minal Gal side chains are down-regulated on metacyclic LPG in favor of terminal Ara-containing tetrasaccharides, there are still significant amounts of terminal Gal within the trisaccharide repeats of metacyclic LPG. It is possible that the trisaccharide repeat units contribute little to the binding of either of the developmental forms because they are buried near the core anchor domains or are made inaccessible by adjacent tetrasaccharide repeat units that have more extended side chains. Alternatively, the trisaccharides may contribute to procyclic PG binding but be made inaccessible by elongation or rearrangement of the phosphoglycan during metacyclogenesis, or both.

If the molecular mechanism that controls interactions between *L. major* and *P. papatasi* also operates in other combinations of *Leishmania* and sandfly, then the LPG of other species could undergo similar modifications during metacyclogenesis. Developmental differences in LPG terminal side chain sugars have recently been found for *L. donovani* and *L. amazonensis* (*17*). Because the stage-specific sugars themselves tend to be species specific, their attachment sites within the fly might also vary, and it is these polymorphisms that could account at least in part for the species specificity of vectorial capacity observed in nature.

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Reversal of the Orientation of an Integral Protein of the Mitochondrial Outer Membrane

Jian-Ming Li and Gordon C. Shore

The NH_2-terminus of the signal-anchor sequence of an integral, bitopic protein of the outer mitochondrial membrane was extended both in amino acid length (from 11 to 38 amino acids) and net charge (from +4 to +8)—changes that confer on the NH_2-terminus characteristics of a strong matrix-targeting signal. The protein was inserted into the outer membrane but in an inverted orientation (N_{cyto}-C_{in}). These findings suggest that, in common with other membrane systems, the orientation of a protein in the outer mitochondrial membrane can be determined by a signal that causes retention of the NH_2-terminus on the cytosolic side of the membrane.

We have focused on a simple bitopic integral protein of the outer mitochondrial membrane in yeast, OMM70 (also called MAS70) (*1*, *2*). The topogenic information in OMM70 resides within a stretch of 29 amino acids at the NH_2-terminus, which anchors the protein in the outer membrane by a predicted 19–amino acid transmembrane segment (amino acids 11 through 29) in the N_{in}-C_{cyto} orientation, where the NH_2-terminus is in the mitochondrion and the COOH-terminus is in the cytoplasm (*1*, *3*). A hybrid protein, pOMD29, was created by fusing amino acids 1 through 29 of OMM70 in-frame to a cytosolic reporter protein, dihydrofolate reductase (*4*) (Fig. 1). The protein pOMD29 was efficiently imported into the outer membrane of intact mitochondria with the expected transmembrane orientation (N_{in}-C_{cyto}) (*3*, *4*). It did not target to endoplasmic reticulum (ER) microsomes. The transmembrane segment (amino acids 11 through 29) was essential for targeting, whereas the positively charged amphiphilic NH_2-terminus contained little targeting information for import but cooperated with the transmembrane segment to enhance the overall efficiency of targeting and insertion (*4*).

We suggest that the topogenic domains of pOMD29 (OMM70) operate as the functional equivalent of the signal-anchor sequence (*5*) found in type II and type III proteins (*6*) inserted into the ER, in which the signal (targeting) sequence is coincident with, or overlaps, the membrane anchor segment. An important consequence of a signal-anchor function is that the sequence that specifies targeting and initial translocation across the membrane is also the sequence that abrogates this process and results in lateral release of the segment to the surrounding lipid bilayer (*7*). Proteins like OMM70 contain a signal-anchor sequence selective for the outer membrane; this allows for the analysis of determinants (*8–10*) that specify transbilayer orientation of the protein.

Import of pOMD29 into the outer membrane of intact mitochondria from rat heart was dependent on adenosine triphosphate (ATP) (Fig. 2) and protease-sensitive surface components (*3*). After centrifugation at the end of import incubations, input pOMD29 sedimented only in the presence of mitochondria (Fig. 2), and, of the mito-

Department of Biochemistry, McIntyre Medical Sciences Building, McGill University, Montreal, Canada H3G 1Y6.

chondrial-bound fraction, a large amount was resistant to extraction by 0.1 M Na_2CO_3 (pH 11.5) (Fig. 2), which is indicative of a protein that is integrated into a membrane lipid bilayer (*11*). In contrast, both the matrix-located mature form and the surface-bound precursor form of pre–ornithine carbamyl transferase (pOCT) after import in vitro were completely extracted by this procedure (Fig. 3) (*12*). The bulk of the pOMD29 polypeptide after insertion into the outer membrane was accessible to exogenous trypsin (Fig. 2), which would be expected if the protein retained the N_{in}-C_{cyto} orientation of OMM70. Under identical conditions, trypsin did not detectably reduce the activity of succinate cytochrome c oxidoreductase (*13*) in the intermembrane space (*3*).

