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Chapter 1

Mitochondrial Protein Import: Specific Recognition and Membrane Insertion of Precursor Proteins

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I. INTRODUCTION

Eukaryotic cells are divided into numerous membrane-bounded compartments ("organelles"), each of which contains a unique and specific set of proteins. Most of the organellar proteins are synthesized as precursor proteins on cytosolic polysomes and thus have to be transported to their functional destination (Wickner and Lodish, 1985). The question of how proteins are directed to their specific target membrane and how they are translocated into and across organellar membranes poses a central theme of modern cell biology. The cytosolic precursor proteins carry specific targeting sequences that are assumed to bind to complementary structures ("receptors") on the surface of organelles. The translocation

into and across the membranes may be mediated by proteinaceous components and/or lipids of the membranes.

The biogenesis of organellar proteins is even more complex in case of mitochondria. Two membranes (outer and inner mitochondrial membranes) limit two soluble compartments, namely, the intermembrane space and the matrix. Whereas some mitochondrial proteins are coded for by mitochondrial genes and are synthesized in the matrix, >90% of the proteins are coded for by nuclear genes and are imported from the cytosol (Pfanner and Neupert, 1987a; Attardi and Schatz, 1988; Hart *et al.*, 1989).

It was first shown for mitochondrial protein import that translocation of precursor proteins across membranes is not mechanistically coupled to synthesis of the protein on ribosomes, both *in vivo* and *in vitro* (Hallermayer and Neupert, 1976; Harmey *et al.*, 1976, 1977; Hallermayer *et al.*, 1977). Many precursor proteins carry amino-terminal extension sequences ("presequences") of about 20–80 amino acid residues. Presequences contain signal information for translocation into mitochondria (Horwich *et al.*, 1985; Hurt and van Loon, 1986). With some precursor proteins, targeting sequences were also found in non-amino-terminal (carboxyl-terminal) regions of the polypeptide (Pfanner *et al.*, 1987b,c; Smagula and Douglas, 1988). The precursor proteins are recognized by specific receptors on the mitochondrial surface and are then inserted into the outer membrane (see later). Further translocation into the inner membrane occurs predominantly at sites of close contact between the mitochondrial outer and inner membranes ("contact sites") (Schleyer and Neupert, 1985; Schwaiger *et al.*, 1987; Rassow *et al.*, 1989). Hydrophilic (proteinaceous) components apparently represent essential parts of contact sites (Pfanner *et al.*, 1987a; Vestweber and Schatz, 1988). Presequences are proteolytically cleaved by the processing peptidase in the mitochondrial matrix (Hawlitschek *et al.*, 1988), the proteins are sorted to their final intramitochondrial location (Hartl *et al.*, 1987), and are often assembled into multisubunit complexes (Schmidt *et al.*, 1983; Lewin and Norman, 1983). Protein import requires energy in (at least) two different forms. Adenosine triphosphate is involved in unfolding of precursor proteins in the cytosol and/or in release of precursor proteins from cytosolic cofactors (Pfanner *et al.*, 1987d, 1988a; Eilers and Schatz, 1988; Murakami *et al.*, 1988). The electrical potential ($\Delta\Psi$) across the inner mitochondrial membrane is needed for the initial transfer of precursors into or across the inner membrane (Pfanner and Neupert, 1985; Schleyer and Neupert, 1985).

This article focuses on the problems of specific recognition and membrane insertion of mitochondrial precursor proteins. Harmey *et al.* (1977) had proposed that "some mechanism of selective recognition of precursor proteins by the mitochondria" exists. A detailed functional analysis in recent years has led to the characterization of receptor sites and of a membrane insertion site for precursors

and thereby provided the basis for identification of components of the mitochondrial protein import apparatus.

II. RECEPTOR PROTEINS ON THE MITOCHONDRIAL SURFACE

A. Functional Characterization of Receptor Sites

Pretreatment of isolated mitochondria with proteases diminished subsequent import of *in vitro*-synthesized precursor proteins (Gasser *et al.*, 1982; Argan *et al.*, 1983). A mild pretreatment with proteases inhibited not only import but also binding of precursors to mitochondria (Riezman *et al.*, 1983; Zwizinski *et al.*, 1984). The mitochondrial membranes were shown to remain intact under these conditions, suggesting that proteinaceous surface components are involved in import of precursor proteins. These components are assumed to perform the function of receptor sites. The import of various precursors exhibited a differential sensitivity toward pretreatment of mitochondria with specific proteases, supporting a model in which several distinct receptor sites exist on the mitochondrial surface (Zwizinski *et al.*, 1984).

