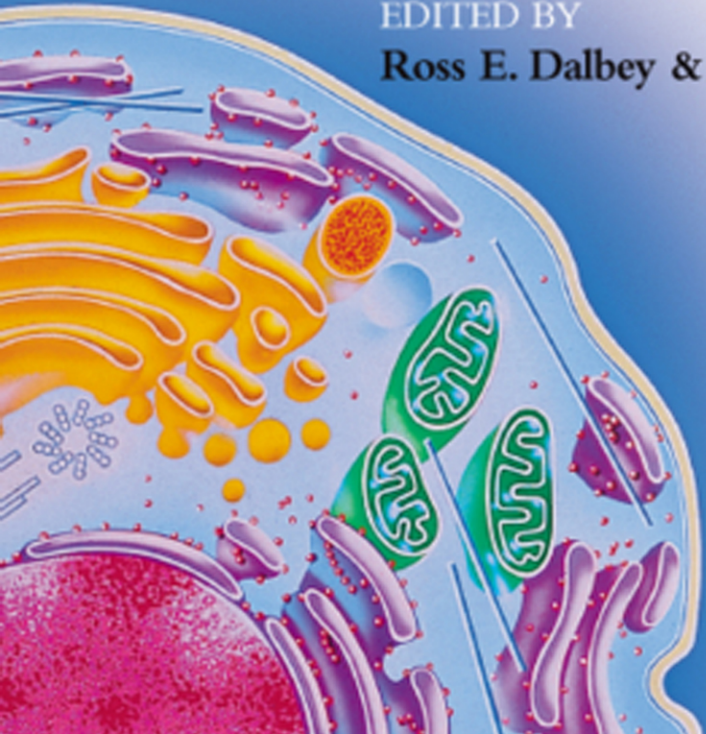
Protein Targeting Transport & Translocation

EDITED BY

Ross E. Dalbey & Gunnar von Heijne



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Foreword

Thirty years ago, in 1971, we published a hypothesis in which we suggested that secretory proteins contain a shared amino-terminal sequence element. A cytosolic binding factor was predicted not only to bind this sequence but also to mediate the attachment of the translating ribosome to the endoplasmic reticulum (ER) membrane. Following completion of translation, the ribosomal subunits were proposed to join the pool of free ribosomal subunits, ready to begin a new round of translation.

This hypothesis attempted to explain the observation that mRNAs for secretory proteins are translated on ER-bound ribosomes and not on free ribosomes. It emphasized the idea that all ribosomes are created equal and opposed a then popular notion that ribosomes might differ in their composition and in their ability to select various mRNAs for translation. The fact that a shared amino-terminal sequence element was not discernible among the few secretory proteins that had been sequenced at that time did not deter us from advancing our proposals. It seemed conceivable to us that such a shared sequence element might be transient in nature and be cleaved off before chain completion and hence be absent in the mature secretory protein.

Earlier it had been established that nascent chains of ER-bound ribosomes are 'vectorially' discharged to the *trans* side of the membrane (the lumen of microsomal vesicles) after incubation with puromycin. Vectorial discharge was thought to proceed through a 'discontinuity' in the membrane. This discontinuity, however, remained undefined until 1975. In what was then dubbed the signal hypothesis, the ideas proposed in 1971 were further amplified to include an ER embedded channel that consists of integral membrane proteins and that functions specifically to allow the passage of nascent secretory proteins to the *trans* side of the ER membrane. The amino-terminal sequence of the nascent secretory protein in concert with several sites on the large ribosomal subunit were envisaged to serve as ligands to assemble (or open) the protein-conducting channel. The concept of a protein-conducting channel made up of integral membrane proteins remained the most contentious aspect of the signal hypothesis for more than 15 years until definitive electrophysiological experiments in 1991 and 1992 established its existence.

