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Recruitment of an alternatively spliced form of synaptojanin 2 to mitochondria by the interaction with the PDZ domain of a mitochondrial outer membrane protein

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Synaptojanin 1 is an inositol 5′-phosphatase highly enriched in nerve terminals with a putative role in recycling of synaptic vesicles. We have previously described synaptojanin 2, which is more broadly expressed as multiple alternatively spliced forms. Here we have identified and characterized a novel mitochondrial outer membrane protein, OMP25, with a single PDZ domain that specifically binds to a unique motif in the C-terminus of synaptojanin 2A. This motif is encoded by the exon sequence specific to synaptojanin 2A. OMP25 mRNA is widely expressed in rat tissues. OMP25 is localized to the mitochondrial outer membrane via the C-terminal transmembrane region, with the PDZ domain facing the cytoplasm. Overexpression of OMP25 results in perinuclear clustering of mitochondria in transfected cells. This effect is mimicked by enforced expression of synaptojanin 2A on the mitochondrial outer membrane, but not by the synaptojanin 2A mutants lacking the inositol 5′-phosphatase domain. Our findings provide evidence that OMP25 mediates recruitment of synaptojanin 2A to mitochondria and that modulation of inositol phospholipids by synaptojanin 2A may play a role in maintenance of the intracellular distribution of mitochondria.

Keywords: dynamin/inositol 5′-phosphatase/mitochondria clustering/phosphoinositide/signal anchor sequence

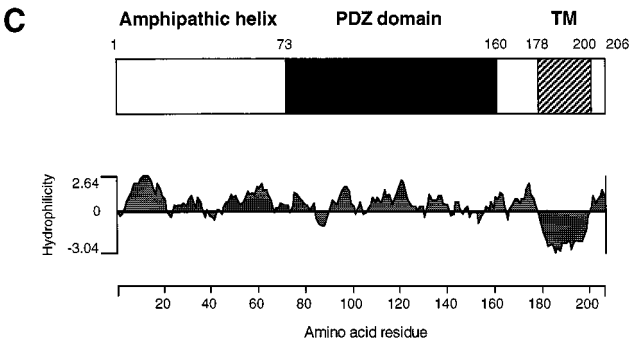
Introduction

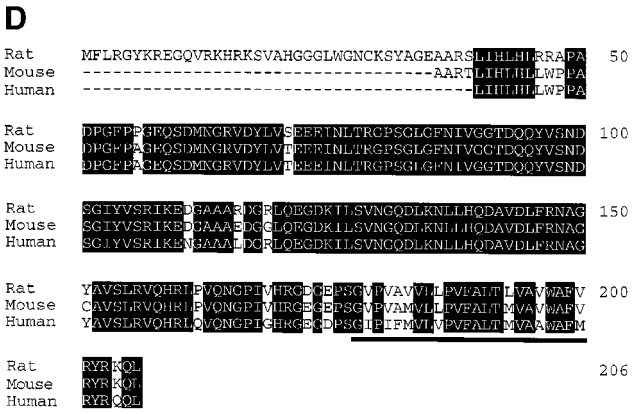
Growing evidence has indicated that inositol metabolism plays pivotal roles in membrane trafficking processes within cells, in addition to its well established role in intracellular signaling pathways. An emerging concept is that various phosphoinositide stereoisomers, with phosphate groups at different positions of the inositol ring, are recognized by specific proteins that are involved in generation of membrane vesicles and in their docking to acceptor compartments. Thus, changes in phosphoinositide levels in a spatially and temporally regulated manner are means by which membrane trafficking events are controlled. These mechanisms seem to underlie the requirements for several enzymes and proteins responsible for synthesis, intracellular transport and breakdown of distinct phosphoinositides in specific membrane trafficking steps (Roth and Sternweis, 1997; Martin, 1998, and references therein).

We have recently identified a neuronal inositol polyphosphate 5′-phosphatase, synaptojanin 1 (McPherson *et al*., 1994a, 1996) and a more broadly expressed form, synaptojanin 2 (Nemoto *et al*., 1997). Synaptojanins dephosphorylate inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate at the D-5 position of the inositol ring *in vitro* (McPherson *et al*., 1996; Nemoto *et al*., 1997; Woscholski *et al*., 1997) and define a subfamily of inositol 5′-phosphatases with a unique three-domain structure: an N-terminal domain homologous to *Saccharomyces cerevisiae* Sac1p (Cleves *et al*., 1989; Novick *et al*., 1989), a central inositol 5′-phosphatase domain and a C-terminal proline-rich domain. Synaptojanin 1 and dynamin 1 are highly enriched in nerve terminals, where they are localized on coated endocytic intermediates (McPherson *et al*., 1994b; Haffner *et al*., 1997). Both proteins undergo dephosphorylation in response to nerve terminal depolarization (Robinson *et al*., 1993; McPherson *et al*., 1994b; Bauerfeind *et al*., 1997), and it has been shown recently that such depolarization-induced dephosphorylation promotes the assembly of several proteins for compensatory endocytosis in nerve terminals (Slepnev *et al*., 1998). Synaptojanin 1 and dynamin 1 specifically interact with the SH3 domains of Grb2 (Gout *et al*., 1993; McPherson *et al*., 1994a), amphiphysin 1 and 2 (David *et al*., 1996; Micheva *et al*., 1997; Ramjaun *et al*., 1997), members of the SH3p4/8/13 protein family (endophilins) (de Heuvel *et al*., 1997; Ringstad *et al*., 1997), intersectin (Yamabhai *et al*., 1998) and syndapin I (Qualmann *et al*., 1999). These properties suggest that synaptojanin 1, together with dynamin 1, functions in the retrieval of the synaptic vesicle membrane by clathrin-coated vesicles. Inositol phospholipids have been reported to bind to several proteins implicated in this process with high affinity, and in some cases modulate their biochemical properties. Examples include the α-subunit of clathrin assembly protein complex AP2, the neuron-specific clathrin assembly protein AP180, the C2B domain of synaptotagmin and the pleckstrin homology domain of dynamin 1 (reviewed in Cremona and De Camilli, 1997). Furthermore, we and others have recently shown that deletion of yeast synaptojanin-like genes causes a severe defect in receptor-mediated and fluid phase endocytosis (Singer-Krüger *et al*., 1998; Stolz *et al*., 1998). These results, together with those of Luo and Chang (1997), provided the first direct evidence for a role for synaptojanins in endocytic membrane trafficking in *S.cerevisiae*.