Precursor proteins bind to the mitochondrial surface in the absence of a membrane potential across the inner membrane. After reestablishing a membrane potential, the precursor proteins are imported from the binding sites without prior release from the membranes (Zwizinski *et al.*, 1983; Riezman *et al.*, 1983; Pfanner and Neupert, 1987b; Pfanner *et al.*, 1987d). This type of binding is termed specific (productive) binding and depends on the presence of surface proteins ("receptors") (Zwizinski *et al.*, 1984), whereas nonproductive (unspecific) binding also occurs to protease-pretreated mitochondria. Unspecific binding may occur to lipids of the outer membrane.

The precursor of the mitochondrial outer membrane protein porin was prepared in large amounts (Pfaller *et al.*, 1985) and bound to isolated mitochondria such that the mitochondrial import sites for porin and other precursor proteins (see Section III) were saturated by the porin precursor. The precursor of the inner membrane protein ADP/ATP carrier could still bind to its proteinaceous surface sites (receptor); that is, binding of ADP/ATP carrier to its receptor was not competed for by porin (Pfaller *et al.*, 1988). Since the affinities of those two precursors for interaction with mitochondria are in a similar range (Pfaller and Neupert, 1987; Pfaller *et al.*, 1988), porin and ADP/ATP carrier seem to use distinct receptor sites.

In summary, functional characterization suggests the following properties of specific mitochondrial import receptors. (i) Receptors are exposed on the mito-

chondrial surface. (ii) A specific receptor recognizes only a subset of precursor proteins; it is not involved in import of all precursor proteins. (iii) Receptors are required for specific binding of precursors to the mitochondrial surface.

B. Identification of Receptors

In the past no receptor protein for import of mitochondrial precursor proteins could be identified despite numerous efforts in several laboratories. This led to speculations that the initial steps of mitochondrial protein import may not involve receptor proteins on the mitochondrial surface (Roise *et al.*, 1986; Hurt and van Loon, 1986), although functional evidence clearly suggested the existence of specific receptor sites (summarized in Section II,A; Pfanner *et al.*, 1988b).

We have started a systematic and basic approach with the aim of identifying the mitochondrial import receptors. Since the mitochondrial outer membrane contains only a relatively small number of proteins (~25), we tried to produce monospecific antibodies against each of these proteins and to test the effect of these antibodies on the import of precursor proteins. Outer membrane vesicles were isolated from mitochondria of the fungus *Neurospora crassa*. The proteins were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to nitrocellulose. Twenty-five distinct bands were excised and used for generation of polyclonal antisera in rabbits. We obtained monospecific antisera against many of the outer membrane proteins (Söllner *et al.*, 1989).

In a first screening we investigated the effect of immunoglobulin G (IgG), which was prepared from the antisera, on the import of ADP/ATP carrier and porin in the assumption that these two precursor proteins use different receptor sites (see Section II,A). Immunoglobulin G was prebound to mitochondria, the mitochondria were reisolated, and the import of *in vitro*-synthesized precursors was tested. Most of the IgG had no significant effect on import of precursor proteins, including IgG against porin, the major protein of the outer membrane. Immunoglobulin G directed against a mitochondrial outer membrane protein of 19 kDa (MOM19) inhibited the import of porin, but not of ADP/ATP carrier. Thus MOM19 which is exposed on the mitochondrial surface appeared to be a possible candidate for a mitochondrial import receptor (Söllner *et al.*, 1989).

