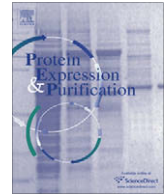




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Single-step production of functional OEP24 proteoliposomes

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ABSTRACT

The pea chloroplastic outer envelope protein OEP24 is a voltage-dependent channel that can function as a general solute channel in plants. OEP24 is a close functional homologue of VDAC which, in mammalian cells, modulates the permeability of the outer mitochondrial membrane. Here, we describe the production in a one-step reaction of active OEP24 in proteoliposomes or in soluble form using a cell-free expression system. We combine evidence from electrophysiological experiments, biophysical characterization, and biochemical analysis demonstrating that OEP24 is present as a functional channel in liposomes. Thus, production of OEP-containing proteoliposomes may provide a helpful tool for deciphering the role of the OEP family members.

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Introduction

Channel proteins are trans-membrane proteins of diverse architectures found in all kingdoms. The ancestral relationship between Gram-negative bacteria, plastids and mitochondria is attested by the presence of similar porin-like channel proteins, such as OEP24 and VDAC, in their outer membranes [1–3].

Chloroplasts are peculiar plants organelles of prokaryotic origin representing a barrier for the trafficking of proteins and metabolites. A number of specific and highly regulated proteins of the outer envelope (OEP)¹ from pea chloroplasts have been identified with various functions for the diffusion of distinct solutes and in metabolic processes. OEP members are a growing β -barrel group of membrane proteins which includes OEP16, OEP21, OEP24 and the recently described OEP37. Each of them plays a specific role in the chloroplast metabolism and is characterized by a particular electrophysiological profile. They represent high-conductance solute channels with different specificities. OEP16 forms a cation-selective channel permeable to amino acids and amines, and has been involved in the NADPH: protochlorophyllide (Pchl) oxidoreductase S (PORA) import in chloroplasts of *Arabidopsis thaliana* [4]. In contrast, OEP21 forms an anionic channel regulated by triose phosphate and ATP coming from the intramembrane space [5].

OEP37, the latest identified member of the family, is a cation-selective, high-conductance channel, which is sensitive to peptides and involved in seed development and germination [6].

OEP24 was identified and characterized as a 24 kDa anionic channel containing seven putative amphiphilic β -strands. OEP24 forms homodimers in its active state and acts as a general solute channel with behaviour similar to that described for porins of gram-negative bacteria and mitochondria [7]. Differently from other OEPs involved in translocation of proteins and amino acids trafficking, OEP24 is the unique non-selective channel described in the outer membrane of chloroplasts [7].

Interestingly, recognizing the similar channel features between OEP24 and mitochondrial VDAC proteins, Röhl and colleagues [8] demonstrated that transformation of yeast lacking the endogenous VDAC1 with OEP24 gene, leads to the recovery of the wild type phenotype. This complementation indicates that both proteins may play some similar functions and that, surprisingly, OEP24 can be expressed and targeted to the yeast mitochondria. To further characterize the biochemical properties of OEP24, and its potential as a mitochondrial target, we decided to exploit an optimized cell-free expression system to produce proteoliposomes containing recombinant OEP24.

Production of membrane proteins (MPs) by classical over-expression systems represents a tremendous problem due to the highly hydrophobic content of the proteins. We previously demonstrated that cell-free expression systems is adequate for rapid production of active recombinant soluble membrane proteins or proteoliposomes containing membrane proteins [9–11]. In this study, we first over-express OEP24 in the presence of synthetic liposomes or detergent and then, demonstrate that the protein

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¹ Abbreviations used: OEP, outer envelope protein; PORA, protochlorophyllide (Pchl) oxidoreductase S; MPs, membrane proteins; DDM, *n*-dodecyl- β -D-maltoside; CD, circular dichroism; BSA, bovine serum albumin.

produced is functional as a channel and organized in the right conformation. The proteoliposomes are internalized into mammalian cells leading to caspase 9 activation indicating apoptosis activation via the mitochondrial pathway. Furthermore, our results suggest the possibility to use this approach as a general strategy for the characterization of biochemical properties of pore proteins.