Synaptojanin 2 undergoes extensive alternative splicing in the C-terminal proline-rich region to generate multiple isoforms (Khvotchev and Südhof, 1998; Seet *et al*., 1998). Our initial characterization of synaptojanin 2 (now referred to as synaptojanin 2A) showed that synaptojanin 2A is associated predominantly with the particulate fraction, while synaptojanin 1 is localized mainly in the soluble fraction in transfected Chinese hamster ovary (CHO) cells,

and that the different protein–protein interactions in their proline-rich region are likely to direct synaptojanin 1 and 2A to different subcellular compartments (Nemoto *et al*., 1997). To explore the biochemical basis of the previous findings, in this study we performed a yeast two-hybrid screening for synaptojanin 2A-specific interacting proteins and we have identified a novel mitochondrial outer membrane protein with a single PDZ domain, OMP25. We show that the PDZ domain of OMP25 binds to the unique motif in the C-terminal sequence specific to synaptojanin 2A among several alternatively spliced forms of synaptojanin 2. By this interaction, OMP25 mediates the recruit-





ment of synaptojanin 2A to mitochondria, where it causes an alteration of morphology of the mitochondrial network. OMP25 represents the first identified component in mammalian mitochondria that appears to play a role in the proper distribution of mitochondria. Our observations implicate the specific pools of inositol phospholipids in regulation of the dynamics of intracellular organelles and extend the roles of inositol metabolism in cell physiology.

Results

Identification of OMP25, a synaptojanin 2A-interacting transmembrane protein with a single PDZ domain

We have searched for proteins that specifically interact with the proline-rich region of synaptojanin 2A and not with that of synaptojanin 1 by yeast two-hybrid screening. Thirty-six clones were obtained and they were independent isolates of four overlapping cDNAs encoding a portion of a novel protein. We refer to this protein as OMP25 (for outer membrane protein of 25 kDa). A full-length cDNA for OMP25 was isolated by screening of rat brain cDNA libraries and a PCR-mediated procedure. The composite cDNA sequence for rat OMP25 is 5.2 kb long (Figure 1A). An extraordinarily long 3′-untranslated region (3′-UTR) is AU-rich and contains multiple AUUUA repeats (underlined in Figure 1A), which are implicated in the instability of mRNAs and often found in transiently expressed mRNAs encoding cytokines and proto-oncogene products (Treisman, 1985; Shaw and Kamen, 1986), suggesting that expression of OMP25 may be dynamically regulated. An open reading frame (ORF) of 621 bases encodes a protein of 206 amino acids with a predicted M_r of 22 564 and pI of 9.46. The putative initiator codon at nucleotides 456–458 is preceded by an in-frame termination codon in the 5′-UTR at nucleotides 312–314. Sequence analysis revealed that OMP25 has a single PDZ domain (amino acids 73–160) that is most similar to the first and second PDZ domains of DLG/SAP97 (Lue *et al.*, 1994; Müller *et al.*, 1995) and PSD-95/SAP90 (Cho *et al.*, 1992; Kistner *et al.*, 1993) (Figure 1B). The region preceding the PDZ domain, especially the N-terminal portion (amino acids 1–50), is rich in basic amino acid residues (pI = 11. 43) and predicted to adopt the amphipathic α-helix structure (data not shown). Near the C-terminus, OMP25 has a 23 amino acid stretch of hydrophobic residues that is predicted to form a membrane-spanning region, as indicated by the hydrophilicity profile (Figure 1C). Searches for OMP25-related sequences showed the presence of highly homologous expressed sequence tags (ESTs) in a variety of human and mouse tissues, which seem to be derived from the OMP25 homologs (Figure 1D).

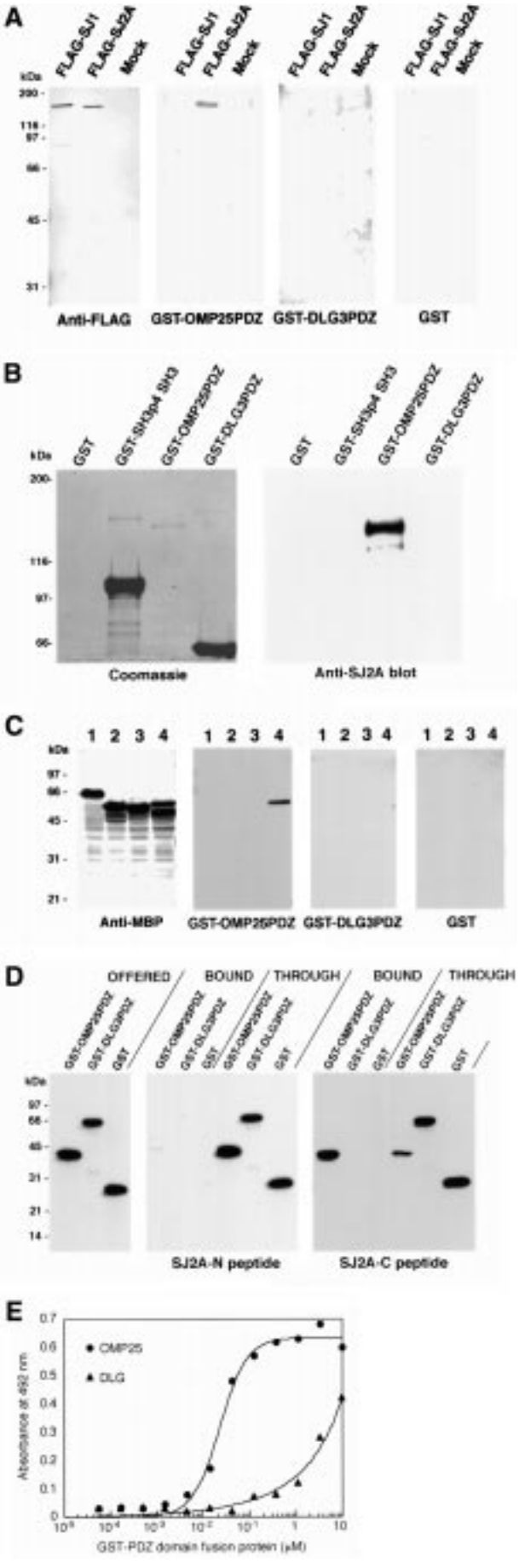
The C-terminal 16 amino acid residues from the synaptojanin 2A-specific exon sequence are sufficient for selective binding to OMP25

Inspection of the synaptojanin 2A sequence revealed that the C-terminal region of synaptojanin 2A conforms to a tS/TXV motif (see Discussion). We prepared GST fusions of the PDZ domain of OMP25 (GST–OMP25PDZ) and of all three PDZ domains of DLG (GST–DLG3PDZ) as a control to study the biochemical interaction between synaptojanin 2A and OMP25. We first tested whether the specific interaction is mediated by the PDZ domain of OMP25 by blot overlay of the extracts from cultured cells transiently expressing FLAG-tagged synaptojanin 1 or 2A. As shown in Figure 2A, while the extracts contained comparable amounts of recombinant synaptojanin 1 and 2A as judged by immunoblotting with anti-FLAG antibody, GST–OMP25PDZ bound to synaptojanin 2A, but not to synaptojanin 1. By contrast, GST–DLG3PDZ bound to neither synaptojanin 1 nor synaptojanin 2A under the conditions used. Furthermore, a 140 kDa protein was affinity purified from rat brain extract selectively by GST–OMP25PDZ and the protein was shown to be synaptojanin 2A by immunoblotting, illustrating the highly specific interaction between synaptojanin 2A and the PDZ domain of OMP25 (Figure 2B).