The first evidence in support of a transient amino-terminal extension in secretory proteins was obtained in 1972 when mRNA for the light chain of

IgG was translated in a membrane-free translation system. However, it could still be argued that the detected amino-terminal sequence extension is not a signal for translocation, but serves other functions, e.g. it might facilitate folding of nascent secretory protein. It was only in 1975, when we succeeded in developing an *in vitro* coupled translation–translocation system that compelling evidence for the function of the amino-terminal extension as a signal for membrane translocation was obtained. The amino-terminal extension of the light chain of IgG was found to be cleaved only when translation occurred in the presence of added microsomal vesicles, but not when the microsomal vesicles were added after translation. This indicated that the microsomal membrane contained an embedded signal peptidase with its active site exposed on the *trans* side of the membrane. Most importantly, the signal-peptidase-processed nascent chains were found to be protected from externally added proteases, indicating that they were translocated into the lumen of the microsomal vesicles to which the added proteases had no access.

Once this coupled *in vitro* translation–translocation system was set up, it was only a matter of time to identify the cast of characters that are involved in translocation of secretory proteins across the ER. The first component to be isolated in 1978/1980 was the binding factor, whose existence was predicted in 1971. Unexpectedly this binding factor turned out to be a ribonucleoprotein particle, consisting of an RNA and six distinct proteins. As predicted in 1971, this binding factor, now termed signal recognition particle (SRP), recognized the signal sequence and bound the translating ribosome to the microsomal membrane. Thereafter, a heterodimeric membrane protein that is located only in the ER and that functions as an SRP receptor was isolated and characterized. Hence the components involved in signal sequence recognition and targeting to the ER were defined. Next, the enzyme that cleaves off the signal sequence was isolated and shown to consist of a complex of five distinct integral ER membrane proteins. A most important advance for the subsequent characterization of the protein-conducting channel was the demonstration in 1989 that protein translocation occurred faithfully in proteoliposomes that were reconstituted after detergent solubilization of microsomal membranes. Finally, the identification and characterization of the protein-conducting channel was accomplished by genetic and biochemical methods. Recent reconstitution of isolated protein conducting channels with RNCs (ribosome–nascent chain complexes) and subsequent analysis by cryo-electron microscopy and three-dimensional image reconstruction at 15.4 Å resolution revealed that the protein-conducting channel is aligned with the tunnel in the large ribosomal subunit and is a rather compact structure that is apparently in intimate contact with the translocating chain. At least four attachment sites to distinct segments of large ribosomal subunit RNA and proteins have been discerned.

Another proposal of the 1975 signal hypothesis was that a nascent integral membrane protein contains a signal sequence that is functionally identical to that of a secretory protein. This signal sequence was suggested to initiate translocation of the nascent membrane protein. An additional sequence element, termed stop-transfer sequence, was proposed to prevent further translocation of the nascent chain to the *trans* side by opening the protein-conducting channel laterally to the lipid bilayer, thereby allowing displacement of the stop-transfer sequence from the aqueous channel to the lipid bilayer. Data supporting these proposals were obtained in 1977/1978, when mRNA of the vesicular stomatitis virus (VSV) membrane glycoprotein (G) was translated in the coupled translation–translocation system. These experiments were paradigmatic as they showed that the asymmetric integration of a membrane protein into the lipid bilayer is not a spontaneous process, as was widely believed at the time, but is catalyzed.

Yet another proposal of the 1975 signal hypothesis was that proteins to be translocated across other intracellular membranes would possess signal sequences that are distinct from those addressed to the ER. Such signal sequences were indeed detected in the late 1970s and early 1980s for translocation across the bacterial plasma membrane, for protein import into mitochondria, chloroplasts and peroxisomes and finally for import and export across the nuclear pore complexes of the nuclear envelope. In many ways, the experiments of the ER translocation system were paradigmatic for the experiments in these other systems. Cell-free translocation systems were set up followed by genetic and biochemical experiments to identify the cast of characters involved in each of the cases. Similar strategies were also used to study intercompartmental transport. The various chapters of this book give us an account of these efforts and where we presently stand.

