinations in a whole range of subcellular compartments. Targeting is mediated by hydrophobic signal sequences or hydrophilic targeting sequences depending upon the compartment, these sequences being often processed. Proteins cannot be translocated through a membrane in a tightly folded stage, they must have a loose conformation, the so-called 'translocation competent state', which is usually kept through interactions with chaperones. In addition to these cytosolic receptor-like components, receptors are also present on the target membranes. Depending upon the organelles and organisms, two different energy sources have been identified, energy rich phosphate bonds (ATP and GTP) and a potential across the target membrane. Besides the signal peptides, various classes of signals have been identified to account for topologies of membrane proteins. Protein secretion in bacterial organisms has been extensively studied. Various classes of proteins use different strategies, some of these may also be used in eukaryotic cells.

Protein translocation; Targeting signal; Transmembrane integration; Protein secretion

1. INTRODUCTION

How do proteins pass through or integrate into membranes? This is one of the fundamental unsolved problems in biology. The great majority of proteins are synthesized in the cytoplasm, although their final destination is varied. In the simple case of a gram-negative bacterial cell, like *Escherichia coli*, there can be 6 different compartments: the cytoplasm, the inner membrane, the outer membrane, the periplasmic space itself comprising a compartment specialized for cell division [1] and the extracellular medium. In the more complex case of an eukaryotic cell about 20 final destinations are possible; besides the cytosol and the extracellular medium, there are many different intracellular organelles, each with a membrane and an intraluminal space. Although each of the various membrane types poses the same problem – how a hydrophilic protein can be transported across a hydrophobic phospholipid bilayer – the cells have not evolved any one universal solution. Many common features are however to be found from one system to the other.

An era of intensive study of the mechanistic details in this field began mainly with the formulation of the signal hypothesis by Blobel and Dobberstein in 1975 [2]. Since the initial research on protein export across the rough endoplasmic reticulum (ER), a wide range of other systems such as bacterial, mitochondrial, glyoxysomal, peroxysomal, chloroplast and cell nucleus systems, have been investigated. Progress in this field has been documented in a number of reviews [3–9].

The aim of this mini-review is to take stock of recent progress and new unifying concepts that have emerged over recent months.

2. TARGETING AND INITIAL PROTEIN-MEMBRANE INTERACTIONS

The final localization of a protein in any given extracytoplasmic compartment or in the extracellular medium requires a correct targeting to the membrane of this compartment and its crossing. This targeting requires a direct or indirect interaction between the protein and a component of the membrane enclosing this compartment through one or more particular protein sequences variously referred to by different authors as signal sequence, leader peptide, presequence, transit sequence, etc. Often, this sequence consists of an N-terminal extension. In general, although this is not always the case, the N-terminal extension is enzymatically cleaved once the targeting has been performed.

The same recognition mechanisms apply to non-cytoplasmic proteins and to membrane proteins. In the case of proteins imported into organelles with several

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compartments (mitochondria, chloroplasts) additional targeting sequences are required.

Surprisingly, although missorting of proteins into various organelles is probably a very rare event, targeting sequences are highly degenerate. When random fragments of bacterial or mammalian genomic DNA were used for their ability to encode targeting signals for the ER [10], mitochondria [11], or the bacterial plasma membrane [12], a significant percentage of the random sequences were active, although to varying degrees. However, with the highly sensitive tests used to score these sequences, even weak targeting signals gave a positive response and the results of these studies should not be interpreted to mean that targeting sequences are non-specific.

Hydrophobic, positively charged, and bend-promoting residues are used in almost all signals, but in different patterns, and many but not all signals seem to be designed as membrane-interacting peptides (apolar α-helices, amphiphilic α-helices, amphiphilic β-strands). Thus the possible involvement of specific lipid constituent of the membrane in interactions with targeting peptides should not be overlooked [13–15].

3. HYDROPHOBIC SIGNAL SEQUENCES

Proteins exported across the ER membrane or across the cytoplasmic membrane in bacteria feature a particular sequence (termed signal sequence) localized at the N-terminal part of the protein. In general, during translocation this extension is cleaved on the trans-side of the target membrane leading to the mature protein. However, if removal is blocked, translocation is not usually impaired. These sequences of 15–30 residues consist of a positively charged N-terminus, a central hydrophobic region, and a C-terminal region predominating in polar residues that are often negatively charged when the first residues of the mature protein are included [16]. Their structure is mostly α-helical in the central region, the C-terminal part comprising a β-turn. The specificity of the bacterial leader peptidase which cleaves signal sequence has been rather well elucidated [16]. Tabulation of many signal sequences and analyses of their characteristics have been published [8,17].