To define the region of synaptojanin 2A required for the association with OMP25, we divided the proline-rich region of synaptojanin 2A into four overlapping segments. Each segment was expressed in *Escherichia coli* as a maltose-binding protein (MBP) fusion protein or in *S.cerevisiae* as a LexA fusion protein, and the binding to OMP25 was tested by blot overlay and yeast two-hybrid assays. In the blot overlay, the most C-terminal segment bound to the PDZ domain of OMP25, whereas other segments showed no detectable binding (Figure 2C). Again the PDZ domains of DLG failed to bind to any segments of the proline-rich region of synaptojanin 2A. The results were corroborated by the yeast two-hybrid assay (data not shown).

To narrow down further the region of synaptojanin 2A mediating the specific binding to the PDZ domain of OMP25, a synthetic peptide corresponding to the last 16 C-terminal amino acid residues of synaptojanin 2A, which is embedded in the synaptojanin 2A-specific exon sequence among various alternatively spliced forms of synaptojanin 2 (Khvotchev and Südhof, 1998; Seet *et al*., 1998), was

Fig. 1. Primary structure of rat OMP25. (**A**) Nucleotide and deduced amino acid sequence of rat OMP25 cDNA. The composite nucleotide sequence of rat OMP25 cDNA is shown. The predicted amino acid sequence is shown below in the three letter code. An in-frame termination codon in the 5′-UTR and potential mRNA-destabilizing elements in the 3′-UTRs are underlined. A PDZ domain and a transmembrane region in OMP25 are indicated by bold letters and italics, respectively. Nucleotides are numbered on the left and amino acid residues are numbered on the right. The sequence data are available from DDBJ/EMBL/GenBank under accession No. AF107295. (**B**) The PDZ domains of rat OMP25 (amino acids 73–160), rat DLG/SAP97 (amino acids 223–310, 318–404 and 464–545) and rat PSD-95/SAP90 (amino acids 64–151, 160–246 and 312–393) are aligned by the Clustal method (Higgins and Sharp, 1988). The residues corresponding to the secondary structural elements identified in the third PDZ domain of PSD-95 are indicated according to Doyle *et al.* (1996). (**C**) Schematic representation of the domain structure and the Kyte–Doolittle hydrophilicity profile of OMP25 (Kyte and Doolittle, 1982). OMP25 has a putative transmembrane segment near the C-terminus (amino acids 178–200). (**D**) Alignment of rat OMP25 and the putative mouse and human homologs. The partial amino acid sequences of putative mouse and human OMP25 are derived from the composite sequences of mouse ESTs AA254858, AA221650, AI317677 and AU044272 and human ESTs AA075102, AA369660, AI309021 and W63718, respectively. The amino acid residues shared by all the homologs are shown by black boxes. The residues corresponding to the transmembrane segments are underlined. Missing amino acids are indicated by dashes. Amino acid residues of rat OMP25 are numbered on the right.



coupled to gel beads via the N-terminal end. The beads were incubated with the GST–PDZ domain fusion proteins and the bound protein was eluted and detected by immunoblotting with anti-GST antibody. As a control, a peptide corresponding to the N-terminal end of synaptojanin 2A was coupled to the gel and the binding to the GST–PDZ domain fusion proteins was tested similarly. The C-terminal 16 amino acid peptide was capable of binding to the GST–OMP25PDZ domain, while it did not bind to GST–DLG3PDZ. No binding between the N-terminal peptide and the GST–PDZ domains was detected (Figure 2D).

The relative binding affinity of the PDZ domains in OMP25 and DLG for the C-terminal peptide of synaptojanin 2A was measured by an enzyme-linked immunosorbent assay (ELISA) using a series of concentrations of the GST fusion proteins (Figure 2E). Analysis of the dose–response curve gave an EC_{50} value of 21 nM. This value is comparable with the apparent EC_{50} between the second PDZ domain of SAP102 and the C-terminal peptide of the *N*-methyl-D-aspartate (NMDA) receptor measured by a similar method (Müller *et al*., 1996), further suggesting that this interaction may be physiologically relevant. The assay suggested that GST–DLG3PDZ has an EC_{50} >10 μM, implying that the PDZ domain of OMP25 has at least 100-fold higher affinity for the synaptojanin 2A C-terminal peptide than the PDZ domains in DLG. Binding of the PDZ domains to the synaptojanin 2A N-terminal peptide was negligible (data not shown).

Collectively, these results indicate that the interaction of synaptojanin 2A with OMP25 is mediated by the 2A-specific exon sequence at its most C-terminal end among various alternatively spliced forms of synaptojanin 2.

The PDZ domain of OMP25 recognizes a unique C-terminal motif of synaptojanin 2A

Our *in vitro* protein binding experiments have shown that the C-terminal sequence of synaptojanin 2A binds to the

Fig. 2. Specific binding of the C-terminal region of synaptojanin 2A to the PDZ domain of OMP25. (**A**) Blot overlay of the recombinant synaptojanins by GST–PDZ domain fusion proteins. Lysates from COS-7 cells expressing either FLAG-tagged synaptojanin 1 (FLAG-SJ1) or synaptojanin 2A (FLAG-SJ2A) and mock-transfected cells (Mock) were probed by anti-FLAG antibody or the indicated GST–PDZ domain fusion protein. (**B**) Affinity purification of synaptojanin 2A from rat brain by the PDZ domain of OMP25. A Triton X-100 extract of rat brain was affinity purified onto the indicated GST fusion proteins. Bound proteins were released by Laemmli sample buffer and either stained by Coomassie Brilliant Blue (left panel) or analyzed by immunoblotting with anti-synaptojanin 2A antibody (right panel). The 145 and 100 kDa proteins purified by the SH3 domain of SH3p4 (endophilin 1) have been identified as synaptojanin 1 (145 kDa form) and dynamin 1, respectively (Ringstad *et al*., 1997). In the eluate from GST–DLG3PDZ, a 60 kDa protein is the fusion protein. The 160 kDa protein was not identified in this study. (**C**) MBP fusion proteins of the four overlapping segments of the synaptojanin 2A proline-rich region (numbered 1–4, from the N- to the C-terminus) were probed by anti-MBP antibody or the indicated GST–PDZ domain fusion protein. (**D**) Binding of GST–PDZ fusion proteins to a 16 amino acid C-terminal peptide of synaptojanin 2A. GST–PDZ fusion proteins were incubated with a bead-immobilized peptide corresponding to the C-terminal 16 amino acid residues of rat synaptojanin 2A, or with a control peptide corresponding to the N-terminal 18 amino acid residues of rat synaptojanin 2A. Bound fusion proteins were eluted with Laemmli sample buffer and detected by immunoblotting with anti-GST antibody. (**E**) Dose–response curves of binding of GST–PDZ domain fusion proteins to the synaptojanin 2A C-terminal peptide in an ELISA assay.