Although signal sequences, cognate recognition factors, targeting and passage through a membrane are common to all of the translocation systems, nature has created fascinating and ingenious variations of that general theme. Bacteria are clearly the masters of this game. A more recent example of their virtuosity is that practiced by pathogenic Gram-negative bacteria. These bacteria polymerize a needle-like structure from a single small protein to puncture the plasma membrane of a eukaryotic cell to transfer certain proteins through a very narrow gauge from the bacterial cytosol across three membranes into the eukaryotic cytosol.

Many important questions remain to be answered and several areas of intracellular macromolecular traffic are just in the beginning phases of exploration. It is clear, for example, that nuclear import or export does not end or begin, respectively, with transport across the nuclear pore complex. Export is preceded and import is followed by an intranuclear phase of transport. Another largely unexplored area is how various segments of nascent membrane proteins interact with protein-conducting channels to achieve

the great variety of polytopic orientations in the membrane. The structural analysis of the various transport systems by X-ray crystallography and cryo-electron microscopy has just begun and should continue to provide major new insights into their function.

The field of macromolecular intracellular traffic is by no means in a stationary phase. To the contrary, it has barely entered the logarithmic phase. This book will be an important milestone and a guide to those who enter this exciting phase. No doubt, a deeper understanding of cellular macromolecular traffic systems will ultimately yield a broader understanding into how a cell, any cell, organizes itself.

Günter Blobel
New York, September 2001

Preface

Not since the *Protein Targeting Book* by Tony Pugsley in 1989 has the topic of protein localization been covered in depth in a textbook. We felt, therefore, the time was right to put together an up-to-date book that could be used both by scientists in general and in graduate level and/or advanced undergraduate courses. It is our strong belief that only when a book finds use in teaching is it really worthwhile.

In just the past ten years, there has been an explosion of activity in the protein targeting and transport area. Many major advances have been made just in this time period. For instance, most of the components that comprise that targeting factors and translocation systems have been identified and some of the protein structures have been solved to high resolution. It is now clear that there are diverse and extremely intricate machineries used to move proteins around within the cell.

In 1999, much attention was focused on the protein targeting area when Günter Blobel was awarded the Nobel Prize in Physiology or Medicine 'for the discovery that proteins have intrinsic signals that govern their transport and localization in the cell'. Now, in the early days of the 21st century, only thirty years after Blobel initiated his first ground-breaking experiments, the protein targeting area has proved to be of fundamental importance in areas ranging from biotechnology and molecular biology to apoptosis, immunology, signal transduction, and others. This book is intended to give some impression of this wide significance while at the same time not losing sight of the basic principles of protein targeting.

We would like to offer our sincerest thanks to all the contributors for their hard work and devotion to research which made this book both necessary and possible.

Ross E. Dalbey
Gunnar von Heijne

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1

Introduction/Overview

Ross E. Dalbey and Gunnar von Heijne

All living cells contain proteins that carry out specialized functions within various subcellular membrane or aqueous spaces. Recent estimates suggest that approximately half of all the proteins of a typical cell are transported into or across a membrane. How are proteins synthesized in the cytoplasm of the cell, inserted into or across membranes, and how are they transported to their correct subcellular destinations? Questions such as these have been a central theme in cell biology for nearly four decades, starting with the pioneering work of George Palade (Nobel Laureate in Physiology or Medicine in 1974) that defined the basic structure of the secretory pathway in eukaryotic cells, and continued by among others Günter Blobel (Nobel Laureate in Physiology or Medicine in 1999) who discovered that proteins possess intrinsic signals that govern their localization in the cell.

Bacterial cells have at least one membrane that separates the inside of the cell from its environment. Gram-positive bacteria have only one membrane, and Gram-negative bacteria have an additional outer membrane. Therefore, in Gram-positive cells there are three compartments – the cytoplasm, the plasma membrane, and the extracellular medium – whereas in Gram-negative bacteria there are five – the cytoplasm, the plasma membrane, the periplasm, the outer membrane, and the extracellular medium.