In cotranslational translocation mechanism of proteins across the ER membrane, as soon as the signal sequence emerges from the ribosome it binds to a ribonucleoparticle (the signal recognition particle, SRP, which halts or slows down translation. SRP consists of a 7 S RNA and 6 polypeptides with relative M_rs of 9, 14, 19, 54, 68 and 72 kDa. These components form 3 distinct structural domains in SRP [18]. The 54 kDa protein is required for signal recognition, the 9-plus 14 kDa dimer is essential for elongation arrest and the large domain (68 plus 72 kDa) serves to bind the ternary complex to the docking protein. An SRP-like component has not been detected in yeast, although *Schizosaccharomyces pombe* contains an essential 7 S RNA homologous to the 7 S RNA in mammalian SRP [19].

In prokaryotes, and in particular in *E. coli*, no bacterial SRP-equivalent has been detected. However, *E. coli* 4.5 S RNA has been found to contain a domain structurally homologous to SRP RNA [20] and an *E. coli* 48 kDa protein shows strong homology to putative GTP-binding and signal sequence binding sites, respectively, of the 54 kDa mammalian SRP [21,22]. Moreover, significant sequence homology was found between part of the docking protein (SRα) and a second *E. coli* protein (Fts Y) of unknown function. The homologous region includes a putative GTP-binding domain [21,22].

Genetic studies with *E. coli* suggest that SecA encoding a 92 kDa cytosolic protein with affinity for the inner membrane and signal sequences [23] is a type of surrogate for SRP [24]. The trigger factor (63 kDa) which binds to precursors and bacterial ribosomes also behaves to some extent like SRP [9]. It has also been shown that SecB functions as a cytosolic signal recognition factor for protein export in *E. coli* [25].

4. HYDROPHILIC TARGETING SEQUENCES

The second group of targeting sequences can be termed 'hydrophilic' since it lacks uninterrupted stretches of hydrophobic residues. These sequences are rich in basic and hydroxylated residues and contain few, if any, acidic residues. These hydrophilic signals target proteins into mitochondria and chloroplasts [26–29] and possibly also into peroxisomes [30]. Although many of these sequences have been determined [for a review, see [31]], no significant homologies have been detected. The primary structures of mitochondrial presequences, however, exhibit several common features as mentioned above. In addition, many show a tendency to fold into an amphiphilic α-helix [32] but how the amphiphilicity of presequences contributes to their targeting function is still poorly understood.

5. TRANSLOCATION COMPETENT PREPROTEINS

It is now clear that the translocation of preproteins can occur either late in translation or even post-translationally [33]. However, proteins cannot be translocated through a membrane in a tightly folded state. This was first shown in the case of an overproduced phosphate binding protein (PhoS) in *E. coli*. After the two step cleavage of the signal peptide by cytosolic proteases, the overproduced PhoS protein folded into its trypsin-resistant native conformation and, as a consequence, could not be exported post-translationally to the periplasmic space [34,35]. Further work confirmed this result with various systems [36,37]. The most strik-

ing example was the blocking of precursor proteins into isolated mitochondria by ligands that stabilize the native conformation of the mature moiety [7] or by cross-linking of this moiety by internal disulfide bridges [36,37]. A partially unfolded stage during translocation is also strongly suggested by various lines of evidence. For example, precursor proteins trapped during their import into isolated mitochondria by low temperatures, by antibodies against the mature moiety, or by disulfide bridges appear to be partly extended, with their NH_2-termini exposed to the matrix and part of their mature region exposed to the mitochondrial surface [37,38]. Denaturation with urea or destabilization by point mutation in the mature region accelerate import into isolated mitochondria [39,40].

It follows logically that a translocation competent conformation of precursors must be maintained in the cytosol. In this conformation, the targeting signal must remain exposed. With maltose-binding protein and ribose-binding protein, it has been demonstrated that precursor forms refold more slowly than the corresponding mature forms [41]. However, additional factors appear to be required in various organisms. These proteins which prevent undesirable interactions have been termed chaperones but even the ribosome itself may be thought of as a chaperone.

