Contact sites between inner and outer membranes: structure and role in protein translocation into the mitochondria

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Contact sites between both mitochondrial membranes play a predominant role in the transport of nuclear-coded precursor proteins into mitochondria. The characterization of contact sites was greatly advanced by the reversible accumulation of precursor proteins in transit (translocation intermediates). It was found that the sites are saturable, apparently contain proteinaceous components and mediate extensive unfolding of the polypeptide chain in translocation. Some components of mitochondrial contact sites are currently being identified.

Introduction

Most of the several hundred mitochondrial proteins are encoded by nuclear genes and are synthesized as precursor proteins in the cytosol [1–3]. Signal sequences, often found in amino-terminal presequences of the precursors, direct the proteins to mitochondria [4,5]. The precursors are recognized by specific receptor proteins on the mitochondrial surface; two receptors were identified recently and termed MOM19 and MOM72 (for mitochondrial outer membrane proteins of 19 and 72 kDa, respectively) [6,7]. The precursors are inserted into the outer mitochondrial membrane [8], and are translocated into and across the inner membrane mainly at sites of close contact between both membranes [9]. The entrance of precursors into the inner membrane depends on the membrane potential $\Delta\Psi$ across the membrane [10]. In the matrix, the inner soluble subcompartment of mitochondria, the presequences are proteolytically removed [11] and the imported proteins interact with the heat shock protein hsp60 in an ATP-dependent process [12]. Several precursor proteins are redirected into and across the inner membrane, thereby following the conserved sorting and assembly pathways that were already established in the prokaryotic ances-

tors of mitochondria [13,14]. Precursor proteins following this 'conservative sorting pathway' often possess a bipartite presequence the first part of which is cleaved off in the matrix and the second part directs the retranslation across the inner membrane.

Contact zones are the major site for import of precursor proteins, both of evolutionary conserved proteins [13] and of proteins that were probably introduced by the eukaryotic cell [15]. The structural and functional characterization of mitochondrial contact sites is thus of high importance in order to understand the import of mitochondrial proteins and the biogenesis of mitochondria in general. Here we summarize the progress made in recent years, mainly by the use of translocation intermediates.

Mitochondria possess sites of close contact between both membranes

From electron microscopic studies, it is well known that mitochondria possess zones of close contact between outer and inner membranes [16]. However, no specific function could be assigned to these sites. Butow and colleagues [17] found ribosomes associated with the cytosolic face of contact sites and concluded that precursor proteins were synthesized at these ribosomes and were imported at contact sites. It took about a decade until experimental evidence was found that precursor proteins were indeed imported at contact sites [9] (see below).

Contact sites occupy about 7–15% of the mitochondrial outer membrane surface [7,18]. As far as it can be

assessed by electron microscopy, the two membranes are not fused at contact sites. Rather two intact bilayers are always visible that are separated by an optically non-dense structure. The distance from the cytosolic side of the outer membrane to the matrix side of the inner membrane is about 18 nm [18]. By saturation of contact sites with precursor proteins it was calculated that about 100–5000 translocation sites were present in one isolated mitochondrion [18,19]. It is so far unclear as to whether the morphologically visible contact sites are part of a large and coherent network ('contact stripes') or whether multiple non-coherent contact sites exist.

Role of contact sites in import of precursor proteins

Various methods were developed for the accumulation of precursor proteins in mitochondrial contact sites: import of precursor proteins at low temperature [9,15,20]; lowering the levels of ATP in the import reaction [21]; prebinding of specific antibodies to portions of the precursor proteins [9,20]; and, induction of a stable tertiary structure in a domain of a precursor protein [18,19,22]. The underlying principle of these methods is that a (usually carboxyl-terminal) portion of a precursor protein is either not or only partially unfolded and thus cannot be inserted into the mitochondrial membranes. The remaining (amino-terminal) part of the precursor is inserted into the membranes and the presequence is cleaved off by the processing peptidase in the mitochondrial matrix. The hereby accumulated precursor proteins reach from the cytosolic side across outer and inner membranes into the matrix, demonstrating that the membranes must be in such close contact that they can be spanned by a single polypeptide chain. Labelling of precursor proteins accumulated in contact sites with specific antibodies and protein-A-gold particles confirmed that the biochemically characterized translocation contact sites were identical with the morphologically visible sites [20].

Most of the studies on protein import through contact sites were done in vitro by employing isolated mitochondria from fungal cells, *Neurospora crassa* and the yeast *Saccharomyces cerevisae*. Recently we could accumulate a precursor protein in contact sites in vivo. A hybrid protein between an amino-terminal portion of a mitochondrial precursor protein and the cytosolic enzyme dihydrofolate reductase (DHFR) was expressed in yeast cells and imported into mitochondria. By addition of a specific ligand a stable tertiary structure of the DHFR-part was induced in vivo and the precursor was trapped in contact sites. As expected, the entire DHFR remained on the cytosolic side whereas the amino-terminal part was inserted into the mitochondrial membranes and the presequence was processed in the matrix, demonstrating the importance of contact sites

for protein import in vivo (Wienhues, U., Griffiths, G., Becker, K., Schleyer, M., Guiard, B., Tropschug, M., Pfanner, N. and Neupert, W., unpublished data).