In contrast to most bacterial cells, eukaryotic cells contain, in addition to the plasma membrane, internal membranes (Figure 1.1). These internal membranes are structural components of organelles and vesicles. Proteins embedded in membranes or localized in the aqueous spaces surrounded by membranes give rise to the specialized functions carried out in these compartments. Thus, the nucleus houses the machinery for DNA replication, transcription and RNA splicing; the mitochondrion specializes in respiration that produces adenosine triphosphate (ATP) for the cell; the chloroplast contains the proteins that are responsible for photosynthesis and the

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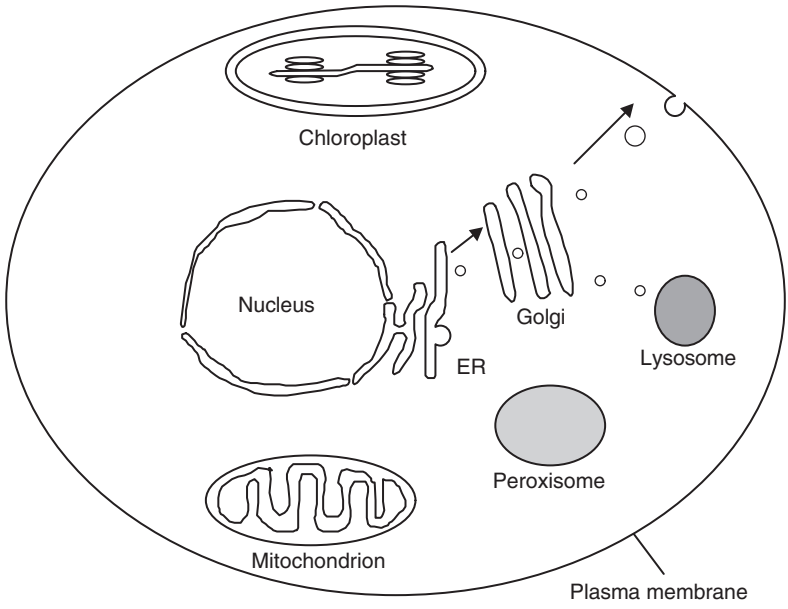

Figure 1.1 Eukaryotic cells. The organelles and membranes are shown for a typical eukaryotic cell. Each of the organelles has a specialized function. Most proteins are synthesized in the cytoplasm on ribosomes. Some proteins are directly targeted from the cytoplasm. This includes proteins directed to the ER, mitochondria, chloroplast, or peroxisome by intrinsic signals within their polypeptide chain. Some proteins that are targeted to the secretion pathway via the ER are further sorted to the Golgi, lysosome/vacuole, secretory vesicles, plasma membrane, or the extracellular medium. Chloroplast and mitochondrion have their own genomes that synthesize a small number of proteins. The chloroplast-synthesized proteins either remain in the stroma or are exported to the thylakoid membrane or thylakoid lumen. In mitochondria, the newly synthesized proteins remain in the matrix or are exported to the inner membrane in mitochondria.

synthesis of energy-rich compounds from carbon dioxide and water; the Golgi apparatus contains enzymes that modify sugars attached to exported proteins; the lysosome/vacuole contains digestive enzymes responsible for intracellular digestion, and the peroxisome houses enzymes for fatty acid oxidation and for producing and metabolizing hydrogen peroxide.

Most proteins are synthesized in the cytoplasm of the cell, except for a small number that are encoded in the mitochondrial and chloroplast genomes. This raises the question of how proteins are transported from the cytoplasm to other destinations within or outside of the cell. Approximately 20% of the proteins in a typical cell are located in the non-cytoplasmic aqueous spaces bounded by a membrane. An additional 25–30% of the proteins are located within a membrane.