In bacteria, at least three proteins may function as chaperones: trigger factor [42], groEL [43] and SecB [44]. Recent evidence demonstrated that members of the 70 kDa heat shock protein (hsp) family were involved in ATP-dependent unfolding of precursor proteins destined for import into both mitochondria and dog pancreas microsomes (for a review, see [45]). Additional cytosolic components sensitive to alkylation and yet to be indentified may also be partly required [46,47].

6. TARGET RECEPTORS ON ORGANELLE MEMBRANES

All proteins destined to be translocated across membranes appear to be recognized by receptor-like components not only in the cytosol as described above but also on the target membrane. Since these components, as in the ER system for example, act sequentially, they may constitute some type of 'proof-reading mechanism', thus greatly enhancing the specificity of the system. In the ER, the ternary complex (ribosome, nascent precursor, SRP) is recognized by the docking protein (DP or SRP receptor) which consists of two polypeptides, a 72 kDa subunit (DPα) and a 30 kDa subunit (DPβ) (for reviews, see [3–6]).

The existence of putative GTP-binding sites within SRP, DP [21,22], and the demonstration that GTP binding and its hydrolysis are the key events to the coupled release of SRP from ribosome-bound nascent chains and the transfer of nascent chain to the signal sequence receptor (see below) into the membrane [48], suggested a possible mechanism of proof-reading to J. Rothman [49].

The existence of protein receptors on the mitochondrial surface is now well documented (for a review, see [31]). A least two proteins that may function as receptors have been recently characterized: a 19 kDa protein [50] and a 42 kDa [37] outer membrane protein which is a component of the yeast mitochondrial import site. Distinct protein groups may have different receptors as demonstrated with the ADP/ATP carrier in mitochondria [31] and with apocytochrome C which may not use these receptors (for a review, see [51]). In chloroplasts, anti-idiotypic antibodies against antibodies recognizing the presequence of an imported chloroplast protein inhibited protein import into chloroplasts and identified a 31 kDa protein at contact sites between the two envelope membranes [52].

In bacteria, although the existence of cytosolic receptors (Trigger factor, SecB, GroEL, SecA) is well documented, the existence of a membrane receptor has not yet been firmly established (for a review, see [53]). However, some results [54] suggest that SecY, a multispanning membrane protein of 443 amino acids [55], may interact directly with the signal sequences of exported proteins. Genetic studies also suggested direct interaction between SecA and SecY [24] and thus suggested some receptor role for SecY. However, it is still not clear whether SecY participates directly in the export process as a translocator or a docking system which recognizes nascent polypeptide-SecA complexes before the final translocation step. Recent results suggest it may fulfill both functions [56].

7. TRANSLOCATION MECHANISM

There has been a long debate on whether proteins move across membranes co-translationally or post-translationally. However, there is now strong evidence against a strict coupling between protein translocation and protein synthesis since import of protein into chloroplasts, mitochondria and peroxisomes and export of proteins to the periplasmic space can occur post-translationally (for a review, see [7]). It has now been demonstrated that even the ER can translocate completed polypeptide chains in yeast or partially completed chains in dog pancreas microsomes as long as they are attached to ribosomes [57–59].

For small proteins with a content of less than approximately 75 amino acid residues (including the signal peptide), the molecular requirements for membrane transport are different in that this transport is fully post-translational, ribosome-independent, SRP-independent, or SecA-SecY-independent in bacteria (for a review, see [53,60]). It is important to note in this context that approximately 40 amino acid residues of a nascent polypeptide are buried within the ribosome and that a typical signal peptide contains 20–30 amino acid

residues. Therefore small precursor proteins cannot make use of the complex translocation machinery efficiently and they apparently have evolved with constraints on the primary structure of their mature part which allow them to escape using this machinery.

The nature of the translocation process is still unclear and still subject to some controversy with opposing proposals that the signal sequence recognizes lipid or protein in the membrane and that the mature portion of the protein is then translocated through either the bilayer itself or through specific protein translocators. The balance of evidence currently favours the idea that the mature portion at least of the exported protein traverses the bilayer in largely unfolded form through an aqueous channel formed by one or more protein translocators as proposed by Singer et al. (1987) [61].