We substituted amino acids 1 through 11 of the signal-anchor sequence of pOMD29 with the NH_2-terminus of pOCT, which resulted in a significant increase in both length and net positive charge of the region upstream of the hydrophobic core of the signal anchor (Fig. 1). The NH_2-terminus of pOCT was chosen because it comprises a matrix-targeting signal (*14*), and the *Neurospora* homolog of OMM70 (MOM72) uses the same import receptor as that used by matrix-destined proteins (*15*). Nevertheless, to rule out the possibility that the potent matrix-targeting signal of pOCT might, in part, override the targeting information that resides in the hydrophobic core of the pOMD29 signal-anchor and result in more than one import pathway being followed in isolated mitochondria by the new protein (designated pO-OMD) (Fig. 1), we carried out import in the presence of carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) to collapse the electrochemical potential, $\Delta\psi$, across the mitochondrial inner membrane. Unlike the import of proteins destined for the inner membrane or matrix, import of all outer membrane proteins examined to date, including pOMD29 (*3*) (Fig. 2), did not require $\Delta\psi$ (*2*, *16*).

After import and insertion of pO-OMD into the outer membrane of intact mitochondria (Fig. 3), the protein demonstrated a very different sensitivity to exogenous trypsin compared to pOMD29. Whereas the bulk of the pOMD29 polypeptide was degraded by trypsin (Fig. 2), pO-OMD was only partially clipped to a polypeptide of slightly smaller size (Fig. 3). Both the trypsin-clipped and unclipped forms of imported pO-OMD were resistant to extraction by alkali (Fig. 3), which indicates that both forms were integrated into the lipid bilayer of the outer membrane. Resistance to exogenous trypsin was a direct consequence of import because complete sensitivity to trypsin was otherwise observed if mitochondria were withheld from import reactions (Fig. 3). Full-length pO-OMD, but not the trypsin-clipped form, was precipitated by an

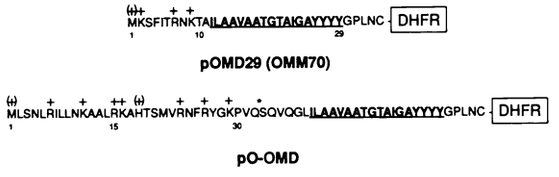

Fig. 1. NH_2-terminal sequence of pOMD29 and pO-OMD. The two proteins are encoded by pSP64 expression plasmids (*3*, *4*), in which the sequence encoding amino acids 1 through 11 of pOMD29 was replaced by 38 amino acids that contained the matrix-targeting signal of pOCT to create pO-OMD (*20*). The identical portion of the membrane anchor sequence in pOMD29 and pO-OMD is underlined. The asterisk denotes the site in the pOCT signal sequence where proteolytic processing would otherwise take place in the matrix (*21*). DHFR, dihydrofolate reductase; + indicates positive charge.

