Mitochondrial import receptors for precursor proteins

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The specific targeting of precursor proteins synthesized in the cytosol to various cell organelles is a central aspect of intracellular protein traffic. Several hundred different proteins are imported from the cytosol into the mitochondria. Recent studies have identified the mitochondrial outer membrane proteins MOM19, MOM72, MOM38 (≈ISP42) and p32 which have a role in initial steps of protein import. The first three components are present in a multi-subunit complex that catalyses recognition and membrane insertion of precursor proteins.

EUKARYOTIC CELLS are divided into a number of membrane-bound compartments, the organelles. The vast majority of proteins destined for the various compartments are encoded by nuclear genes and are synthesized as precursor proteins on cytosolic polysomes[1]. The selective transfer of precursor proteins into the organelles raises some fundamental questions. How are the precursor proteins directed to the correct target organelle? How are precursor proteins translocated into and across biological membranes that are naturally impermeable to macromolecules? Recently, it has become clear that the precursor proteins possess signal sequences that are decoded by receptor-like structures of the organelles. The precursors are inserted into the organelle membranes and are then partially or completely translocated across the membranes.

This review will focus on the biogenesis of mitochondrial proteins[2-5]. More than 95% of mitochondrial proteins are encoded by nuclear genes. After synthesis on cytosolic polysomes, the precursor proteins, many of them carrying a positively charged amino-terminal presequence, are post-translationally imported into mitochondria. The presequences contain targeting (signaling) information to direct the precursors into the organelle. After recognition by receptors on the outer membrane surface, the precursors are translocated into and across the membranes at sites of close contact between mitochondrial outer and inner membranes. In the mitochondrial matrix, the presequences are proteolytically removed by the mitochondrial processing peptidase. The proteins are sorted to their final submitochondrial destination and assembled into functional complexes.

Mitochondrial protein import has been analysed mainly in the fungus *Neurospora crassa* and the yeast *Saccharomyces cerevisiae*. Most studies utilized cell-free systems containing radiolabeled precursor proteins that were synthesized *in vitro* and isolated mitochondria. The general validity of the import mechanisms has been demonstrated in studies with mammalian mitochondria[6].

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Translocation intermediates of mitochondrial precursor proteins

An important step towards a molecular understanding of mitochondrial protein uptake was the generation of translocation intermediates on the import pathways of precursor proteins[3,4,7]. In particular, a reversible arrest of precursor proteins at the level of binding to the mitochondrial outer membrane was achieved by a combination of two procedures: (1) interference with the unfolding of precursor polypeptides by lowering the temperature in the import reaction or depleting the import system of ATP (ATP is probably required to release cytosolic cofactors from precursor proteins[8,9]); and (2) dissipation of the electrical potential ($\Delta\psi$) across the inner membrane, thereby preventing the insertion of precursors into the inner membrane.

As an example, Fig. 1 shows the import pathway of the ADP/ATP carrier, one of the most abundant mitochondrial proteins, into the inner membrane[10,11]. In the first step, the precursor protein interacts with a binding site on the mitochondrial surface. This is followed by insertion of the precursor into a saturable (proteinaceous) site in the mitochondrial outer membrane. This second step depends on the presence of ATP and a higher temperature. The precursor is then transferred into the mitochondrial inner membrane at areas of close contact between both membranes; an electrical potential ($\Delta\psi$) is required for the initial entrance of the ADP/ATP carrier into the inner membrane. Finally, the precursor is completely translocated into the inner membrane and functional dimers are assembled[12].

Competition studies with the precursor for porin, an outer membrane protein, revealed that nearly all precursor proteins analysed used the same site for insertion into the outer membrane [see (3) in Fig. 1]. This site was consequently termed the 'general insertion protein' (GIP)[7,13]. That the binding of the ADP/ATP carrier to its surface receptor was not competed for by the precursor of porin suggested the presence of at least two different receptor sites on the mitochondrial surface[7,13].