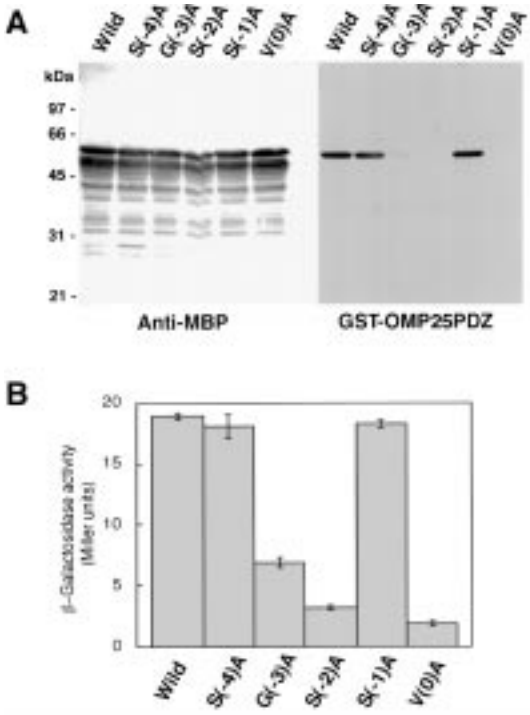


Fig. 3. Alanine replacement mutagenesis of the C-terminal region of synaptojanin 2A. (**A**) Blot overlay. The five most C-terminal amino acid residues of synaptojanin 2A (-SGSSV) in segment IV of the proline-rich region were replaced by alanine residues and expressed as MBP fusion proteins. The wild-type and mutant proteins were probed by anti-MBP antibody or GST–OMP25PDZ. (**B**) Yeast two-hybrid assay. The wild-type and the segment IV mutants were expressed as the LexA fusion protein in AMR70. The strains were mated with L40 expressing the GAL4 activation domain fusion protein of the PDZ domain of OMP25. The activity of β-galactosidase in the resultant dipolid strains was measured in triplicate and expressed as the mean values ± SD in Miller units.

PDZ domain of OMP25, but not to the PDZ domains of rat DLG, despite their close similarity. We therefore addressed the structural requirement for the specific binding of synaptojanin 2A to the PDZ domain of OMP25 by mutagenesis analysis. A series of C-terminal mutant proteins in which the residues at different positions of the five most C-terminal amino acid residues of synaptojanin 2A (-SGSSV) were replaced with alanine residues were expressed and their binding to the PDZ domain of OMP25 was analyzed by blot overlay and yeast two-hybrid assays (Figure 3A and B). Replacement of the serine residue in position –2 or the C-terminal valine residue abolished the binding to the PDZ domain, whereas replacement of the serine residues in position –1 or –4 did not affect the binding, indicating that the residues in the C-terminus and position –2 are essential for binding to the OMP25 PDZ domain, in agreement with studies on other PDZ domain-mediated interactions.

Somewhat unexpectedly, when the glycine residue in position –3 was replaced by an alanine residue, the binding of the mutant protein to the PDZ domain of OMP25 was diminished significantly, suggesting that this residue is also important for the effective binding of the ligand to the OMP25 PDZ domain. A recent study on the binding specificities of several PDZ domains by the oriented peptide library technique showed that the PDZ domains of mouse DLG bind preferentially to peptides with an ES/TXV/I motif, with a glutamate residue at position –3 (Songyang *et al*., 1997). The difference in the preferred recognition motifs at the –3 peptide position may account in part for the observed specific interaction between the C-terminus of synaptojanin 2A and OMP25. Remarkably, the C-terminus of human synaptojanin 2A deduced from an EST (accession No. AI014555) ends in -SGSSV, supporting the importance of the glycine residue at position –3.

OMP25 mRNA is widely distributed in rat tissues

We performed a series of Northern blot experiments to determine the tissue distribution of OMP25 mRNA and to verify the authenticity of the OMP25 cDNA. We used three probes derived from the coding region and the distal and middle parts of the 3′-UTR of OMP25 cDNA (Figure 4). The probe corresponding to the PDZ domain of OMP25 detected RNA species of 6.0, 3.6, 3.4 and 1.0 kb with varying relative abundance in all the adult rat tissues tested (Figure 4A). A probe from the distal 3′-UTR detected only the largest 6 kb transcript (Figure 4C), while a probe from the middle 3′-UTR hybridized to the largest 6 kb and the middle 3.6 and 3.4 kb bands and not to the smallest 1 kb band (Figure 4B). These results indicate that the isolated OMP25 cDNA corresponds to the largest 6 kb transcript, and other smaller transcripts arise by alternative splicing or alternative usage of polyadenylation sites within the 3′-UTR. Thus, OMP25 mRNA is widely expressed in rat tissues as multiple transcripts.

OMP25 is localized to mitochondria via the C-terminal transmembrane region and its overexpression induces clustering of mitochondria

To investigate the possible biological function of OMP25, we expressed OMP25 with a hemagglutinin (HA) epitope tag at the N-terminus in CHO cells and examined its intracellular localization by indirect immunofluorescence. Overexpressed OMP25 was localized on punctate and rod-like structures. An increased level of expression correlated with coalescence of these structures in the perinuclear region, and in highly overexpressing cells they formed one or a few masses. The structures were found to be coincident with mitochondria as detected by antibody against cytochrome *c* reductase (complex III) or cytochrome *c* oxidase (complex IV) (Figure 5). The structures were also recognized by MitoTracker, a mitochondria-specific fluorescent dye whose uptake depends on the mitochondrial membrane potential (Poot *et al*., 1996), confirming the mitochondrial localization of OMP25 and the preservation of the mitochondrial membrane potential despite the grossly altered distribution and morphology. OMP25 in the transfected cells did not co-localize with markers for other organelles tested including the endoplasmic reticulum, the Golgi and endosomes (data not shown).

To determine the region required for targeting of OMP25 to mitochondria, we expressed epitope-tagged truncated proteins and examined their localization by indirect immunofluorescence. The protein lacking the N-terminal basic region (OMP25ΔN) exhibited a distribution indistinguishable from that of intact OMP25 and induced mitochondrial clustering (Figure 6A, a and b). In contrast, the

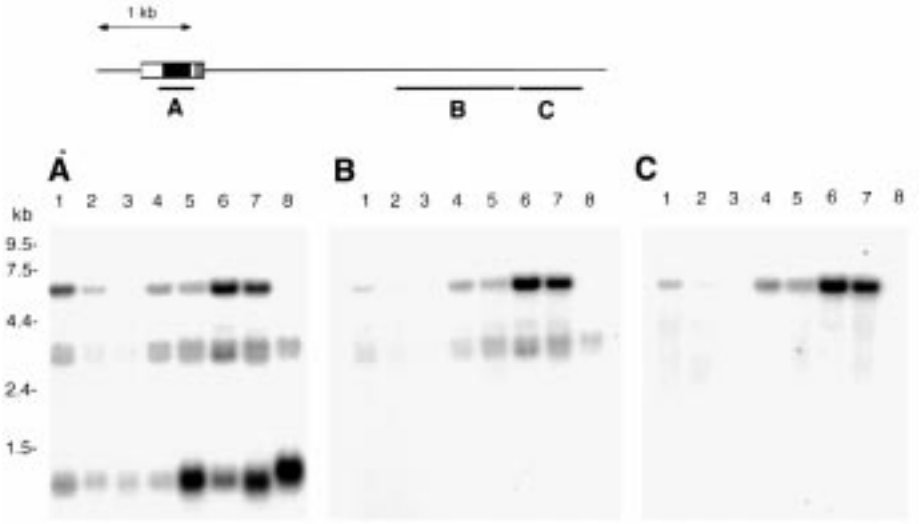


Fig. 4. Tissue distribution of rat OMP25 mRNA. OMP25 transcripts in various adult rat tissues were detected by Northern blot analysis with the probes derived from the open reading frame (**A**), the middle (**B**) and the distal (**C**) parts of the 3′-UTR of the isolated OMP25 cDNA, as indicated at the top. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. RNA size markers are indicated in kb on the left.