Proteins are imported directly from the cytoplasm into the endoplasmic reticulum (ER), mitochondria, peroxisomes and chloroplasts by mechanisms that use a targeting sequence and a translocation machinery. Exported proteins are usually made in a precursor form with an amino-terminal signal peptide that directs the protein into the export pathway. Such amino-terminal signal peptides target proteins to the ER membrane where they are recognized by the translocation machinery (see Chapter 5). For peroxisomal proteins, there are two types of targeting sequences directing import: an amino-terminal signal or a carboxyl-terminal targeting sequence (see Chapter 12). Mitochondrial targeting sequences target proteins to the mitochondrial membrane by being recognized by surface exposed mitochondrial receptors (see Chapter 10). The chloroplast targeting signal directs chloroplast proteins to the chloroplast for import into the organelle (see Chapter 11).

In addition to importing proteins from the cytoplasm into the organelle, mitochondria and chloroplasts also export proteins from the mitochondrial matrix or the chloroplast stroma where proteins are encoded by their respective organellar genomes. It is not surprising that mitochondrial and chloroplast export machineries share some important features with those found in bacterial cells since these organelles descended from bacterial progenitors millions of years ago.

Proteins that are localized to the Golgi, lysosome/vacuole, and plasma membrane are first inserted into the ER. Within the ER, disulfide bonds are introduced into the proteins by a protein disulfide isomerase (see Chapter 7). Additionally, a 14-residue oligosaccharide core is attached to glycoproteins containing asparagine-linked sugars. The oligosaccharyl core is processed initially in the ER and then further trimmed and modified in the Golgi apparatus. Misfolded proteins in the ER are recognized and retrotranslocated out of the ER lumen into the cytoplasm where the protein is ubiquinated and degraded by the proteasome (see Chapter 9). Proteins that are folded correctly move from the ER to the *cis* Golgi and further along the secretory pathway by vesicular transport.

The details of how vesicles are formed at the ER and move through the Golgi stacks (from *cis* to *trans*) are being actively worked out. The SNARE hypothesis (see Chapter 16) proposes that vesicles mediate trafficking in the anterograde – forward – direction from the *cis* Golgi cisternae to the *medial* Golgi cisternae and then from the *medial* Golgi cisternae to the *trans* cisternae. The donor vesicle fuses with its target vesicle using a number of proteins (NSF, SNAP, SNARE, etc.). A competing hypothesis states that the vesicles do not mediate movement of cargo through the stacks. Rather, the *cis* Golgi cisternae mature into the *medial* cisternae; the *medial* cisternae then mature into the *trans* cisternae. In this model, the cisternae mature because certain Golgi components contained within the cisternae are removed by retrograde vesicle transport (see Chapters 15 and 16).

Transport into and out of the nucleus is unlike the mechanism for insertion into the ER, mitochondria, chloroplast and peroxisome in that it occurs via large aqueous pores that span both nuclear membranes. These nuclear pore complexes are huge structures that support two-way trafficking (Chapter 13). Proteins imported into the nucleus typically contain a positively charged nuclear localization signal. A number of soluble factors are also required for transport.

A good understanding of protein targeting and translocation is important for many areas in biology and medicine. It has applications in biotechnology, where growth hormones, insulin, interleukins and coagulation factor VIII, to name but a few, have been engineered to be secreted into the culture media. In immunology, knowledge of the secretion pathway has been very useful for the understanding of how peptides from antigens are displayed by the major histocompatibility complex proteins on the cell surface. In programmed cell death, protein translocation to and from the plasma membrane, mitochondria and the nucleus is critical for regulating apoptosis. Lastly, nuclear trafficking is very important for signal transduction and cell cycle regulation.

This book brings together a number of important topics in the protein localization field. First, we will describe some of the common techniques used to study protein translocation and transport (Chapter 2). Second, we review the targeting signals within exported proteins that direct the export of proteins to their subcellular compartment (Chapter 3). Third, we review how proteins cross and insert into membranes in bacteria and in the ER of a eukaryotic cell (see Chapters 4–6). Fourth, we will describe how disulfide bonds are introduced into exported or membrane proteins as they enter the ER lumen (Chapter 7). Fifth, we will report on the unfolded protein response where the cells can adapt to the condition where unfolded proteins accumulate in the lumen of the ER (Chapter 8) and quality control mechanisms allowing proteolysis of misfolded proteins (Chapter 9). Sixth, we will describe protein import into the mitochondria, chloroplast and peroxisome (Chapters 10–12). Seventh, we will review the import and export of nuclear proteins and regulation of this process (Chapter 13) and the movement of proteins along the secretion pathway (ER to Golgi to either the vacuole or the plasma membrane) (Chapters 14–16).