Identification and characterization of import receptors

For many years, all attempts to identify mitochondrial import receptors failed. This led to controversial views on the mechanism of mitochondrial protein import. The existence of binding sites with receptor function was suggested by the analysis of translocation intermediates[3,7]. Alternative proposals emphasized a predominant role of a hypothetical precursor–lipid interaction[14]. We applied a systematic approach towards the characterization of the 25 or so mitochondrial outer membrane proteins that can be resolved by SDS-PAGE (polyacrylamide gel electrophoresis). Monospecific antisera against those outer membrane proteins of *Neurospora crassa* mitochondria were generated and immunoglobulins G (IgGs) and F_{ab} fragments were prepared. IgGs or F_{ab} fragments were prebound to isolated mitochondria, and inhibition of the binding and import of various precursor proteins was analysed. Two outer membrane proteins of 19 kDa and

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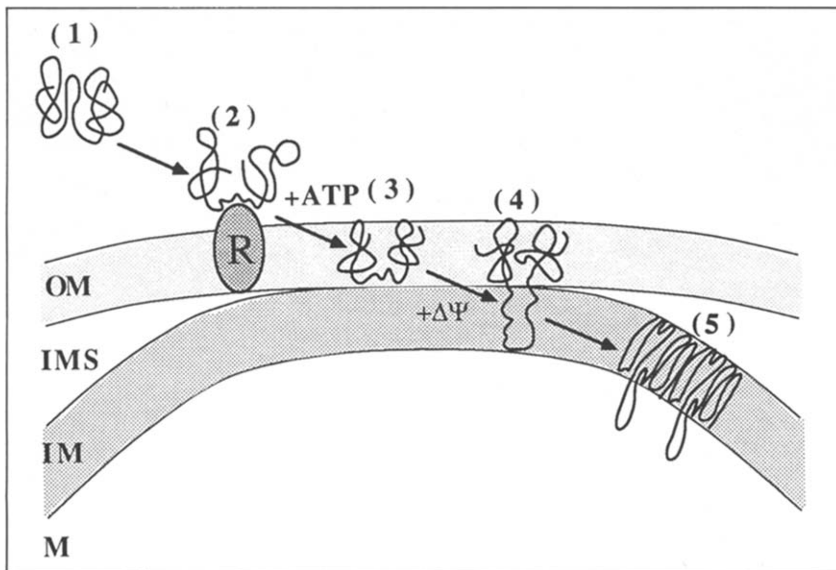

Figure 1
Translocation intermediates on the import pathway of ADP/ATP carrier into the mitochondrial inner membrane (modified from Refs 10, 11). (1) The ADP/ATP carrier is synthesized as precursor in the cytosol. (2) The precursor binds to a receptor protein (R) on the mitochondrial surface and (3) in an ATP-dependent step, is inserted into the outer membrane (OM). (4) Translocation into the inner membrane (IM) requires the electrical potential $\Delta\psi$ and is followed by (5) the assembly to the functional dimeric form. Intermembrane space, IMS; matrix, M.

72 kDa, termed MOM19 and MOM72, were identified that appeared to be components of the protein import machinery[15,16].

To identify the import steps involving MOM19 and MOM72 procedures that allow the generation of translocation intermediates were used (Fig. 1). Both proteins were found to function as receptors required for high-affinity binding of precursor proteins to the mitochondrial surface[15,16]. Covalent cross-linking of surface-bound precursors to MOM19 and MOM72 provided further evidence for a function as import receptors (T. Söllner, W. Neupert and N. Pfanner, unpublished results). Most precursor proteins analysed, including all presequence-carrying precursors, employed MOM19 as a receptor[15] (Fig. 2). The precursor of the ADP/ATP carrier, which contains multiple internal signal sequences, mainly used MOM72[16,17]. The precursor of the ADP/ATP carrier that accumulated on the mitochondrial surface, but not that imported, was found in a complex with MOM72 (as determined by its co-immunoprecipitation with MOM72 after lysis of mitochondria)[16].