protein without the transmembrane region (OMP25ΔC) was localized diffusely throughout the cytoplasm and did not perturb mitochondrial distribution (Figure 6A, c and d). Consistent with the immunofluorescence localization, subcellular fractionation of the transfected cells showed that intact OMP25 and OMP25ΔN were associated with the particulate fraction, whereas OMP25ΔC was recovered in the soluble fraction (Figure 6B). These results indicate that the C-terminal transmembrane region of OMP25 is essential for localization of OMP25 to mitochondria, while the N-terminal region is dispensable, although the positively charged and amphipathic helical nature of the region bears some resemblance to mitochondrial presequences (Roise and Schatz, 1988).

To test further whether the C-terminal transmembrane region is sufficient for mitochondrial targeting, we expressed green fluorescent protein (GFP) in fusion with the transmembrane region of OMP25. As shown in Figure 6C, the GFP fusion protein co-localizes with mitochondria and does not affect the mitochondrial distribution, demonstrating that the transmembrane region of OMP25 contains all the essential information for mitochondrial targeting and has the ability to direct a heterologous protein to mitochondria.

OMP25 is an integral protein in the mitochondrial outer membrane with the PDZ domain facing the cytoplasm

The transient expression study of epitope-tagged OMP25 constructs indicates that OMP25 is localized to mitochondria via its C-terminal transmembrane region. The presence of an integral membrane protein with a PDZ domain in an intracellular organelle is unprecedented. To rule out the possibility that the addition of a negatively charged epitope sequence might affect the observed localization of OMP25, we tested the mitochondrial localization of OMP25 by independent approaches.

We probed the localization of anti-OMP25 immunoreactivity in rat liver mitochondria (Figure 7A). An antibody against the N-terminal peptide from OMP25 recognized a 25 kDa band in the mitochondrial fraction. The immunoreactive protein could not be extracted by alkali carbonate treatment (Fujiki *et al.*, 1982) and was degraded by proteinase K treatment, indicating a mitochondrial membrane localization with the bulk of the protein exposed to the cytosol.

Radiolabeled OMP25 was synthesized *in vitro* by using rabbit reticulocyte lysate in the presence of [^{35}S]methionine and incubated with freshly isolated rat liver mitochondria (Figure 7B). The *in vitro* translated protein appeared as a single major band at 25 kDa upon SDS–PAGE, and the protein was found to be imported to mitochondria. No change in the molecular mass of OMP25 was observed upon import, which indicates the lack of a cleavable presequence in OMP25. Most of the imported protein was recovered in the pellet after alkali carbonate extraction, indicating the insertion of the protein into the mitochondrial membrane. The inserted protein was degraded by mild proteinase K treatment under the condition where cytochrome *c* oxidase subunit IV (COXIV), an inner membrane protein with its C-terminal portion protruding into the intermembrane space (Zhang *et al.*, 1988), was protected. Similarly to other mitochondrial outer membrane proteins (Shore *et al.*, 1995), the import of OMP25 was independent of a membrane potential across the inner membrane $\Delta\Psi_m$, as dissipation of $\Delta\Psi_m$ by an uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not affect the import of OMP25, whereas it blocked the import of ornithine carbamoyltransferase (OCT), a mitochondrial protein targeted into the matrix by its presequence, which is cleaved upon mitochondrial import (Nguyen *et al.*, 1986).

Taken together with immunofluorescence localization, we concluded that OMP25 is inserted into the mitochondrial outer membrane via the C-terminal transmembrane region, while the remainder of the protein, including the PDZ domain, is exposed to the cytoplasm.

OMP25 recruits synaptojanin 2A to mitochondria

To test the interaction of OMP25 with synaptojanin 2A within cells, we co-transfected CHO cells with synapto-