Materials and methods

Recombinant expression of OEP24 proteoliposomes and soluble OEP24

Full-length OEP24 cDNA was cloned into the pVEX 2.4b-NdeI vector (Roche Applied Science) using NdeI and XhoI restriction sites. To express recombinant OEP24, a RTS 500 *Escherichia coli* HY kit (Rapid Translation System, Roche Applied Science) and the ProteoMaster device (Roche Applied Science) was used according to the protocol for 1 ml reaction [12].

To synthesize liposomes containing OEP24 (L-Os), synthetic liposomes were directly added to the RTS reaction mixture at 1:1 ratio (v/v, 5 mg/ml lipid final concentration). Synthetic lipids: were prepared as described in [11,12] and were dissolved in CHCl₃/MeOH (1:1). Liposomes were obtained by evaporation of CHCl₃/MeOH (SpeedVac System, Thermo Savant) and the lipid film resuspended in DEPC-treated water at the final concentration of 10 mg/ml. Sonication (3 times for 1 min on ice; Branson Sonic Power, Smithkline Company Brentford, Middlesex, UK) and filtration through a 0.20 µm filter led to liposome constitution.

Production of soluble OEP24 is performed by adding 2.5 mM of *n*-dodecyl-β-D-maltoside (DDM) to the reaction mix.

Purification of OEP24 proteoliposomes and soluble OEP24

After 72-h reaction in the ProteoMaster device (Roche Applied Science) settled at 990 rpm, 25 °C, the mixtures containing L-Os and soluble OEP24 in DDM were centrifuged at 13,000g, 20 min at 4 °C. For L-Os the supernatant was discarded and the pellet, containing proteoliposomes, was resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5); for the soluble OEP24, the supernatant with the soluble recombinant protein was kept for purification and the pellet, rich in insoluble proteins from the bacterial lysate, was discarded.

To purify L-Os, the resuspended pellet was loaded onto a discontinuous sucrose gradient and processed as previously described [11,12]. Briefly: 60%, 25% and 10% sucrose solutions were prepared in 50 mM Tris, pH 7.5. After ultracentrifugation for 1 h at 200,000g at 4 °C (Beckman L2 65B ultracentrifuge, rotor SW2) 1-ml fractions are collected from the top to the bottom of the gradient. The gradient fractions were then loaded onto a 15% polyacrylamide gel, transferred on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and analyzed by Western blotting using a monoclonal anti-HRP-conjugated antibody (Sigma Aldrich, 1:5000 in TBS-T 0.1%, milk 4%). Fractions' purity was checked by coomassie blue staining [12].

Purification of soluble OEP24 was performed using MagneHis (Promega) and the MagneSphere Magnetic Separation Stand (Promega). Beads (300 µl of beads solution per 1 ml reaction) are pre-equilibrated with 50 mM Tris-HCl, pH 7.5, 0.5 mM DDM; (Tris-DDM) for 10 min at 4 °C before the reaction mixture addition. After a 1-h incubation at 4 °C on a rotating wheel, the complex was washed (5 min at 4 °C) first in Tris-DDM, then in Tris-DDM containing 20 mM imidazol, and in Tris-DDM containing 50 mM imidazol. Recombinant soluble OEP24 was eluted first in Tris-DDM containing 0.5 M imidazol and 0.5 M NaCl, and then in Tris-DDM containing 1 M imidazol and 0.5 M NaCl. Elutions were dialyzed O/N against PBS, 0.5 mM DDM at 4 °C to remove

NaCl and imidazol. The yield and the fractions purity of each preparation were checked by coomassie blue staining as previously described [9].

Circular dichroism of soluble OEP24 and OEP24 liposomes

Circular dichroism (CD) experiments were performed on a Jobin Yvon CD6 circular dichroism spectropolarimeter. Data were recorded between 190 and 260 nm at 25 °C with an interval of 1 nm and an integration time of 8 s, using cells of 0.1 cm. The protein concentration was 0.2 mg/ml. Each spectrum was corrected for buffer containing 0.5 mM DDM for soluble OEP24 and with empty liposomes for the proteoliposomes samples.