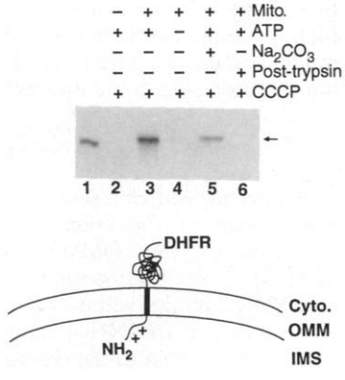

Fig. 2. Insertion of pOMD29 into the outer membrane of intact mitochondria in the N_{in}-C_{cyto} orientation. The translation product [^{35}S]pOMD29 in reticulocyte lysate was incubated with purified mitochondria (Mito.) from rat heart at 30°C for 30 min under standard import conditions (lanes 3 through 6) (*2*), either in the presence (lanes 3, 5, and 6) or absence (lane 4) of ATP (*18*). The reactions contained 1.0 μM CCCP. After import, reaction mixtures were treated on ice with (lane 6) or without (lanes 2 through 5) trypsin (0.2 mg/ml) for 20 min (*18*), layered over a 30% (w/v) sucrose cushion, and recovered by centrifugation. Mitochondria (lanes 3 through 6), or the membranes derived from the mitochondria after vigorous extraction with 0.1 M Na_2CO_3 (pH 11.5) (*11*) (lane 5), were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Lane 1, 20% of input [^{35}S]pOMD29; lane 2, import reactions lacking mitochondria. The arrow denotes pOMD29. The predicted topology of pOMD29 in the outer membrane is shown in the lower panel, in which the cytoplasm (Cyto.), outer mitochondrial membrane (OMM), and intermembrane space (IMS) are indicated. DHFR, dihydrofolate reductase.

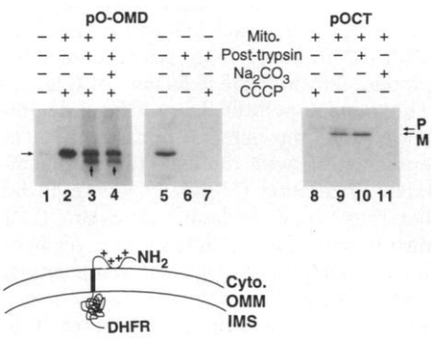

Fig. 3. Insertion of pO-OMD into the outer membrane of intact mitochondria in the N_{cyto}-C_{in} orientation. Import reactions that contained [^{35}S]pO-OMD (lanes 1 through 7) or [^{35}S]pOCT (*12*) (lanes 8 through 11), together with the various treatments, were carried out as described in Fig. 2. After centrifugation (lanes 1 through 4 and 8 through 11), pellets were analyzed by SDS-PAGE and fluorography. Alternatively, import reactions were carried out in the absence of mitochondria and treated with (lanes 6 and 7) or without (lane 5) trypsin, and aliquots were analyzed directly by SDS-PAGE without prior centrifugation. Horizontal arrow (left), full-length pO-OMD; vertical arrows, trypsin-clipped pO-OMD; arrows labeled P and M, precursor and mature forms of pOCT, respectively. The predicted topology of pO-OMD in the outer membrane is shown in the lower panel. Abbreviations are as in Fig. 2.

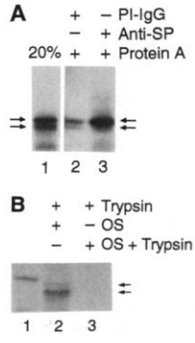

Fig. 4. Accessibility of newly imported [^{35}S]pO-OMD to exogenous trypsin. Import reactions were treated with trypsin (Figs. 2 and 3). (**A**) The trypsin-treated import products (Fig. 3) were incubated either with preimmune immunoglobulin G (PI-IgG) (lane 2) or with antibody against amino acids 1 through 27 of pOCT (anti-SP) (*12*, *17*) (lane 3). Immune complexes were recovered with immobilized protein A and analyzed by SDS-PAGE and fluorography (*12*). Lane 1, 20% of imported pO-OMD after treatment with trypsin. (**B**) After trypsin treatment, mitochondria were subjected to osmotic shock and brief sonication to rupture the outer membrane (*3*, *18*), in the presence of either trypsin (20 μg/ml) and soybean trypsin inhibitor (40 μg/ml) (OS) (lane 2) or trypsin (20 μg/ml) without inhibitor (OS + trypsin) (lane 3), incubated on ice for 10 min, and analyzed by SDS-PAGE and fluorography. Lane 1, 20% of input [^{35}S]pO-OMD. Upper and lower arrows in (A) and (B) designate full-length and trypsin-clipped pO-OMD (Fig. 3), respectively.

antibody directed against amino acids 1 through 27 of pOCT (*17*) (Fig. 4A). Clipping by trypsin, therefore, occurred at the NH_2-terminus of pO-OMD. However, rupture of the outer membrane of the mitochondria to allow access of trypsin to the intermembrane space (*4*, *18*) resulted in degradation of both the clipped and unclipped protein (Fig. 4B). Taken together, these results indicate that pO-OMD was inserted into the outer membrane of intact mitochondria in vitro in the N_{cyto}-C_{in} orientation, with most of the protein facing the intermembrane space.