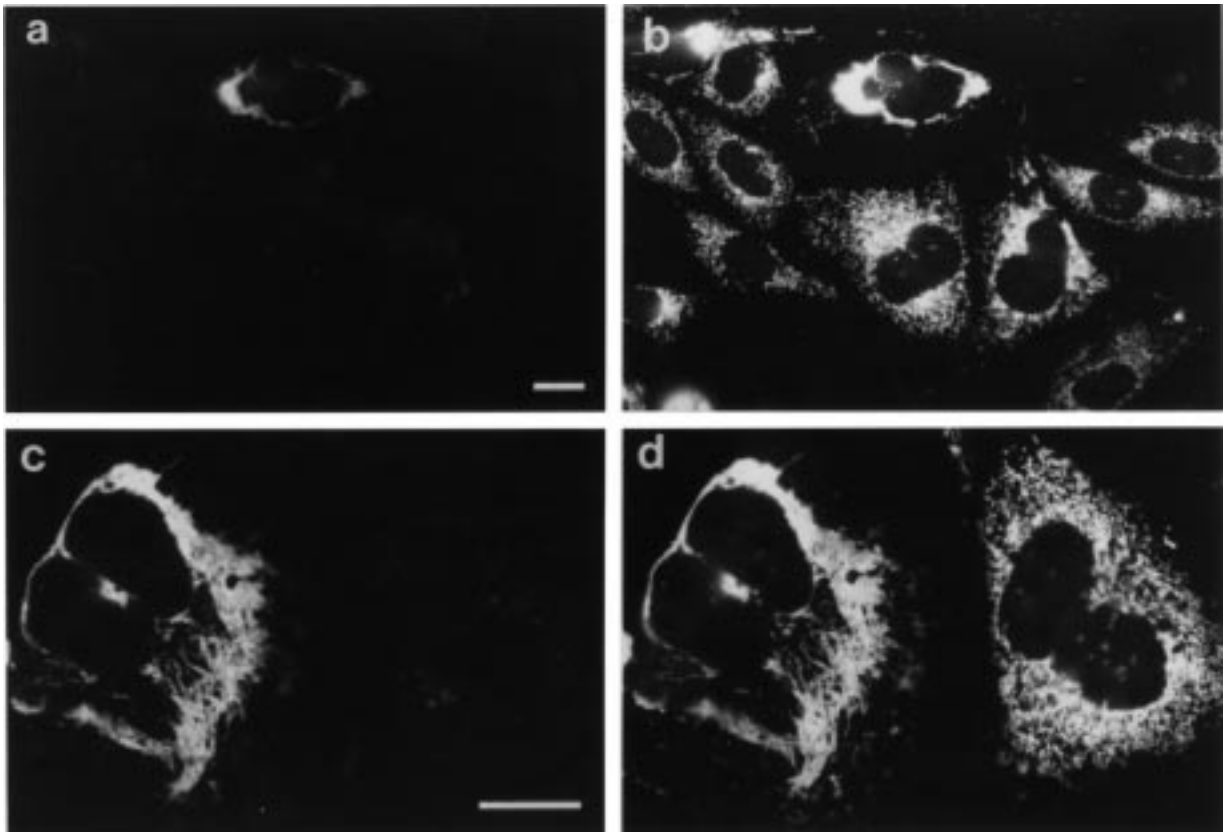


Fig. 5. Co-localization of HA-tagged OMP25 with mitochondria in CHO cells. Immunofluorescence of CHO cells transfected with the HA epitope-tagged OMP25 construct. The cells were double-stained with mouse monoclonal anti-HA antibody (a and c) and rabbit polyclonal antibody against cytochrome *c* oxidase (b and d). Scale bars correspond to 10 μm. Note the perinuclear clustering of mitochondria in transfected cells (**a** and **b**) which starts from bundling of mitochondria in cells expressing low levels of OMP25 (**c** and **d**).

janin 2A and OMP25. When synaptojanin 2A was co-expressed with OMP25, synaptojanin 2A was co-localized with OMP25 on the mitochondria as defined by Mito-Tracker staining, whereas synaptojanin 2A was distributed throughout the cytoplasm when overexpressed alone (Figure 8 and data not shown). We observed that the alterered morphology and distribution of mitochondria are more pronounced in the cells expressing OMP25 and synaptojanin 2A than in the cells expressing OMP25 alone. Similar results were obtained by co-expression of synaptojanin 2A and OMP25ΔN (data not shown). These results indicate that synaptojanin 2A is recruited to mitochondria by the interaction with the PDZ domain of OMP25.

Modified synaptojanin 2A with a mitochondrial anchor sequence bypasses the requirement for OMP25 for mitochondrial clustering

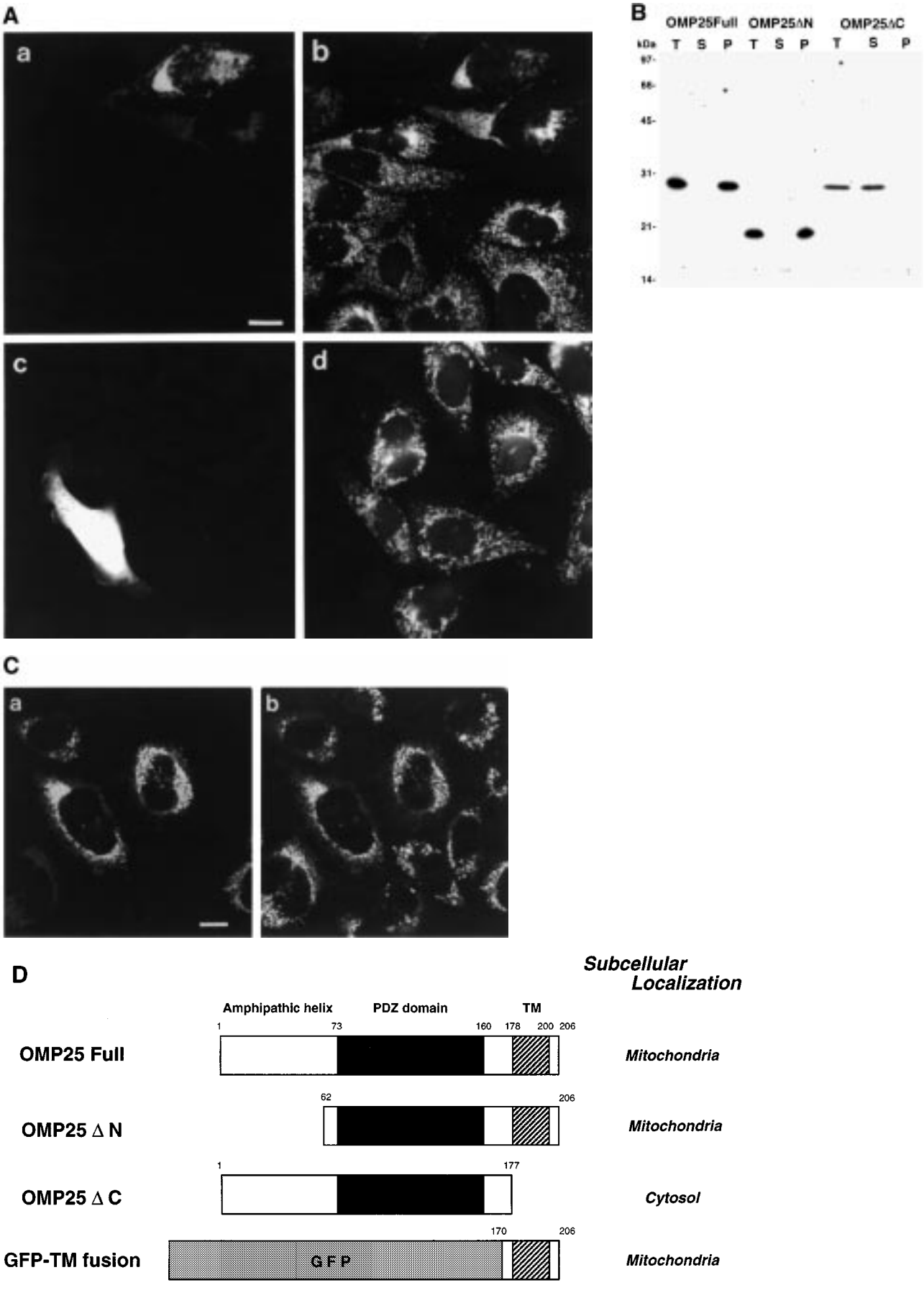
We further tested whether the enforced expression of synaptojanin 2A on the mitochondrial outer membrane results in a change in distribution of mitochondria independently of OMP25. An expression plasmid was constructed that produces synaptojanin 2A fused with the transmembrane domain of OMP25, in order to target synaptojanin 2A to the mitochondrial outer membrane without the assistance of OMP25. The modified synaptojanin 2A tagged with the transmembrane region of OMP25 was directed to mitochondria, and yielded an altered distribution of mitochondria in an expression level-dependent way (Figure 9B, a and b). In highly overexpressing cells, mitochondria collapsed into a paranuclear mass.

To test if the morphological changes depend on the inositol 5′-phosphatase activity of synaptojanin 2A, we prepared plasmids for expression of either the Sac1 homology region or the proline-rich region of synaptojanin 2A fused to the same transmembrane region (Figure 9A). Both of these chimeras, which lack the inositol 5′-phosphatase domain, were targeted to mitochondria but neither of them induced the morphological changes of mitochondria, although occasionally we observed a moderate clustering of mitochondria in some of the cells highly overexpressing the Sac1 domain chimera (Figure 9B, c–f). We conclude that the central 5′-phosphatase domain, and most likely the catalytic action of synaptojanin 2A, is required to alter the distribution and morphology of mitochondria.