A single isolated *Neurospora* mitochondrion carries roughly 50–200 MOM19 molecules and a similar number of MOM72 molecules (for comparison, porin, the most abundant outer membrane protein, has about 4000–8000 copies per mitochondrion). The localization of the receptors on the outer membrane was studied by electron microscopy after labeling with specific antibodies and protein A-gold particles, MOM19 and MOM72 are distributed over the entire mitochondrial surface with a slight (MOM19) or strong (MOM72) enrichment in contact sites[15,16]. About 50% of MOM72 molecules, but only about 15% of MOM19 molecules are present in contact zones which comprise 7–15% of the outer membrane surface. The primary structures of the two receptors were determined by DNA sequencing[17] (H. Schneider, T. Söllner, N. Pfanner and W. Neupert, unpublished). Both proteins possess a putative membrane-anchoring sequence at the amino terminus and a large hydrophilic domain (including the carboxyl terminus) that obviously protrudes into the cytosol. Further analysis of the receptor molecules is underway. At present, we know that treatment of mitochondria with the protease elastase generates a membrane-bound 17 kDa fragment of MOM19. This 17 kDa fragment is able to mediate the import of F_1-ATPase subunit β ($F_1\beta$), but not that of several other proteins studied, perhaps suggesting that different portions in the MOM19 molecule interact with various precursors[15]. Treatment of MOM72 with very low concentrations of protease releases a large soluble fragment of about 60 kDa that may contain the binding site for the ADP/ATP carrier[16].

By applying the procedure developed with *N. crassa* mitochondria, the import receptor MOM72 of yeast mitochondria was identified[17,18]. It was found that yeast MOM72 was identical to a 70 kDa outer membrane protein (MAS70) that had previously been used to study the biogenesis of mitochondrial outer membrane proteins without obtaining evidence for its function as import receptor[19]. Mitochondria from a yeast mutant lacking MOM72 are impaired in specific binding and import of the ADP/ATP carrier. However, a small amount (25–30%, compared to wild-type mitochondria) of the ADP/ATP carrier is still imported, apparently explaining the viability of the mutant cells[17,18]. The residual import predominantly occurs via MOM19. With *N. crassa* mitochondria depleted of MOM72 by a selective protease treatment, the residual import of the ADP/ATP carrier was inhibited by antibodies against MOM19 (Ref. 17). Moreover, a minor fraction of import of various precursors may depend on MOM72, although MOM72 is not essential for import of presequence-carrying precursors[17,18]. Thus both receptors may possess overlapping specificity (see Fig. 2).

As fusion proteins that consist of a mitochondrial presequence and a non-mitochondrial passenger protein use MOM19 as an import receptor, it is expected that the presequences interact with MOM19, which acts as a presequence receptor. However, it cannot be excluded that the mature part of a precursor protein also participates in the binding to MOM19. In any case, proteins that are synthesized without a cleavable presequence must contain targeting information in the mature part. For instance, internal sequences of the ADP/ATP carrier have to interact with MOM72 and MOM19.

In an elegant approach using anti-idiotypic antibodies, Blobel and colleagues[20] identified a 32 kDa yeast mitochondrial protein (p32) as a putative import receptor. Antibodies were raised against a mitochondrial presequence and the antigen-binding sites of these antibodies were assumed to mimic the binding sites of receptors (and other proteins interacting with