Giant proteoliposomes preparation

Giant proteoliposomes were prepared as described [13] with a few modifications. 10 µl of L-Os were added to a 2 ml solution of 250 mM KCl, 1% octylglucoside, 1 mg/ml alectin, 10 mM Hepes (pH 7.4). After 20 min equilibration at room temperature, 320 µg of SM2 Bio-Beads (Bio-Rad) were added to the solution and incubated overnight at 4 °C to remove the detergent. The Bio-Beads having settled to the bottom, the supernatant was removed and centrifuged 25 min at 90,000 rpm. The pellet was resuspended in 15 µl 10 mM Hepes (pH 7.4). To form giant liposomes, a single cycle of dehydration/rehydration was performed. Rehydration was done in 250 mM KCl, 10 mM Hepes (pH 7.4).

Patch clamp recordings

A 7-µl sample of giant liposomes was deposited in a Petri dish used as a patch clamp chamber. The chamber was then filled with 7 ml of 250 mM KCl, 10 mM Hepes (pH 7.4). Channel activity was recorded using the patch clamp technique in the inside-out conformation [14]. Patch pipettes were filled with a solution containing 250 mM KCl, 5 mM MgCl₂, 2 mM CaCl₂ and 10 mM Hepes (pH 7.4). The currents were recorded using a Biologic RK-300 amplifier, filtered at 300 Hz, sampled at 1 kHz and analyzed with Clampfit 10 (Axon).

Analysis of caspase 9 activation

HCT116 (human colon carcinoma cells) were cultured in 6-well plates in McCoy's 5A medium (GIBCO, Invitrogen Corporation) modified with fetal bovine serum, 10% and incubated with different concentrations of L-Os. After 24 h treatment, cells were washed twice in PBS and suspended in 100 µl of FastBreak Cell Lysis reagent (Promega, Madison, WI, USA) with protease inhibitors (Complete Mini protease cocktail, Roche, IN) and incubated for 30 min on ice. Cells were then centrifuged at 12,000g for 15 min at 4 °C and the supernatant recovered. Protein concentration was determined with a colorimetric dye-binding assay (BCA Assay, Pierce, Rockford, IL, USA). For Western blot analysis, aliquots containing 50–75 µg of proteins were separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked with 4% milk in 0.1% TBS-Tween (TBS-T) for 1 h at RT, incubated with primary antibodies recognizing the cleaved forms of caspase 9 (1:500 in PBS-Tween 0.1%, 5 mg/ml BSA, Calbiochem, CA), and subsequently incubated with an anti-rabbit peroxidase-labelled secondary antibody (Amersham Corp., Arlington Heights, IL, USA) 1:10000 in TBS-T. Positive immunoreactive bands were detected using the Lumi-Light chemiluminescence kit (ECL, Amersham, Buckinghamshire, UK).

Evaluation of cell viability: MTT assay

The MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the yellow MTT and form dark blue formazan crystals which are accumulated within healthy cells. 5000 HCT116 cells were seeded in a 96-well plate and treated 24 h with L-Os (0.18; 0.35; 1.07 and 2.08 μ M). MTT was then added to each well (100 μ g) and cells were incubated for a further 5 h at 37 °C. The medium was removed and DMSO (200 μ l) added to each well to break cells and liberate the solubilised blue crystals. After 5 min of gentle shaking, the colour was quantified at 562 nm using a multiwell scanning spectrophotometer (Multiscan Spectrum, Thermo Labsystem). Differences between samples and treatments were evaluated by variance analysis (two-tails, paired values) followed by the protected least-significant difference test. An asterisk (*) indicates a difference statistically significant with $P \leq 0.05$ compared to the control.

Results and discussion

In vitro expression of OEP24 and proteoliposomes

OEP24 belongs to a class of proteins located at the outer chloroplast membrane. While various inner-envelope carrier proteins show distinct substrate specificity, outer-envelope channels apparently lack such specificity and it remains unclear how transport is regulated through the outer membrane. In chloroplasts, the total number of proteins indispensable for plastid metabolism is partially unknown even though recent proteomics studies have highlighted new members of chloroplast proteins [15,16]. We decide to test the expression of full-length OEP24 from pea chloroplast using our optimized heterologous *E. coli* cell-free protein synthesis system. The recombinant tagged OEP24 protein expressed in absence of liposomes or detergent was easily detectable by Western blotting using a monoclonal anti-his antibody (Fig. 1A). When produced in the presence of liposomes (Fig. 1B) or DDM (Fig. 1C), the yield of purified recombinant OEP24 proteoliposomes or soluble OEP24 ranged between 0.2 and 0.5 mg/ml. To produce