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2

Methods in Protein Targeting, Translocation and Transport

Ross E. Dalbey, Minyong Chen and Martin Wiedmann

INTRODUCTION

Protein targeting, translocation and transport mechanisms have been studied extensively by scientists over the last 30 years using biochemical, genetic, cell biological, molecular biological, and electron microscopic techniques. In this chapter, we will cover some of the key techniques used to study protein export. We have divided them into four categories: *in vivo*, genetic, *in vitro*, and cell biology techniques. The *in vivo* techniques are necessary to examine the fate of a protein within an intact cell and often take advantage of mutants that were identified using genetics. The genetic section is separate from the *in vivo* section because of the premier importance it plays in the protein transport area. Genetics have unraveled most of the protein components that make up the translocation machinery involved in protein export. A powerful role is also played by the *in vitro* techniques where the functions of the purified proteins are defined and where the goal is to reconstitute transport events in a test tube. Finally, cell biology techniques exploiting electron microscopy and fluorescence light microscopy have allowed the researcher to follow the fate of a protein within a cell.

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IN VIVO STUDIES: PULSE-CHASE STUDIES WITH WHOLE CELLS AND SUBCELLULAR FRACTIONATION

Bacteria

Almost all proteins exported to the outer membrane and periplasmic space of *Escherichia coli* are made in a precursor form containing an amino-terminal extension peptide called a signal peptide. The export of these proteins requires the Sec machinery comprising SecA, SecY, SecE, SecG, SecD, SecF (Schatz and Beckwith, 1990; Wickner et al., 1991), and YajC (Duong and Wickner, 1997). Also needed for export is the electrochemical membrane potential (Geller et al., 1986) and ATP hydrolysis (Chen and Tai, 1985; Geller et al., 1986).

The use of drugs and Sec mutants to study protein export

To examine export *in vivo*, cells are typically labeled with [^{35}S]-methionine for a short time (15 s) and chased with non-radioactive methionine for various times. The labeled proteins are immunoprecipitated with antiserum to the respective protein, and analyzed by SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and phosphorimaging. In these pulse-chase experiments, preproteins are rapidly inserted into the membranes and processed by signal peptidase, an integral membrane protease that removes signal sequences. The addition to a bacterial culture of carbonyl cyanide *p*-chlorophenylhydrazone (CCCP), an uncoupler of the membrane electrochemical potential, causes accumulation of non-translocated preproteins at the membrane (Daniels et al., 1981; Date et al., 1980). The addition of azide, an inhibitor of SecA, causes Sec-dependent proteins to accumulate (Oliver et al., 1990). The effects of these drugs on the export of preproteins is tested by examining whether the precursor form of the exported protein accumulates. Usually, the precursor form that accumulates is easily detected by SDS–PAGE and fluorography.

Using Sec mutants is instrumental with *in vivo* studies for determining whether a protein is exported by the Sec machinery. For instance, thermosensitive (t.s.) mutations in SecA (Oliver and Beckwith, 1981) and SecY (Ito et al., 1983) and cold-sensitive (c.s.) mutations in SecE (Schatz et al., 1989), SecG (Nishiyama et al., 1994) and SecD (Gardel et al., 1987) have been isolated. T.s. and c.s. mutants are grown at the non-permissive temperature for certain times to deplete (synthetic mutants) or to inactivate (folding mutants) the Sec protein. When the cells are grown at the non-permissive temperature the kinetics of protein translocation can be investigated. If the newly synthesized preproteins accumulate at the non-permissive temperature, the protein is Sec-dependent. If no translocation defect is observed in these conditional mutants, it is useful to analyze a SecE