presequences). Antibodies prepared against the first antibody should thus recognize the binding sites of import receptors. These anti-idiotypic antibodies recognized the integral membrane protein p32 and two further proteins (p65 and p63) with unknown function. F_{ab} fragments directed against p32 inhibited import of precursor proteins into isolated mitochondria, and a complex between a precursor protein and p32 could be formed after lysis of mitochondria with detergent[20]. The location of p32 appears to be in mitochondrial contact zones. Unexpectedly, the primary sequence of p32 (deduced from the DNA sequence) was identical to that reported for the mitochondrial phosphate carrier, a protein of the inner membrane[21,22]. Moreover, in a similar approach Blobel and colleagues[23] identified a 36 kDa protein (p36) of the chloroplast envelope as an import receptor. The primary sequence of p36 turned out to be identical to the sequence of the phosphate translocator, an inner membrane protein of the chloroplast envelope[24]. Among the possible explanations for these surprising findings are: (1) that proteins exist with a dual localization in the outer and inner membranes, having a dual function; (2) protein families exist with members in different locations that possess similar epitopes for binding of antibodies; or (3) there is a cross-reactivity of antibodies between phosphate carriers and import components in the outer membrane. In support of the last explanation, MOM38, which is part of the general insertion protein (see below), contains a segment with similarity to a sequence in the phosphate carrier; MOM38 is about 30- to 100-fold less abundant than the phosphate carrier. A yeast mutant lacking p32 was unable to grow on non-fermentable carbon sources and was impaired, yet not fully blocked, in mitochondrial import of some precursor proteins[21]. The phenotype of the mutant is thus compatible with both lack of an import receptor and a defect in mitochondrial phosphate transport (leading to defects in mitochondrial energy metabolism) and does not clarify the issue[22].

Ono and Tuboi[25] purified a 29 kDa protein from rat liver mitochondria by its ability to bind to a chemically synthesized presequence. F_{ab} fragments against the 29 kDa protein inhibited import of precursor proteins, and the protein was suggested to be localized in membrane contact zones. A relation or even identity of this putative import receptor to p32 has to be proven. Gillespie[26] cross-linked a synthetic presequence to a 30 kDa membrane protein of rat heart mitochondria. The specificity of this cross-link approach is unclear, particularly as the 30 kDa protein turned out to be an ADP/ATP carrier[22], the most abundant mitochondrial membrane protein.

Role of the receptors in protein import

Why should mitochondria possess more than one import receptor and why do MOM19 and MOM72 show a complementary, yet overlapping specificity for various precursors? Our considerations will focus on these two receptors, as the properties of the p32/29 kDa protein were determined with other precursors and thus can not