Discussion

The C-terminus of synaptojanin 2A associates with the PDZ domain of OMP25, a novel mitochondrial outer membrane protein

The PDZ domain, also called DHR (disc large homology region) or GLGF repeat, is a conserved motif of ~90 amino acids, which was originally discovered in PSD-95 (Cho *et al*., 1992), Dlg-A (Woods and Bryant, 1991) and ZO-1 (Itoh *et al*., 1993). All these proteins have a similar domain organization, with three N-terminal PDZ domains,


A
a
b
c
d
B
OMP25Full
OMP25ΔN
OMP25ΔC
kDa
T S P T S P T S P
67-
68-
45-
31-
21-
14-
C
a
b
D
Subcellular Localization
Amphipathic helix
PDZ domain
TM
1
73
160
178
200
206
OMP25 Full
Mitochondria
62
206
OMP25 Δ N
Mitochondria
1
177
OMP25 Δ C
Cytosol
170
206
GFP-TM fusion
G F P
Mitochondria

a single internal SH3 domain and a C-terminal guanylate kinase domain that is enzymatically inactive (Kistner *et al*., 1995), and constitute a family of proteins collectively termed MAGUKs (membrane-associated guanylate kinase homologs). The PDZ domains in these proteins bind to the C-termini of cell surface receptors, ion channels and other intracellular signaling molecules and act as scaffolds on which macromolecular complexes are formed (for a recent review, see Fanning and Anderson, 1998). A tS/TXV motif was identified initially in the C-termini of the NMDA receptor NR2 subunits and the Shaker-type potassium channels and was required for binding to the first and second PDZ domains of PSD-95 and related proteins (Kim *et al*., 1995; Kornau *et al*., 1995; Lau *et al*., 1996; Niethammer *et al*., 1996). PDZ domains with various ligand-binding specificities have been identified subsequently in a wide range of proteins in diverse organisms including fungi and bacteria (Pallen and Wren, 1997).

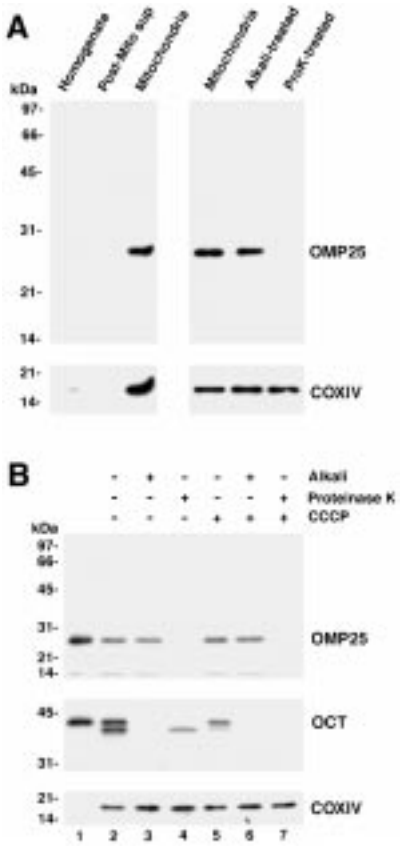


Fig. 7. OMP25 is an integral protein in the mitochondrial outer membrane. (**A**) Localization of OMP25 immunoreactivity in the rat liver mitochondria fraction. Left panel: mitochondria and post-mitochondrial supernatant (Post-Mito Sup) were prepared from rat liver homogenate. Each fraction (50 μg protein/lane) was subjected to immunoblotting with an antibody against the N-terminal peptide of OMP25 or an antibody against cytochrome *c* oxidase subunit IV (COXIV). Right panel: the isolated mitochondria were treated either with 0.1 M Na_2CO_3, pH 11.5 or 0.1 mg/ml proteinase K, as indicated, and subjected to immunoblotting. (**B**) *In vitro* import of OMP25 into the isolated mitochondria. Top panel: OMP25 was synthesized in reticulocyte lysate in the presence of [^{35}S]methionine (lane 1, 10% of the radiolabeled precursor protein) and incubated with the isolated rat liver mitochondria in the presence (lanes 5–7) or absence (lanes 2–4) of 1.0 μM CCCP. After the import, mitochondria were recovered by centrifugation (lanes 2 and 5). Portions were either resuspended in 0.1 M Na_2CO_3, pH 11.5, and the alkali-inextractable fractions obtained (lanes 3 and 6), or treated with 0.1 mg/ml proteinase K (lanes 4 and 7). Middle panel: radiolabeled human pre-OCT was synthesized and processed in the same way. Bottom panel: the samples were analyzed by immunoblotting with an antibody against COXIV.

The biochemical characterization in this study indicates that the C-terminus of synaptojanin 2A associates with OMP25 by a PDZ domain-mediated interaction and that the OMP25 PDZ domain largely shares characteristics of group 1 PDZ domains according to the recent classification of PDZ domains by Songyang *et al.* (1997). This is consistent with the high degree of similarity among the PDZ domain in OMP25 and those in PSD-95 and DLG, representatives of group 1 PDZ domains, as depicted in Figure 1B. The alanine replacement mutagenesis of the ligand substantiates the similarity in primary structure and confirms that S(–2) and V(0) are critical residues in the interaction of the synaptojanin 2A C-terminal sequence with the PDZ domain of OMP25. The OMP25 PDZ domain exhibits a novel property of requiring a glycine residue in position –3 of the ligand C-terminus for effective binding, which presumably contributes to its selective recognition of the synaptojanin 2A C-terminal sequence. The full explanation for the difference between the PDZ domain of OMP25 and those in PSD-95 and DLG in the preference for the –3 peptide position awaits more thorough structural analysis.

We demonstrated that OMP25 localizes to the mitochondrial outer membrane via the C-terminal signal anchor sequence (Shore *et al*., 1995). This is based on several lines of evidence. First, the Kyte–Doolittle hydrophobicity plot indicates the presence of a single membrane-spanning region near the C-terminus of OMP25, and deletion analysis showed that the transmembrane region is essential for its localization to the mitochondrial membrane. Secondly, the transmembrane region of OMP25 is capable of directing heterologous proteins exclusively to mitochondria, when attached at the C-termini, as shown by the localization of GFP or synaptojanin 2A chimeric proteins. Thirdly, *in vitro* mitochondria import experiments demonstrated that OMP25 is inserted into the outer membrane of mitochondria in the absence of the membrane potential, with the N-terminal portion facing the cytoplasm. Finally, an antibody raised against the N-terminal peptide of OMP25 recognized a 25 kDa protein in rat liver mitochondria, whose property is consistent with it being a mitochondrial outer membrane protein with an N_{out}–C_{in} orientation.