Some of the components of translocation machineries in various membranes have been identified. In the ER, the ternary complex (consisting of ribosome-nascent precursor-SRP) after binding to the 72 kDa integral docking protein releases the SRP, thereby allowing the signal sequence to interact with a 35 kDa integral ER glycoprotein termed signal sequence receptor (SSR) [62]. This protein is probably part of the putative translocator.

In *E. coli* cytoplasmic membrane the 42 kDa SecY as well as SecD and SecE [63,64] probably constitute the putative translocator.

In chloroplasts and mitochondria (for a review, see [31]) import requires proteins on the surface which may be concentrated at contact sites between the two envelope membranes. A 42 kDa component of the yeast mitochondrial outer membrane which is required for import, has recently been identified [37].

8. ENERGETIC REQUIREMENTS

Depending upon the organelles and organisms, two different sources have been identified: energy-rich phosphate bonds (ATP and GTP) and a potential across the target membrane. ATP is required in the cytosol to maintain nascent polypeptide chains into a translocation competent conformation. The chaperone proteins are often dependent upon ATP for their action either in maintaining a loose conformation or in causing partial unfolding (for a review, see [19,29,37,45]). In fact, ATP appears to be required for ribosome-independent protein transport (small precursors) across all translocation-competent membranes [65].

Since incompletely folded precursor chains can be translocated into mitochondria in the absence of ATP [31,66], it seems that it is not the driving force to transfer the precursor's mature moiety across the membrane. In addition, it has recently been shown that only internal ATP was important for precursor import [67]. The refolding of the transported polypeptide chains may provide some of the driving force and allow unidirectional transport.

GTP appears to be required for the release of SRP from ternary complex (ribosome, nascent chains, SRP) and initiation of translocation in the ER [48]. In bacteria, in vitro studies demonstrated that ATP is also required (for a review, see [68]). The ATP requirement may stem from the SecA-translocation ATPase activity [69] which requires the functions of SecA, SecY and the signal peptide and mature domains of precursor proteins [70].

The second energy source, a potential across the target membrane, is only required for translocation across the inner membrane of bacteria [71,72] and mitochondria (for a review, see [31]); however, it is not required for the ER. With mitochondria, a potential across the inner membrane is necessary to move the NH_2-terminal part of a precursor across both mitochondrial membranes, but it is not necessary for subsequent translocation of the entire precursor into the mitochondria. Therefore, $\Delta\psi$ is not the energy source to move the mature part of precursors across mitochondrial membranes (for a review, see [31]). With bacteria it has been shown in vitro, that a high concentration of SecA allows proton motive force ($\Delta\mu H^+$) independent translocation thus suggesting that $\Delta\mu H^+$ is required for high affinity interaction of SecA with the presumed secretory machinery in the cytoplasmic membrane (SecY, SecD, SecE) [73].

9. PROCESSING OF TARGETING SIGNALS

Processing of targeting signals is usually a late event in the translocation process which is not strictly required for transport in most cases. The hydrophobic signals are cleaved by integral membrane proteases. The best characterized among these enzymes is the leader peptidase of *E. coli* [74]. In this organism another peptidase cleaves specifically the signal peptides of lipoprotein precursors [75]. In the ER, signal peptidase appears to be a glycosylated multimeric protein [76]. The yeast ER signal peptidase is a 18.8 kDa protein which has been cloned and sequenced [77]. Despite structural differences between prokaryotic leader peptidase and canine signal peptidase, the substrate specificities are remarkably similar, since either enzyme can cleave prokaryotic or eukaryotic substrates at the correct peptide bond in vitro [78].

For proteins located into the intermembrane space of mitochondria or for proteins transported across the thylakoid membrane into the lumenal space in chloroplasts, a complex two step processing occurs (for a review, see [31]). Presequences of these proteins usually consist of two domains specifying transfer into the matrix and then to the intermembrane space, or envelope transfer and then thylakoid transfer, respectively. It has recently been shown that the reaction

specificities of the thylakoid processing peptidase and *E. coli* leader peptidase are identical [79].

The hydrophilic signals are removed by soluble metalloproteases. The subunits of these proteases have been cloned and sequenced (for a review, see [31]).