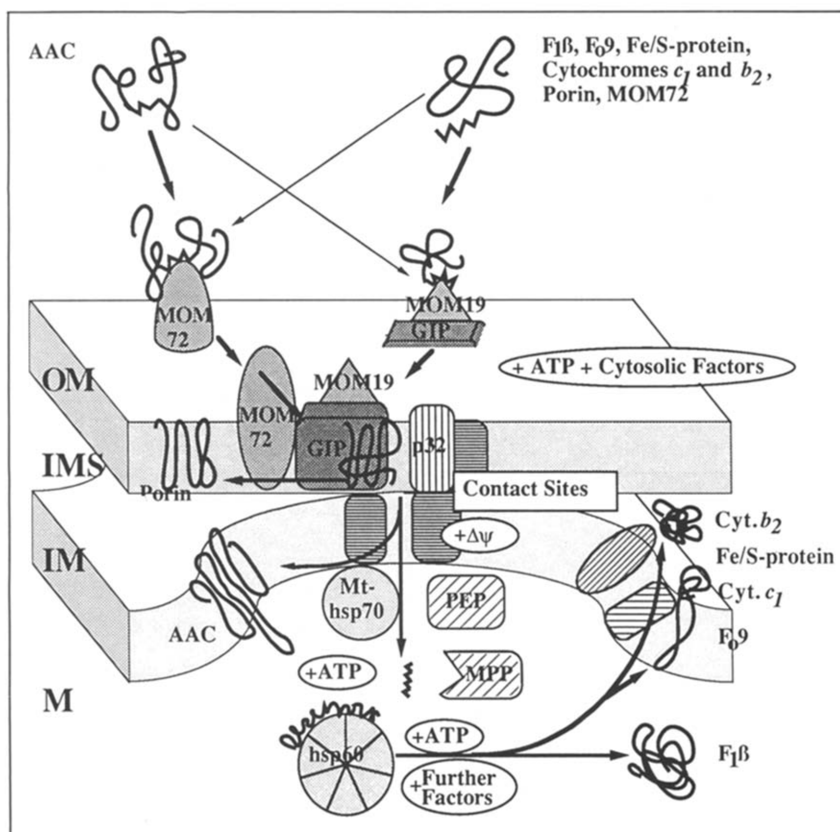

Figure 2

Hypothetical model of mitochondrial protein import. Precursor proteins synthesized on cytosolic polysomes are recognized by receptors on the mitochondrial surface. A complex between the receptor MOM19 and the general insertion protein, GIP, which is (at least partly) formed by the protein MOM38 (≈ISP42), collects most precursor proteins and inserts them into the outer membrane. The precursor of ADP/ATP carrier (AAC) predominantly uses MOM72 as receptor. MOM72, and also MOM19–GIP, are expected to show a lateral mobility in the outer membrane. Upon association of MOM72 with the MOM19–GIP complex, the AAC is donated to GIP and inserted into the outer membrane. GIP and the receptor complex are also involved in the translocation of precursor proteins through contact sites. Since p32 (and the possibly related 29 kDa protein of rat liver mitochondria) seems to be exclusively located in contact sites, it should function at this stage of the import pathway. The precursor proteins are supposed to be translocated through a proteinaceous channel; the membrane potential $\Delta\psi$ is required for the initial entrance of the presequences into the inner membrane. The mitochondrial heat shock protein of 70 kDa (mt-hsp70) binds to the precursor proteins emerging on the matrix side and thereby supports the completion of translocation[36]. The presequences are cleaved off by the enzyme mitochondrial processing peptidase (MPP, a protein of 51–57 kDa) in cooperation with the processing enhancing protein PEP (a protein of 48–52 kDa)[2,3,5]. Imported proteins destined for the matrix are refolded in association with the heat shock protein hsp60, while other proteins are retranslocated to the inner membrane or the intermembrane space (after interaction with hsp60)[3–5]. Abbreviations: AAC, ADP/ATP carrier; F_o9, F_o-ATPase subunit 9; $F_1\beta$, F_1-ATPase subunit β; GIP, general insertion protein/particle; IM, inner membrane; IMS, intermembrane space; M, matrix; MOM19, MOM72, mitochondrial outer membrane proteins of 19 kDa and 70–72 kDa, respectively; MOM38 (≈ISP42), mitochondrial outer membrane protein (import site protein) of 38–42 kDa; OM, outer membrane; p32, yeast mitochondrial membrane protein of 32 kDa.

be directly compared. Many proteins employing MOM19 as a receptor have homologous proteins in prokaryotic cells, suggesting that these proteins were already present in the prokaryotic ancestors of mitochondria, according to the endosymbiont hypothesis of mitochondrial origin. A targeting signal, usually present in an amino-terminal presequence, now directs the proteins from the cytosol via MOM19 into mitochondria[15]. Proteins such as the ADP/ATP carrier are unlikely to possess a prokaryotic equivalent and it is assumed that they are derived from the eukaryotic host cell[12]. It is possible that the internal signals in the ADP/ATP carrier initially recognized MOM19 predominantly until the more efficient high-affinity interaction with MOM72 was developed. In summary, MOM19 seems to function as a 'master receptor' for most mitochondrial precursor proteins while MOM72 has a more specialized role. Additionally, both receptors may function as back-up receptors for each other. The precursor of MOM72 employs MOM19 as its receptor, emphasizing the general role of MOM19 (Ref. 16). It is unknown which receptor, if any, is used by the precursor of MOM19 which, like all outer membrane proteins, is encoded in the nucleus and imported from the cytosol.