Fab fragments directed against MOM19 were prebound to mitochondria and the import of precursors to the four mitochondrial subcompartments was studied (Table I). The import of porin (outer membrane), cytochrome c_1, and Fe/S protein of the bc_1 complex (intermembrane space side of the inner membrane), subunit 9 of F_0F_1-ATPase (inner membrane), and subunit β of the F_0F_1-ATPase (matrix side of the inner membrane) were strongly inhibited. The import of ADP/ATP carrier (inner membrane) was practically unaffected. A series of controls excluded unspecific effects of IgG and Fab fragments against MOM19 (anti-MOM19), such as inactivation of precursor proteins or cytosolic cofactors and

TABLE I
TARGETING PATHWAYS OF MITOCHONDRIAL PRECURSOR PROTEINS

Protein	Functional destination	Present in prokaryotic ancestor	N-terminal targeting sequence	Receptor	Membrane insertion
Cytochrome c_1	Intermembrane space	Yes	Yes	MOM19[a]	GIP[c]
Fe/S protein	Intermembrane space	Yes	Yes		
F_0-ATPase subunit 9	Inner membrane	Yes	Yes		
F_1-ATPase subunit β	Matrix	Yes	Yes		
Porin	Outer membrane	?	?		
ADP/ATP carrier	Inner membrane	No	No	MOM72[b]	GIP
Cytochrome *c*	Intermembrane space	Yes	?	No surface receptor	Endogenous activity of precursor

[a]Mitochondrial outer membrane protein of 19 kDa.
[b]Mitochondrial outer membrane protein of 72 kDa.
[c]General insertion protein.

inhibition of later transport steps (translocation from the outer into the inner membrane, the membrane potential-dependent step, and proteolytic processing of precursor proteins) (Söllner *et al.*, 1989). As a further control, the import of cytochrome *c* was tested. The precursor, apocytochrome *c*, spontaneously inserts into the outer mitochondrial membrane (Rietveld *et al.*, 1985; Stuart *et al.*, 1990) and does not use a protease-accessible surface receptor (Nicholson *et al.*, 1988). As expected, anti-MOM19 did not inhibit the import of apocytochrome *c*.

Inhibition of protein import by anti-MOM19 occurred at the level of specific binding of precursors to the mitochondrial surface. Thus MOM19 fulfills all the functional criteria established for a mitochondrial import receptor: it is exposed on the mitochondrial surface; it is involved in the import of a subset of precursor proteins, and it is required for specific binding of precursors to mitochondria. We conclude that the outer membrane protein MOM19 is identical to (or closely associated with) a specific import receptor (Söllner *et al.*, 1989).

With similar procedures we found IgG and Fab fragments against a mitochondrial outer membrane protein of 72 kDa (MOM72). Anti-MOM72 selectively inhibited the import of ADP/ATP carrier, but not of other mitochondrial precursor proteins tested. The inhibition of import occurred at the level of specific binding of ADP/ATP carrier to the mitochondrial surface, whereas other import steps were unaffected (Söllner *et al.*, 1990). We conclude that MOM72, which is exposed on the mitochondrial surface, represents a specific import receptor for ADP/ATP carrier and probably similar precursors (Table I).

C. Import of Precursor Proteins Bypassing Receptor Sites

Blocking of receptor sites by specific antibodies or degradation of receptors by treatment of mitochondria with proteases strongly reduced the import rates of mitochondrial precursor proteins (Pfaller *et al.*, 1989; Söllner *et al.*, 1989). This suggests a crucial role of receptor sites for the efficiency of protein import.

A residual import of precursors, however, can also occur when the surface receptors are blocked or degraded. This low efficient import still exhibits several basic features of mitochondrial protein import, including dependence on ATP and membrane potential $\Delta\Psi$ and translocation via contact sites (Pfaller *et al.*, 1989). Precursor proteins are obviously able to bypass surface receptors and enter the mitochondrial import pathways at a later stage. The very low efficiency suggests that bypass import does not significantly contribute to the import processes under physiological conditions (Pfanner *et al.*, 1988c).

The existence of bypass import, at least under certain experimental conditions (high amounts of precursor proteins), led to a series of very surprising findings. Nonmitochondrial "targeting" sequences, such as a chloroplast signal sequence (Hurt *et al.*, 1986) or sequences of a cytosolic protein (Hurt and Schatz, 1987), could direct proteins into mitochondria albeit with a low efficiency (summarized in Pfanner *et al.*, 1988c). The main common property of these nonmitochondrial signals was the abundance of positively charged amino acid residues, which appears to be an essential requirement for the $\Delta\Psi$-dependent insertion into the inner membrane. Import directed by nonmitochondrial signals was not affected by pretreatment of mitochondria with proteases. These "targeting" signals apparently bypass the surface receptors (Pfaller *et al.*, 1989). Mitochondrial import receptors therefore specifically interact with authentic mitochondrial targeting sequences; receptors are responsible for the selectivity of protein import.

