SUPPLEMENTARY ONLINE DATA
Membrane integration of a mitochondrial signal-anchored protein does not require additional proteinaceous factors

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EXPERIMENTAL

Isolation of mitochondria by enzymatic spheroblastation

Preparation of crude mitochondria from cultures grown in lactate medium was performed as described previously [1]. Cells were harvested (3000 *g*, 5 min, 20°C), washed once in water, resuspended in 2 ml/g of cell weight of resuspension buffer [100 mM Tris base and 10 mM DTT (dithiothreitol), pH 9.4] and sedimented again. Cells were then washed with spheroblasting buffer (1.2 M sorbitol and 2 mM potassium phosphate buffer, pH 7.2) and cell walls were subsequently digested at 30°C for 45 min in 6 ml/g of cell weight of the spheroblasting buffer supplemented with 1.1 mg/ml zymolyase (Seikagaku). All further steps were performed on ice or at 4°C. Spheroblasts were collected by centrifugation (2000 *g*, 5 min) resuspended in 100–200 ml of homogenization buffer (0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF, 0.2% fatty-acid-free BSA (Sigma), 10 mM Tris/HCl, pH 7.4) and lysed by ten strokes in a tight fitting Potter glass-glass homogenizer. Cell lysates were clarified by two centrifugation steps as described before and mitochondria were sedimented by centrifugation (18 000 *g*, 12 min). Mitochondrial pellets were resuspended in 30 ml of SEM buffer (250 mM sucrose, 1 mM EDTA and 10 mM Mops-KOH, pH 7.2) containing 2 mM PMSF and clarified twice by centrifugation (2000 *g*, 5 min). Mitochondria were re-isolated by centrifugation (18 000 *g*, 12 min), resuspended in SEM buffer, aliquoted, snap-frozen in liquid nitrogen and stored at −80°C.

CD spectroscopy

The CD spectra of the peptide were recorded in an Aviv 202 spectropolarimeter in a thermostatically controlled quartz optical cell with a path length of 1 mm. Spectra were recorded at a wavelength range of 190–260 nm at 1 nm intervals with an average time of 6 s and three repetitions. The peptide was scanned at a concentration of 10 μM in two different environments: 5 mM Hepes buffer (pH 7.4), and the above buffer with 1% LPC. The signals of the buffer and 1% LPC before adding the peptide were subtracted from the signals after peptide addition.

Polarized ATR-FTIR analysis of the peptides

To determine the orientation of the peptide in lipid multibilayers, we used polarized ATR-FTIR spectroscopy. Spectra were recorded with a Bruker equinox 55 FTIR spectrometer equipped with a deuterated triacylglycerol sulfate detector coupled to an ATR device as described previously [2]. Briefly, prior to sample preparations, the TFA counter ions, which associate with the peptides, were replaced with chloride ions through several freeze-drying steps of the peptides in 0.1 M HCl. After the ion exchange, a mixture of phospholipid (1 mg) alone or with peptide (100 μg) was deposited on a germanium prism. The aperture angle of 45° yielded 25 internal reflections. Lipid/peptide mixtures were prepared by dissolving them together in a 1:2 MeOH/CH$_2$Cl$_2$ mixture and drying under vacuum for 15 min. Polarized spectra were recorded and the respective spectrum of pure phospholipid in each polarization was subtracted to yield the difference spectra in order to determine the amide I absorption peaks of the peptide. For each spectrum 60 scans were collected with a resolution of 4 cm^{-1}.

ATR-FTIR data analysis

Prior to curve fitting, a straight baseline passing through the ordinates at 1700 and 1600 cm^{-1} for the peptide, or 2800 and 3000 cm^{-1} for the lipids was subtracted. To resolve overlapping bands, we processed the spectra using PEAKFIT software (Jandel Scientific). Second-derivative spectra were calculated to identify the positions of the component bands. These wavenumbers were then used as initial parameters for curve fitting with Gaussian component peaks. Positions, band widths and amplitudes of the peaks were varied until (i) the resulting bands shifted by not more than 2 cm^{-1} from the initial parameters, (ii) all of the peaks had reasonable half-widths (<20–25 cm^{-1}) and (iii) there was good agreement between the calculated sum of all the components and the experimental spectra ($r^2 > 0.99$). The ATR electric fields of incident light were calculated as follows [3]:

$$E_x = \frac{2\cos\theta\sqrt{\sin^2\theta - n_{21}^2}}{\sqrt{(1 - n_{21}^2)[(1 + n_{21}^2)\sin^2\theta - n_{21}^2]}}$$

$$E_y = \frac{2\cos\theta}{(1 - n_{21}^2)}$$

$$E_z = \frac{2\sin\theta\cos\theta}{\sqrt{(1 - n_{21}^2)[(1 + n_{21}^2)\sin^2\theta - n_{21}^2]}}$$

where θ is the angle of incidence between the light beam and the prism normal (45°) and n_{21} is the reflective index of the Ge