Do cytosolic cofactors play a role in the specific binding of precursor proteins to receptors, in analogy to the function of the signal recognition particle (SRP) in targeting precursor proteins to the endoplasmic reticulum? The purified precursor of porin did not require the addition of cytosolic factors for the efficient interaction with MOM19 and subsequent import[15], demonstrating that at least in this case cofactors were not essential for binding to the receptor. Moreover, p32 and the 29 kDa protein apparently interact directly with presequences without need for cytosolic factors[20,25]. Murakami and Mori[27] purified a 50 kDa cytosolic protein from rabbit reticulocyte lysate that interacts with presequences and stimulates protein import into isolated mitochondria. This presequence binding factor (PBF) is suggested to keep precursor proteins in an import-competent (loosely folded) conformation[27], probably in cooperation with heat shock proteins of 70 kDa (hsp70s) and further components[8,9,27–29]. A possible role of PBF in the targeting of precursors to mitochondrial surface receptors has not been examined so far.

We have previously described a pathway, termed the 'bypass import'[30,31], by which various precursor proteins can enter the mitochondrial import machinery at a post-receptor stage. This import route obviously lacks the specific recognition step. It also allows a low level of import of proteins carrying (positively charged) non-mitochondrial signal sequences, for example, a chloroplast signal sequence. In view of its very low efficiency, bypass import is probably of little significance under physiological conditions, in agreement with the generally observed high specificity of the bulk of mitochondrial protein uptake[30]. However, this residual import might represent a primitive form of protein import, allowing import with few constraints to the specificity of signals (the positively charged amino acid residues in the 'signal sequences' are probably required for responding to the electrical potential across the inner membrane[4]). A co-evolution of specific targeting signals and import receptors may have led to the selective and very efficient uptake mechanism via MOM19, MOM72 and further receptors.

An import pathway that is so far unique is that of apocytochrome *c*, the precursor of the intermembrane space protein cytochrome *c* (Ref. 3). Apocytochrome *c* possesses an endogenous activity to insert itself efficiently into lipid membranes and thus is independent of surface receptors. The covalent addition of heme by the enzyme cytochrome *c* heme lyase, which is exposed to the mitochondrial intermembrane space, traps the protein in this subcompartment.

A mitochondrial receptor complex and the general insertion protein

How are precursor proteins transferred from the receptors MOM19 and MOM72 to the general insertion site GIP (Ref. 13)? Upon lysis of mitochondria with non-ionic detergent, a high molecular weight complex was isolated that contained MOM19, MOM72, and two further proteins of 38 kDa and 22 kDa, termed MOM38 and MOM22 (Ref. 32). The two major components of this 'mitochondrial receptor complex' are MOM19 and MOM38, which are present in a molar ratio of roughly 1:1, whereas only a fraction of the MOM72 (and MOM22) molecules are found in the complex, in agreement with the (partially) differing distribution of MOM19 and MOM72 over the outer membrane. This receptor complex is not only involved in recognition of precursors, but also in membrane insertion and translocation of precursors. The ADP/ATP carrier accumulated at the GIP-site was co-purified with the receptor complex, indicating that GIP is present in the complex. MOM38 apparently is, or is part of, the GIP. MOM19, MOM22 and MOM72 are degraded by low concentrations of protease added to mitochondria, whereas the GIP (by functional criteria) and MOM38 (by intactness of the protein) show a high and identical protease resistance[32]. MOM19 would thus be in direct contact with GIP, allowing the rapid transfer of precursor proteins from the bound state (at MOM19) to GIP, thereby facilitating the insertion into the outer membrane.

We propose that the MOM19–MOM38 complex is dynamic in its location, diffusing laterally in the outer membrane and collecting precursor proteins from all over the mitochondrial surface, eventually delivering them to contact sites (Fig. 2). A similar dynamic location is expected for MOM72. MOM72 present in the receptor complex would represent the fraction of the receptors that are in the process of donating precursor proteins to GIP (i.e. MOM38) for insertion into the outer membrane[32].