Yeast mitochondria with disrupted outer membrane are able to translocate precursor proteins directly across the inner membrane (Ohba and Schatz, 1987a; Rassow, Pfanner, and Neupert, in preparation). The nature and function of these inner membrane import sites is unknown. They might for example be related to the translocation sites that are (permanently or transiently) present in contact sites between both membranes (Schwaiger *et al.*, 1987). Further studies are required to decide how specific these import sites are and if they contain components for recognition of mitochondrial precursor proteins.

III. THE "GENERAL INSERTION PROTEIN"

After interaction with specific surface receptors, mitochondrial precursor proteins are inserted into the outer membrane. Studies on the import pathways of

ADP/ATP carrier and porin suggested the existence of a new functional component for protein translocation across membranes, a membrane insertion site (Pfanner and Neupert, 1987b; Pfaller and Neupert, 1987). Precursor proteins that are inserted into the outer membrane are not accessible to specific antibodies or to low concentrations of proteases added to the mitochondria, in contrast to precursors that are bound to the surface receptors (Söllner *et al.*, 1988), suggesting that the membrane insertion sight is buried in the outer membrane.

The membrane insertion sites are saturable; the determined number of sites is practically identical for ADP/ATP carrier and porin and is in a similar range as the number of receptor sites (Pfaller and Neupert, 1987; Pfaller *et al.*, 1988). Precursor proteins inserted into the outer membrane are extractable from the membranes by "hydrophilic perturbants" or "protein denaturants" such as carbonate ions or urea (Pfanner and Neupert, 1987b; Pfaller and Neupert, 1987). The precursor proteins thus may be inserted into a proteinaceous membrane environment.

The precursor of porin competed for the import of nearly all other mitochondrial precursor proteins tested, including cytochrome c_1, Fe/S protein, F_0-ATPase subunit 9, F_1-ATPase subunit β, and ADP/ATP carrier (Table I). Competition of import specifically occurred at the level of insertion of precursors into the outer membrane (Pfaller *et al.*, 1988) and not for the interaction with receptor sites (see Section II,A). We concluded that the various precursor proteins competed for interaction with the same component of the protein import apparatus, namely, a common membrane insertion site. The only precursor protein the import of which was not competed for was apocytochrome *c* (Table I); this fits well with the bulk of evidence suggesting that cytochrome *c* uses a very unique import pathway (Nicholson *et al.*, 1988).

The common membrane insertion site, termed the general insertion protein (GIP), has not been identified so far. The receptors MOM19 and MOM72 can form a high molecular weight complex that contains two other outer membrane proteins. One of these proteins, termed MOM38 (molecular weight 38 K), exhibits the properties expected of GIP (Pfaller, Söllner, Griffiths, Pfanner, and Neupert, in preparation). A recent finding on protein import into yeast mitochondria also may be of interest for the identification of GIP. Antibodies directed against 45-kDa mitochondrial proteins inhibit import of precursor proteins when bound to mitochondria that had been pretreated with proteases (Ohba and Schatz, 1987b). Where the antibodies bound to intact mitochondria, the inhibition of import was only marginal; the antibodies obviously do not block receptor proteins that are exposed on the mitochondrial surface. Since the inactivated component(s) appears to be protected against proteases, it may be buried in the outer membrane and has to be "freed" from other proteins by the pretreatment with proteases in order to be accessible to the antibodies. We speculate that a component that is recognized by the anti-45-kDa antibodies is related to GIP (Pfaller *et*

al., 1988). Vestweber *et al.* (1989) reported that these antibodies also recognized a protein of 42 kDa, termed ISP42 (import site protein). Inhibition of import was found to be caused by the anti-ISP42 antibodies. Moreover, a fraction of precursor proteins that was accumulated in contact sites was cross-linked to ISP42. MOM38 of *Neurospora crassa* and ISP42 of yeast thus may be related to GIP.