L-Os in a one-step reaction, synthetic liposomes (a mixture of DOPC:DOPE:DMPA:Cholesterol) were directly added to the reaction mixture. Synthesis of OEP24 in the presence of liposomes suggests that addition of synthetic lipids is compatible with the expression of OEP24 and results in a favourable association between the hydrophobic domain of this protein and lipids (Fig. 1B). Purification of L-Os was performed onto a discontinuous sucrose gradient as previously described [11]. To check the purity of the proteoliposomes, the fractions content was analyzed by coomassie blue staining and Western blotting using an anti-his antibody (Fig. 1B). Interestingly, expression of OEP24 in the presence of liposomes can result in the formation of homo-oligomers as previously described for purified OEP24 from pea chloroplasts [7]. Such oligomeric organization have been also observed in the case of the mitochondrial VDAC1 suggesting that, due to the high rate of protein synthesis with the *in vitro* system, multiple copies of channel proteins associate either during or shortly after synthesis [11].

We next examined the expression of OEP24 in the presence of a nonionic detergent, *n*-dodecyl- β -D-maltoside (DDM). Nonionic and zwitterionic detergents have been previously shown to be compatible with the cell-free expression systems at concentrations ranging up to 5 times their CMC. The presence of a major homogenous band at 24 kDa detected in the purified fractions by coomassie blue staining indicated that soluble OEP24 is pure at 95% (Fig. 1C). These results indicate that the cell-free expression system renders feasible the synthesis of OEP24 either in a soluble form or integrated into the lipid bilayer of synthetic liposomes (Fig. 1B and C).

Circular dichroism of soluble OEP24 and OEP24 liposomes

The secondary structure of the purified proteins either in proteoliposome or in soluble form was assayed by circular dichroism. The far-UV circular dichroism (CD) spectrum is a powerful technique to estimate the fraction of a molecule that presents α -helix, β -sheet and β -turn conformations. CD spectroscopy can be used to analyze proteins in solution or solutions containing membrane proteins embedded in membranes such as liposomes. To determine if recombinant OEP24 protein displayed secondary structures in detergents or embedded into the lipid bilayer, purified soluble OEP24 in the presence of DDM and OEP24 proteoliposomes were tested by CD. As shown in Fig. 2, CD experiments indicated a secondary structural organization reflecting high β -sheet content for purified soluble OEP24 and OEP24 inserted into liposome (L-Os). The OEP24 CD spectra were typical of folded protein even if

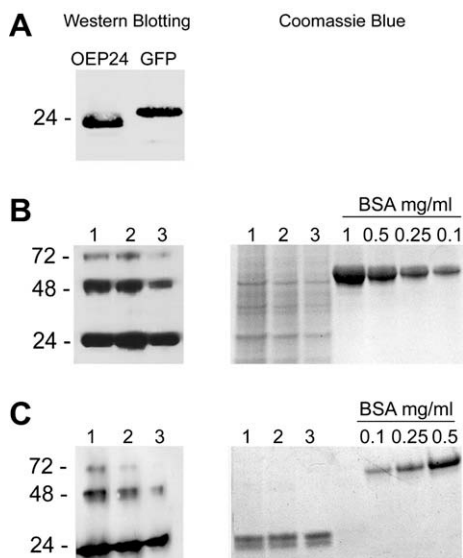


Fig. 1. (A) Production of OEP24 and GFP (expression control). (B) Purification of OEP24 liposomes. Western blotting (left) and coomassie blue staining of isolated fractions containing L-Os. Bovine serum albumin (BSA) is loaded as reference (right). (C) Western blotting (left) and coomassie blue staining of soluble OEP24 purified in the presence of DDM (right).