The significance of the full-length, trypsin-resistant, and alkali-resistant pO-OMD (Fig. 3) is not clear. One possibility is that it represents a translocation intermediate in transit to the interior of the organelle. If so, it is surprising that it resists extraction by alkali. Alternatively, it may represent pO-OMD in the N_{cyto}-C_{in} orientation but where the NH_2-terminus partially intercalates into the phospholipid headgroups at the surface of the bilayer and remains shielded from trypsin. Previous studies of the pOCT signal sequence, for example, have shown that its amphiphilic character provides the peptide with high-affinity lipid surface-seeking properties (*19*).

The structural determinants that either cause retention of the NH_2-terminus of pO-OMD on the cytosolic side of the outer membrane during import in vitro or allow translocation of the NH_2-terminus of pOMD29 across the membrane, and whether or not the directionality imposed by the structural determinants is related to their strengths as matrix-targeting signals, remain to be determined. It also remains to be determined if retention of the NH_2-terminus on the cytosolic side of the membrane is the result of interactions with a specific membrane protein (receptor) or with the surface of the lipid bilayer or a result of some other process. Our findings are consistent with models generated for the ER and bacterial plasma membrane (*8–10*) in which orientation is determined by sequences that flank the hydrophobic core of a signal anchor (*8*), especially as this pertains to the presence or absence of a retention signal on the NH_2-terminal side of the hydrophobic core, which is made up in part of positively charged residues (*10*).

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Peptides Presented to the Immune System by the Murine Class II Major Histocompatibility Complex Molecule I-A^d

Donald F. Hunt,* Hanspeter Michel, Tracey A. Dickinson, Jeffrey Shabanowitz, Andrea L. Cox, Kazuyasu Sakaguchi, Ettore Appella, Howard M. Grey, Alessandro Sette

Between 650 and 2000 different peptides are associated with the major histocompatibility complex class II molecule I-A^d. Sequences for nine of these were obtained by a combination of automated Edman degradation and tandem mass spectrometry. All of the peptides are derived from secretory or integral membrane proteins that are synthesized by the antigen-presenting cell itself. Peptides were 16 to 18 residues long, had ragged NH_2- and COOH-termini, and contained a six-residue binding motif that was variably placed within the peptide chain. Binding data on truncated peptides suggest that the peptide binding groove on class II molecules can be open at both ends.

Recognition of peptide fragments by T lymphocytes is a central event in the immune response. Peptides are presented to the immune system in association with either class I or class II molecules of the major histocompatibility complex (MHC). Assembly of the peptide-MHC molecule complexes takes place in two different intracellular compartments (*1*, *2*). Peptides that are derived from the degradation of cytosolic proteins and transported into the endoplasmic reticulum are found in complexes with MHC class I molecules (*3–7*). Peptides derived mainly from exogenous proteins that enter the cell by phagocytosis, endocytosis, or internalization of the cell membrane (*8–11*) are processed in acidic endosomal compartments and become associated with MHC class II molecules.

D. F. Hunt, H. Michel, T. A. Dickinson, J. Shabanowitz, A. L. Cox, Department of Chemistry, University of Virginia, Charlottesville, VA 22901.
K. Sakaguchi and E. Appella, Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, MD 20892.
H. M. Grey and A. Sette, Cytel Corporation, 3525 John Hopkins Court, San Diego, CA 92121.

*To whom correspondence should be addressed.

Sequences for twelve 13- to 17-residue peptides eluted from class II I-A^b and I-E^b molecules (*12*, *13*) were determined by a combination of high-performance liquid chromatography (HPLC) and automated Edman degradation. Each sequence was derived from a cell membrane glycoprotein or from protein present in the cell growth medium (*12*). Several of the peptides had the same core determinant and the same NH_2-terminus but varied in length and thus had different COOH-termini. Surprisingly, three of the four parent proteins contributed 50 to 75% of the total number of peptides associated with each of the class II MHC molecules. If this result were typical of antigen-presenting cells, it would suggest that a relatively small number of self peptides participate in the selection process for the development of immature T cells.

We now present data on peptides from another class II molecule, I-A^d, obtained by reversed-phase HPLC and the combination of microcapillary HPLC–electrospray ionization–tandem mass spectrometry (*14*). This latter methodology defines the approximate number, quantity, and molecular