IV. ROLE OF RECEPTORS AND GENERAL INSERTION PROTEIN

Receptor proteins on the mitochondrial surface are responsible for the specificity and selectivity of protein uptake (see Section II,C). They recognize mitochondrial targeting sequences and strongly enhance the import rates of those precursors. Precursor proteins with amino-terminal targeting sequence interact with the receptor MOM19, whereas ADP/ATP carrier, a precursor with several internal targeting sequences (but no N-terminal signal), interacts with MOM72 (Table I). Treatment of mitochondria with the protease elastase generates an 17-kDa fragment of MOM19 that still mediates import of F_1-ATPase subunit β, whereas import of other MOM19-dependent precursor proteins is inhibited (Söllner *et al.*, 1989). Distinct segments of MOM19 may thus be responsible for interaction with the various precursor proteins. This offers the possibility for characterization of functional sites of this import receptor.

Most mitochondrial proteins that were found to require MOM19 for import have equivalents in bacteria and thus were probably already present in the prokaryotic ancestors of mitochondria (Table I) (the evolutionary origin of porin is unknown). According to the endosymbiont hypothesis, after endocytosis of the prokaryotic cell, the (now) mitochondrial genes for these proteins were transferred to the nucleus (see Hartl *et al.*, 1987, for a discussion). An amino-terminal targeting sequence directed the proteins back to mitochondria. We propose that MOM19 was used as surface receptor for those precursor proteins. On the other hand, proteins exist that were most likely not present in the prokaryotic ancestor. The ADP/ATP carrier was probably established in the eukaryotic cell (Klingenberg, 1985). Its targeting sequences are not located at the amino terminus of the precursor protein (Pfanner *et al.*, 1987b; Smagula and Douglas, 1988), and the precursor uses a different surface receptor, MOM72 (Söllner *et al.*, 1990).

The receptors MOM19 and MOM72 then transfer the precursor proteins to the GIP in the outer membrane. The receptors themselves may possess some activity for membrane insertion of precursor proteins and thereby facilitate the action of GIP. Alternatively, receptors may only be able to bind precursor proteins, and the insertion into the outer membrane is solely performed by GIP (possibly in cooperation with lipids of the outer membrane). As described earlier, MOM19, MOM72, and probably GIP can be detected in a protein complex in the outer membrane (Pfaller *et al.*, in preparation). The receptors and GIP may not func-

tion as independent entities in the outer membrane. Their possible assembly into a multisubunit complex may help in coordination of their activities; it may even be a prerequisite for some of their functions.

Beyond GIP, the import pathways diverge; some precursors assemble into the outer membrane (porin), whereas most precursors move on to contact sites between both membranes and then to the other mitochondrial subcompartments (Pfaller *et al.*, 1988; Hartl *et al.*, 1989). Since translocation of proteins across the mitochondrial membranes occurs predominantly at contact sites, receptors and GIP may be concentrated in contact site regions of the outer membrane to ensure efficient and rapid translocation of precursor proteins. It might well be that, in addition, receptors and GIP are distributed over the entire mitochondrial surface in order to increase the probability for the initial high-affinity binding of precursor proteins and to collect the precursors for transfer to contact sites. This implies lateral diffusion of receptors and/or GIP in the outer membrane.

V. SUMMARY AND PERSPECTIVES

Functional characterization of initial steps of mitochondrial protein import provided the tools for identification of two mitochondrial outer membrane proteins, MOM19 and MOM72, as specific receptors for precursor proteins. Bound precursor proteins are transferred to a common membrane insertion site in the outer membrane, the "general insertion protein" (GIP).

Future research will address the role of functional domains of the receptors and the type and specificity of interaction with precursor proteins. The existence of (at least) two distinct membrane-bound receptors for precursor proteins appears to be of relevance for protein translocation across membranes in general; it may have implications on other organelles such as chloroplasts and the endoplasmic reticulum.

A GIP for the entry of precursor proteins into a membrane is most likely not only present in mitochondria, but may also be found in several other biological membranes. For instance, the SecY (PrlA) protein, an integral protein of the cytoplasmic membrane of *Escherichia coli* that is involved in export of proteins (Watanabe and Blobel, 1989; Wickner, 1989), might have a similar role.

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