To obtain information on the conformation of precursor proteins in transit through contact sites, we again employed hybrid proteins carrying DHFR at the carboxyl-terminus. The DHFR-part was stabilized by binding of methotrexate and the precursor thus arrested in contact sites of isolated mitochondria. By use of a series of constructs with various lengths of the amino-terminal portion (that was derived from the precursor of mitochondrial cytochrome b_2) we found that about 50 amino acid residues were sufficient to span both mitochondrial membranes at contact sites. This unexpectedly low number of amino acid residues suggests a high degree of unfolding of the polypeptide chain in transit (Rassow, J., Hartl, F.-U., Guiard, B., Pfanner, N. and Neupert, W., unpublished data).

Only the initial entrance of a precursor protein into the inner membrane that is usually mediated by the positively charged presequence depends on the membrane potential, $\Delta\psi$. The completion of transport involving translocation of the major portion of the polypeptide chain can occur in the absence of $\Delta\psi$ [9,18]. This supports the model of an electrophoretic effect of the membrane potential on the presequences of precursor proteins or of a voltage-gated channel allowing transit of the presequence [10]. Import of most precursor proteins appears to require ATP on the cytosolic side, probably to confer a transport-competent conformation to the precursor proteins [23,24]. We recently found that the actual unfolding of precursor proteins and their translocation through contact sites is independent of the ATP levels. We conclude that ATP in cooperation with cytosolic cofactors is involved in preventing the misfolding of precursor proteins. The extensive unfolding of precursors during membrane translocation appears to be performed by the membrane-bound mitochondrial import machinery independently of ATP (Pfanner, N., Rassow, J., Guiard, B., Söllner, T., Hartl, F.-U. and Neupert, W., unpublished data).

Components of mitochondrial contact sites

By fractionation of mitochondria into submitochondrial vesicles, vesicle populations enriched in contact sites could be prepared, indicating that contact sites are stable structures [20,25]. In agreement with this, the calculated number of contact sites per one mitochondrion does not significantly change under various metabolic conditions [18,20]. Translocation contact sites are saturable with precursor proteins [18,19], and precursor proteins accumulated in contact sites apparently are embedded in a hydrophilic environment that is accessible to protein denaturants such as urea [26,27]. We propose that proteins represent essential compo-

nents of contact sites, both for the structural stability of contact sites and for the function of contact sites in translocation of precursors.

The localization of the import receptors MOM19 and MOM72 with regard to contact sites was analyzed by immunocytochemistry. While MOM19 was about equally distributed across the outer mitochondrial membrane [6], MOM72 was concentrated in contact site regions [7]. MOM72 that acts as import receptor for the inner membrane protein ADP/ATP carrier thus represents the first component with known function that is preferentially located in contact sites. A fraction of MOM19 and MOM72 molecules associate with a 38 kDa outer membrane protein (MOM38) to form a high molecular mass complex, termed the mitochondrial receptor complex (Pfaller, R., Söllner, T., Griffiths, G., Pfanner, N. and Neupert, W., unpublished data). MOM38 appears to be related to the general insertion protein (GIP) that is responsible for the insertion of precursors into the outer membrane [8]. The mitochondrial receptor complex might be preferentially located in contact site regions. The function of two proteins of 100 and 64 kDa that are also enriched in contact sites is unknown [25].

The import pathway of the intermembrane space protein cytochrome c is unique in most aspects. The precursor protein, apocytochrome c, inserts into the outer membrane without the aid of surface receptors or the general insertion protein [28,29]. The enzyme cytochrome c heme lyase that adds heme to the apoprotein on the intermembrane space side represents the only identified component of the import machinery for apocytochrome c [30,31]. Most interestingly, cytochrome c heme lyase appears to be located in or close to contact site regions although all available evidence suggests that there is no direct relation between the import pathway of apocytochrome c and that of precursors using surface receptors. Both, cytochrome c heme lyase and MOM72 seem to be enriched in submitochondrial vesicles containing contact sites (Hergersberg, C., Griffiths, G., Söllner, T., Nicholson, D.W., Stuart, R.A., Pfanner, N. and Neupert, W., unpublished data). These results indicate a general role of contact sites in the import of mitochondrial precursor proteins.

Perspectives

The purification of mitochondrial contact sites will be a most important aim in the near future. This should allow the identification and characterization of the various components that are probably required for the structure and function of contact sites. We speculate that structural proteins keep the two membranes in close contact and that the centre of contact sites is formed by specific proteinaceous pores that mediate the translocation of precursor proteins. The reconstitution of a functional translocation pore will be a major goal in the field of intracellular protein sorting. Components such as cytochrome c heme lyase are probably peripherally associated with the translocation machinery. We propose that contact sites represent the entry (and exit) gate of mitochondria with a possible involvement also in the transport of lipids, RNA and other molecules. The characterization of mitochondrial contact sites may thus lead us beyond the problems of protein sorting into numerous aspects of mitochondrial biogenesis and function in general.

Acknowledgements

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