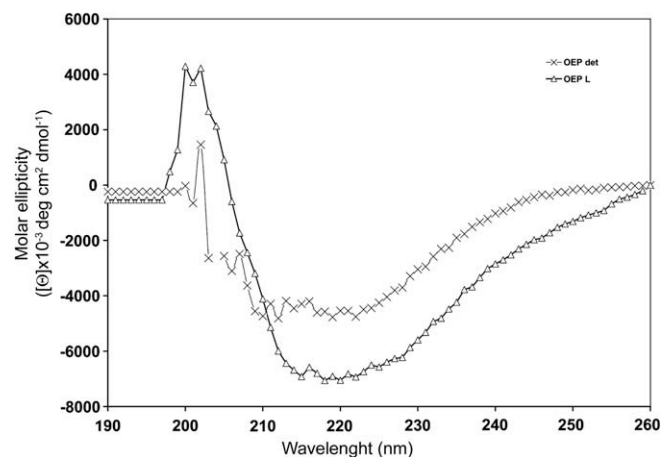


Fig. 2. Circular dichroism of OEP24 liposomes (OEP L; triangles) and the soluble form of OEP24 (OEP det; crosses) in 0.5 mM DDM. As control, empty liposomes were used.

the recording below 200 nm was difficult due to the properties of the chemicals used to make liposomes and to the properties of the detergent (Fig. 2). The CD spectra of cell-free expressed L-Os had a molar ellipticity peak of negative amplitude ($-7000 \text{ deg cm}^2 \text{ dmol}^{-1}$) centred on 217 nm which is typical of a protein containing a large amount of β -strands (Fig. 2). These results obtained by subtracting the signal of empty liposomes as control, correspond to previous observations with reconstituted OEP24 in liposomes showing OEP24 as a β -strand rich protein [7]. These structural similarities confirm that L-Os produced in one-step reaction with the cell-free expression system are a suitable source of porins for CD analysis. The CD spectra of soluble OEP24 in DDM showed a different profile from OEP24 embedded into the lipid bilayer (L-Os) suggesting a different protein conformation in the presence of DDM (Fig. 2). Soluble OEP24 profile appeared as an α -helix-like CD spectrum. This change could be due to a slight increase in the helical fraction of the protein or due to a structural reorganisation of the β turns that connects β -strands due to the interaction of the molecule with the detergent. In the case of β -rich protein, this phenomenon has been already demonstrated and is well established [17]. According to our data, the membrane protein OEP24 produced in a cell-free expression system either in soluble form or integrated into liposome is properly folded; differences in the spectra are due to the physical conditions surrounding the proteins, lipids or detergent.

Electrophysiological measurements

The growing interest in porins from chloroplast is due to their properties and functions. In fact, beside plant physiology, there is an interesting evolutionary context that unites membrane proteins from gram-negative bacteria, plastids and mitochondria [18].

Until recently, the outer envelope membrane was considered freely permeable to solutes with molecular mass smaller than 10 kDa but identification of different members of the OEPs family clearly unveils a more complex picture of the chloroplast metabolism [7,19,20]. Chloroplasts, like mitochondria, require the presence of transporters and channels with different specificity and selectivity to coordinate and modulate their biosynthetic functions with an asymmetrical distribution of proteins in the inner and outer membranes. In the OEPs family, OEP16 is permeable to amino acids and not to sugars, organic acids and sugar phosphates [19], OEP21 is anion-selective channel ATP-regulated, OEP37 forms a rectifying, high conductance, cation-sensitive channel [6]. OEP24 is the only general solute channel identified so far [7].

In order to confirm that recombinant OEP24 synthesized in the presence of liposomes is functional as a channel, giant liposomes were generated by fusing OEP24 liposomes with asolectin vesicles and the function was assayed with the patch clamp technique. Single channel currents could be observed at both positive and negative membrane potentials (Fig. 3A). Multiple conductance levels were observed (Fig. 3A and C). The most frequently detected conductances were 83 and 300 pS (Fig. 3B) and the latter value appears to match that described in the literature for the other OEP porin members [7]. L-Os are fully active in transport of solutes and present multiple conductance levels (Fig. 3). The highest conductance (300 pS) was already described [7] but we observed and characterized a new lower conductance of 83 pS. This conductance was also detected in other members of the OEPs family [6] and confirms the correct folding and organization of the proteins into liposomes.