10. CORE GLYCOSYLATION OF SECRETORY PROTEINS

Elegant genetic studies carried out in yeast have allowed elucidation of the stepwise assembly of the core oligosaccharide transferred to secretory proteins (for a review, see [77]). The 11 yeast genes that are required for the proper assembly and trimming of core oligosaccharides have been identified and the effects of blocks into this stepwise assembly on protein secretion have been evaluated. The results imply that it is not the exact structure of the core oligosaccharide that is important for protein secretion and cell growth, but rather a bulk of chemical property inherent to the monosaccharide units. Perhaps core sugars increase the polarity of secretory proteins, thereby reducing aggregation or deleterious associations with membrane [77].

11. TRANSMEMBRANE INTEGRATION SIGNALS

Besides the signal peptides various classes of signal have been identified to account for topologies of membrane proteins. Stop transfer or anchor signals are used to prevent the protein from being fully secreted and provide it with a transmembrane topology. Uncleaved signal peptides typically featuring positively and negatively charged residues on both sides of the 20 residue apolar segment and start-stop signals, have been described by various authors. Possible mechanisms of assembly of polytopic proteins have been proposed (for a review, see [8]).

12. PROTEIN SECRETION TO THE EXTRACELLULAR MEDIUM IN BACTERIAL CELLS

Natural extracellular proteins of prokaryotes include a variety of enzymes which degrade large polymers (nucleic acids, protein, polysaccharides, lipids, etc.) to create smaller molecules which can be transported and metabolized by the bacterium. Bacteria also secrete a variety of toxins which are active against eukaryotic or prokaryotic cells. Gram-positive bacteria secrete proteins directly across the cytoplasmic membrane to the extracellular medium with or without a stop-over at the extracellular face of the cell envelope (for a review, see [80]). Gram-negative bacteria need to overcome the additional permeability barrier of the outer membrane. Two types of strategy are mainly used. Either additional steps in the signal-dependent export pathway are used or a completely different strategy is employed for getting out of the cell. The signal-dependent pathway has been rather well defined in at least 3 cases. With pullulanase produced by *Klebsiella pneumoniae*, many gene products are needed for the final step [81]; with proteins secreted by the general secretion pathway in *Pseudomonas aeruginosa* several *xcp* gene products are also required (for a review, see [82]). The IgA protease contains a C-terminal domain allowing transfer of the enzymatic domain across the outer membrane of *Neisseria gonorrhoeae* by forming a pore [83].

A whole class of proteins uses a different strategy which was first observed with hemolysin (HlyA) in *E. coli*. Two gene products (HlyB and HlyD) are required for secretion of HlyA to the medium. This secretion does not involve SecA and SecY and the toxin molecule lacks an N-terminal signal sequence (for a review, see [84]). In fact, HlyA carries a C-terminal signal sequence and is part of a growing family of such proteins including proteins targeted to peroxisomes and glycosomes in eukaryotes, in addition to other bacterial toxins and some proteases. HlyB and HlyD are two membrane proteins which probably constitute a membrane-bound translocator specific for hemolysin export or other polypeptides carrying the C-terminal signal domain of HlyA. HlyB resembles the P-glycoprotein, or multi-drug resistance protein (Mdr), and both proteins are emerging as prototypes of a new family of surface transport pump using ATP in prokaryotes and eukaryotes, respectively.

Colicins are bacterial toxins produced and secreted by *E. coli* and its relatives. A small lipoprotein, termed lysis protein, simultaneously expressed with group A colicins, causes release of the toxin through a two-step mechanism involving a direct permeabilisation of the inner membrane and an indirect permeabilization of the outer membrane through activation of a normally dormant phospholipase (for a review, see [81,85]).

13. IMPORT OF PROTEINS INTO BACTERIA

To kill sensitive *E. coli* cells, colicin must be imported. The import mechanism is to a significant extent similar to protein import into mitochondria. The toxins must first bind to an outer membrane receptor, they are then transported, probably through a translocator composed of several proteins to their intracellular targets (for a review, see [86]). However, it has been demonstrated that this translocation may not require any form of energy (μH^+ or ATP) in the case of colicin A [87].

14. CONCLUSIONS

In the field of protein translocation across membranes, we have reached a very interesting stage where the actors have been selected and most of the play has been written but the order of the events and the details

of the scenario still need to be settled before it becomes a good story.

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