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(taken to be 4.03) divided by the reflective index of the membrane (taken to be 1.5). Under these conditions, E_x, E_y and E_z are 1.40, 1.52 and 1.64 respectively. The electric field components together with the dichroic ratio (defined as the ratio between absorption of parallel ($A_\parallel$) and perpendicular ($A_\perp$) polarized light, $R^{\text{ATR}} = A_\parallel / A_\perp$ are used to calculate the orientation order parameter, f, by the following formula:

$$R = \frac{A_\parallel}{A_\perp} = \frac{E_x^2}{E_y^2} + \frac{(E_z^2/E_y^2)(f\cos^2\alpha + (1-f)/3)}{(f\sin\alpha)/2 + (1-f)/3}$$

where α is the angle between the transition moment of the amide I vibration of the α-helix and the helix axis. We used the value of $27°$ for α as was previously suggested [3,4]. The orientation order parameter f allows calculating the 'average' angle of the peptide α-helices relative to the membrane normal by the following formula:

$$f = \frac{1}{2}[3(\cos^2\gamma)] - 1$$

Lipid order parameters were obtained from the lipid symmetric ($2850\,\text{cm}^{-1}$) and asymmetric ($2921\,\text{cm}^{-1}$) stretching mode using the same equations differing only by setting $\alpha = 90°$ [3].

Fluorescence measurements with a stopped-flow setup

The kinetics of peptide binding to LUVs was measured in a stopped-flow fluorimeter. The measurements were done at 25 °C with a slit width of 5 nm. The λ_{ex} was 467 nm and emission was detected using a cut-off filter of 520 nm. Every reaction was repeated at least six times, and the average signal was considered as the representative signal for the reaction. The association rate constants were measured in PBS under pseudo-first-order rate conditions. The data were fitted using a double exponent equation:

$$F(t) = \Delta F_1 \exp(^-k_{\text{obs1}}t) + \Delta F_2 \exp(^-k_{\text{obs2}}t) + F_\infty$$

where k_{obs1} and k_{obs2} are the observed rate constants for the first and second components of a double-exponential reaction and ΔF_1 and ΔF_2 are the amplitudes for the first and second components of a double-exponential reaction.

MC simulations

MC simulations of the OM45-SA peptide and its double mutant R4E, K26E were performed using the MCPep server (available online at http://bental.tau.ac.il/MCPep/). The membrane was represented as a smooth hydrophobic profile of native width of 30 Å (1 Å = 0.1 nm), corresponding to the hydrocarbon region. A negative surface charge was located on both sides of the membrane at a distance of 20 Å from the midplane. Its magnitude was estimated based on the relative fraction of charged lipids in the mitochondrial OM, i.e. 13 % (PI) + 2×4 % (CL) + 2 % (PS) = 23 %. The membrane was embedded in an aqueous solution of 0.1 M monovalent salt and pH 7.0, corresponding to physiological
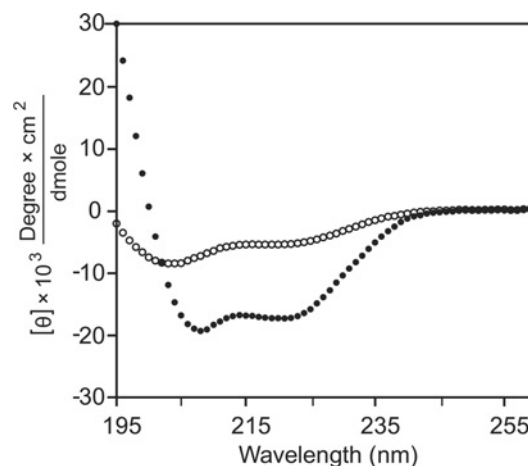
Figure S1   CD spectra of the OM45-SA peptide

Freeze-dried samples of peptide were dissolved directly in buffer (pH 7.4) (open circles) or in buffer containing 1 % (w/v) LPC (closed circles). The spectra were taken at peptide concentrations of 10 μM.

Table S1   Peptide secondary structure in buffer (pH 7.4) and in buffer + 1 % LPC determined by CD spectroscopy

	Secondary structure (%)	
	Buffer	Buffer + 1 % LPC
α-Helix	17	58
β-Sheet	32	9
Random coil	33	18
Other	18	15

conditions. The initial peptide structure was constructed as a canonical α-helix and placed either in TM orientation with its principle axis roughly along the membrane normal or surface orientation with its axis roughly in the membrane surface. The MCPep server calculated the free energy of each configuration in comparison with the free energy in the aqueous phase.

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