Caspases 9 activation ad cell viability: MTT assay

In 1999 an intriguing study showed that transformation with an OEP24 plasmid could induce complementation in a yeast strain lacking the VDAC1 isoform and that the protein could functionally

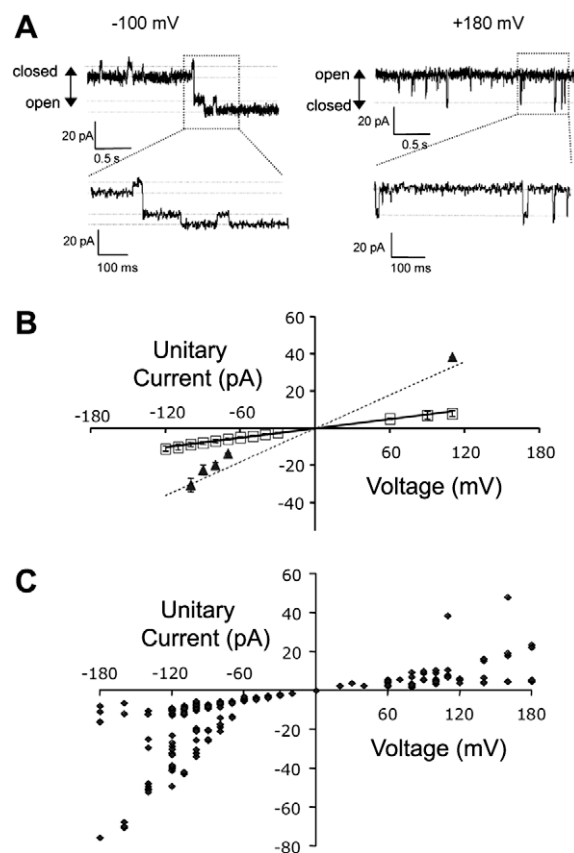


Fig. 3. Electrophysiological characterization of OEP24. Inside-out patch-clamp experiments were performed on giant liposomes incorporating OEP24. (A) Representative recordings of single channels at 2 voltages and 2 different time scales. (B) Unitary current vs. voltage relationship of single channels from one patch (each data point represents the average of at least 3 measurements). Linear regression of the lower current values (squares) yielded an 83 pS conductance (solid line). Larger currents (triangles) were too dispersed for fitting but could correspond to a previously reported 300 pS conductance (dashed line). (C) Unitary currents observed at different voltages in all tested patches.

replace the channel [8]. In mitochondria and chloroplasts, VDAC and OEP24 represent the major solute channel with high conductance and in Gram-negative bacteria different kinds of high-conductance channels have been identified: porin-forming, porin-like channels and ligand-gated channels suggesting a common origin of the two channels from an ancestral porin [1,21]. We recently demonstrated that proteoliposomes containing human VDAC1 protein were able to deliver functional VDAC in different cancer cell lines and to induce apoptosis by activation of the intrinsic pathway which involves release of cytochrome c from mitochondria and activation of the caspase cascade [11]. From these data, we wanted to verify whether L-Os could interact with mammalian mitochondria after cellular uptake.

Caspase 9 activation, an early apoptosis mitochondria-mediated marker, was first checked after cellular uptake of L-Os in HCT116 colon carcinoma cell lines in order to verify a potential pro-apoptotic effect of the L-Os [22]. The anti-caspase 9 antibody recognized, when activated, the cleaved forms at 37/35, 18 and 10 kDa (Fig. 4A). Liposomes containing recombinant VDAC, Bak (a proapoptotic membrane protein) or both proteins synthesized with the cell-free expression system, were used as positive controls [11,23]. As shown in Fig. 4A, L-Os (0.35 and 1.07 μM) induced a weak cleavage of the protease after 24 h treatment compared to the strong activation of caspase 9 induced by Bak (LB 0.15 μM), VDAC (LV 0.3 μM) and both proteins (LVB 0.75 μM).