It is unknown if the association of MOM72 and MOM19–MOM38 preferentially occurs in contact zones or elsewhere in the outer membrane. The function of MOM22 is unknown, yet its exposure on the mitochondrial surface would be compatible with a function in early stages of protein import. Since a fraction of precursor proteins arrested in mitochondrial contact sites are co-purified with the receptor complex, this complex may well form an initial part of the translocation machinery in contact sites[32]. Our working model includes that mitochondrial outer and inner membranes are kept together by structural components, while components of the import machinery diffuse into and out of contact sites with possible cycles of assembly and disassembly.

Chemical cross-linking to a precursor protein arrested in contact sites has identified a 42 kDa protein (ISP42) of yeast mitochondrial outer membranes[33]. This 42 kDa protein was not degraded by a pre-treatment of mitochondria with protease under conditions that are known to inactivate the surface receptors[3,19]. This 42 kDa yeast protein was suggested to be related to MOM38 of *N. crassa* and thus function as general insertion protein[4,13]. The primary se-

quences of MOM38 and ISP42 have recently been determined and are highly similar[32,34], indicating that ISP42 is the equivalent of MOM38. The yeast 42 kDa protein is an essential protein as disruption of its gene is lethal to the cells[34]. Yeast cells with decreased amounts of this protein were impaired in import of mitochondrial precursor proteins, confirming a function of ISP42 in protein import[34]. In light of these findings, the direct translocation of some precursors across the inner membrane of yeast mitochondria with a disrupted outer membrane appears to be an artifact of *in vitro* systems[35] and not relevant in the physiological import routes *via* the outer membrane.

In summary, two different approaches, association with import receptors in *N. crassa* and cross-linking to a precursor in yeast mitochondria, led to the identification of the same protein MOM38/ISP42. This emphasizes the central role of MOM38 in mitochondrial protein import.

Perspectives

The biochemical and molecular identification of mitochondrial import receptors now provides the basis for a detailed characterization of structure and function of the receptor molecules. This includes the definition of critical regions in receptors and precursor proteins required for high-affinity binding (it is still unknown which feature of presequences specifies their actual targeting function). Further studies will be directed towards an identification of additional putative import components in the outer membrane. The mechanism of transfer of precursor proteins to the general insertion protein and other transport components of the outer membrane and eventually the translocation through contact sites must be investigated. It will also be important to know whether the energy for membrane insertion of precursor proteins is derived from conformational changes or whether a supply of metabolic energy is necessary.

The mitochondrial import receptors may possess additional functions besides recognition of precursor proteins. The surprising relation of p32 to the phosphate carrier was discussed above. Moreover, the primary sequences of MOM19 and MOM72 revealed limited similarities of segments of the receptors to proteins with a putative or known cytoskeletal function [regions of similarity to the *CDC23* and *CDC16* gene products in MOM72 (Refs 17, 18), and to α-actinin in MOM19 (H. Schneider, T. Söllner, N. Pfanner and W. Neupert, unpublished)]. In addition to a possible evolutionary relationship, this may indicate that these mitochondrial surface proteins could be involved in the interaction of mitochondria with the cytoskeleton. We assume that both movement of mitochondria along cytoskeletal elements and self-recognition of individual mitochondria prior to fusion require specific components on the mitochondrial surface. MOM72 and MOM19 could therefore be of general importance for the interaction of a mitochondrion with its environment.

Acknowledgements

We are grateful to many colleagues for stimulating discussions and sharing unpublished information with us. Studies of the authors' laboratory were supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 184, project B1) and the Fonds der Chemischen Industrie.

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Solution to Lipsky Acrostic (September)

'. . . the scientist . . . is, in fact, generally a happy person, for he always has something to do. His work, moreover, sometimes brings satisfaction of a kind known by few others, when . . . he at his microscope or at the laboratory bench realizes that what lies before him no other man has ever seen.'

George W. Corner, *Anatomist at Large*, Basic Books, NY 1958, p. 62

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