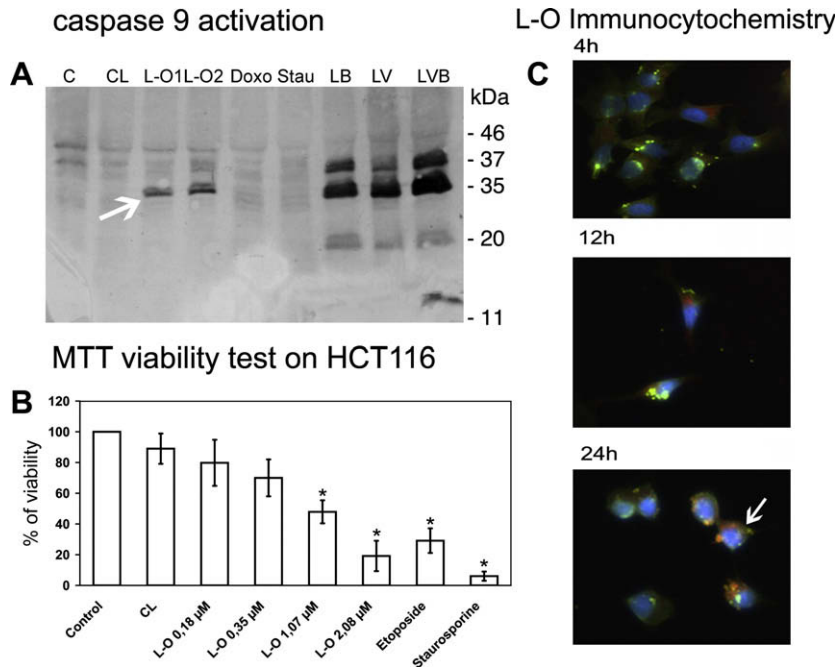


Fig. 4. (A) Apoptotic marker detection: Caspase 9 activation in HCT116 after 24-h treatment on cell lysate. Two different concentrations of proteoliposomes were tested: L-O1 (0.35 μM), L-O2 (1.07 μM). Proteoliposomes containing Bak (LB 0.15 μM), VDAC (LV 0.3 μM) and both proteins (LVB 0.75 μM) are used as positive control. C, control. Untreated cells; CL, empty liposomes control. (B) MTT viability assay. HCT116 were treated with OEP24 proteoliposomes (L-Os) at different concentrations for 24 h. Etoposide (25 μM) and staurosporine (1 μM) were used as apoptosis inducers. The histogram represents the average of $n = 5$ experiments. Untreated cells represent 100% viability. (C) Immunolocalization of exogenous OEP24: HCT cells are incubated for 4, 12, 24 h in the presence of proteoliposomes. Alexafluor 488 (green) is used as secondary antibody against the anti-his used to detect exogenous OEP24. Mitotraker (red) labels mitochondria and DAPI (blue) stain the nucleus. (For interpretation of colour mentioned in this figure the reader is referred to the web version of the article.)

As shown in Fig. 4B, monitoring the cell viability by a simple colorimetric test is an indirect way to confirm apoptosis. HCT116 cells were incubated with increasing concentrations of L-Os and sensitivity to proteoliposomes treatment was recorded after 24 h. A dose-dependent effect on cells viability was detected: at 1.07 μM of L-Os, almost 50% of cell death was reached. Empty liposomes used as negative control did not affect cellular viability (Fig. 4B). These data indicate that L-Os induced cellular death in a dose-dependent manner.

To verify if L-Os localize at the mitochondrial level, we performed a confocal microscopy immunocytochemistry time course assay (Fig. 4C). Fig. 4C shows that the internalization process proceeds along 24 h. After 12 h, cells started to change shape and the green fluorescence was more diffused and co-localized at the mitochondrial site (Fig. 4C, panels 12 and 24 h).

Even if there is a significant difference in the amino-acid composition, OEP24 complements VDAC in yeast [8] and induces apoptosis in mammalian cells when delivered by liposomes. The molecular mechanisms underlying the cellular effect of OEP24 will require further investigation but this pro-apoptotic effect suggests and confirms the conservation of essential functions during the evolution, shared with VDAC and inherited by the ancestral organelle progenitor of mitochondria and chloroplasts [24].

In conclusion, we have demonstrated that OEP24 integrated in synthetic liposomes is fully functional and displays the correct folding and secondary structure typical of porins. Purified L-Os were able to elicit apoptosis in human cancer cells confirming a definite homology in function between OEP24 and VDAC.

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