Fig. 6. The C-terminal transmembrane region is essential for mitochondrial localization of OMP25. (**A**) Immunofluorescence of CHO cells transfected with the HA epitope-tagged construct for OMP25 lacking either its N-terminal region (a and b) or its C-terminal transmembrane region (c and d). The cells were double-stained with mouse monoclonal anti-HA antibody (a and c) and rabbit polyclonal antibody against cytochrome *c* oxidase (b and d). The scale bar corresponds to 10 μm. (**B**) CHO cells were transfected as above and the total homogenates (T) were fractionated into the soluble (S) and the particulate fractions (P). The localization of OMP25 and its truncations was detected by immunoblotting with anti-HA antibody. (**C**) CHO cells were transfected with the construct for GFP fused to the C-terminal transmembrane region of OMP25. The cells were incubated with MitoTracker and then fixed. Fluorescence of GFP (a) and MitoTracker (b) was detected by confocal laser scanning microscopy. The scale bar corresponds to 10 μm. (**D**) Schematic representation of the structure of OMP25 and its variants and summary of the localization.

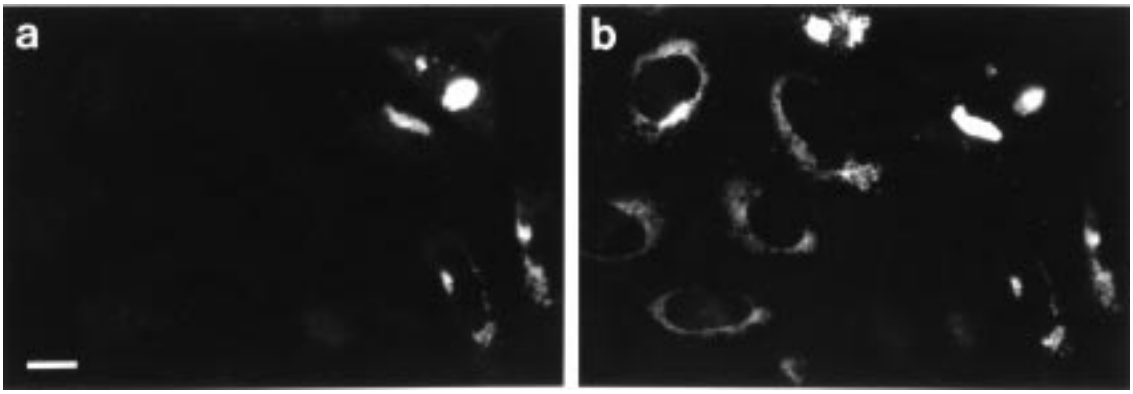


Fig. 8. Interaction of synaptojanin 2A and OMP25 in CHO cells. Immunofluorescence of CHO cells transfected with FLAG-tagged synaptojanin 2A and HA-tagged OMP25 constructs. The cells were double-stained with mouse monoclonal anti-FLAG antibody (**a**) and rabbit polyclonal anti-HA antibody (**b**). The scale bar corresponds to 10 μm.

The localization and orientation of OMP25 enable its PDZ domain to mediate the interaction with synaptojanin 2A in the cytosol. Synaptojanin 2A thus recruited could regulate inositol phospholipid levels on the mitochondrial outer membrane. OMP25 is so far unique among PDZ domain-containing proteins with respect to domain structure and subcellular localization. To our knowledge, this is the first example of an integral membrane protein with a PDZ domain in an intracellular organelle, which recruits a cytosolic protein to the organellar membrane.

Inositol phospholipid metabolism and distribution of mitochondria

A major finding of the present study is that overexpression of OMP25 results in a profound change in architecture of the mitochondrial network. This effect was mimicked by expression of mitochondria-targeted synaptojanin 2A, and not by the synaptojanin 2A mutant proteins without the 5′-phosphatase domain. We therefore suggest that the observed effect was caused by recruitment of endogenous synaptojanin 2A-related protein(s) and the concomitant change in metabolism of inositol phospholipids in the vicinity of mitochondria. At present, the means by which overexpression of OMP25 or synaptojanin 2A induces changes in the distribution of mitochondria is not clear. Obviously, the action of inositol 5′-phosphatases requires the presence of the substrates, inositol polyphosphates or polyphosphoinositides. There are only a few reports dealing with the contents of polyphosphoinositides and the enzyme activities for synthesis and breakdown of phosphoinositides in mitochondria. They are conflicting, probably due to metabolic instability of these compounds, as well as to the difference in the methods and sources which were used for the isolation of mitochondria, measurement of the enzyme activities and detection of phosphoinositides (reviewed in Daum and Vance, 1997). Nevertheless, the available literature shows that polyphosphoinositides, which can serve as substrate for synaptojanin 2A, are quantitatively minor components in the mitochondrial membranes. Therefore, we consider it unlikely that changes in phosphoinositide levels mediated by synaptojanin 2A lead directly to alteration of the structural integrity and the physical properties of the mitochondrial membrane. We hypothesize that changes in phosphoinositides in the vicinity of, or within, the mitochondrial outer membrane influence the activity of a component in the machinery which regulates distribution of mitochondria.

In budding yeast, the distribution of mitochondria is defective in the absence of a normal actin cytoskeleton, and an actin-based motor activity is associated with mitochondria (Drubin *et al*., 1993; Lazzarino *et al*., 1994; Simon *et al*., 1995; Smith *et al*., 1995). Genetic analyses have further identified several genes required for the motility and distribution of mitochondria in *S.cerevisiae* (McConnell *et al*., 1990; Hermann *et al*., 1997; reviewed in Yaffe, 1999). Among them, *MMM1*, *MDM10* and *MDM12* are of special interest to our study, as they encode integral proteins in the mitochondrial outer membrane, and loss of functions of these genes causes collapse of the tubular mitochondrial network into a large round mass (Burgess *et al*., 1994; Sogo and Yaffe, 1994; Berger *et al.*, 1997), somewhat similar to the abnormally packed mitochondria in the cells overexpressing OMP25 or mitochondria-tagged synaptojanin 2A. A potential role for these proteins in attachment of yeast mitochondria to the actin cytoskeleton has been suggested, and a recent report supports this view (Boldogh *et al*., 1998). It is tempting to speculate that OMP25 might have a role in docking of mitochondria to cytoskeletal elements in mammalian cells.

The transport or positioning mechanisms of mitochondria in higher eukaryotic cells are poorly understood and no structural components in mitochondria have been described so far in these processes. In fission yeast and higher eukaryotes, the localization of mitochondria depends on the microtubule cytoskeleton (Heggeness *et al*., 1978; Ball and Singer, 1982; Couchman and Rees, 1982; Summerhayes *et al*., 1983; Yaffe *et al*., 1996), while in budding yeast the microtubule function has little effect on the distribution of mitochondria (Huffaker *et al*., 1988). Kinesin or kinesin-like motor proteins have been found associated with mitochondria in both vertebrates and flies (Leopold *et al.*, 1992; Jellali *et al*., 1994; Nangaku *et al*., 1994; Elluru *et al.*, 1995; Pereira *et al*., 1997; Khodjakov *et al*., 1998). Inactivation of kinesin in human fibroblasts by microinjection of anti-kinesin antibody results in clustering of mitochondria in the perinuclear region (Rodionov *et al*., 1993). Specifically, Hirokawa and colleagues recently demonstrated that targeted disruption of mouse KIF5B, a conventional kinesin, results in clustering of

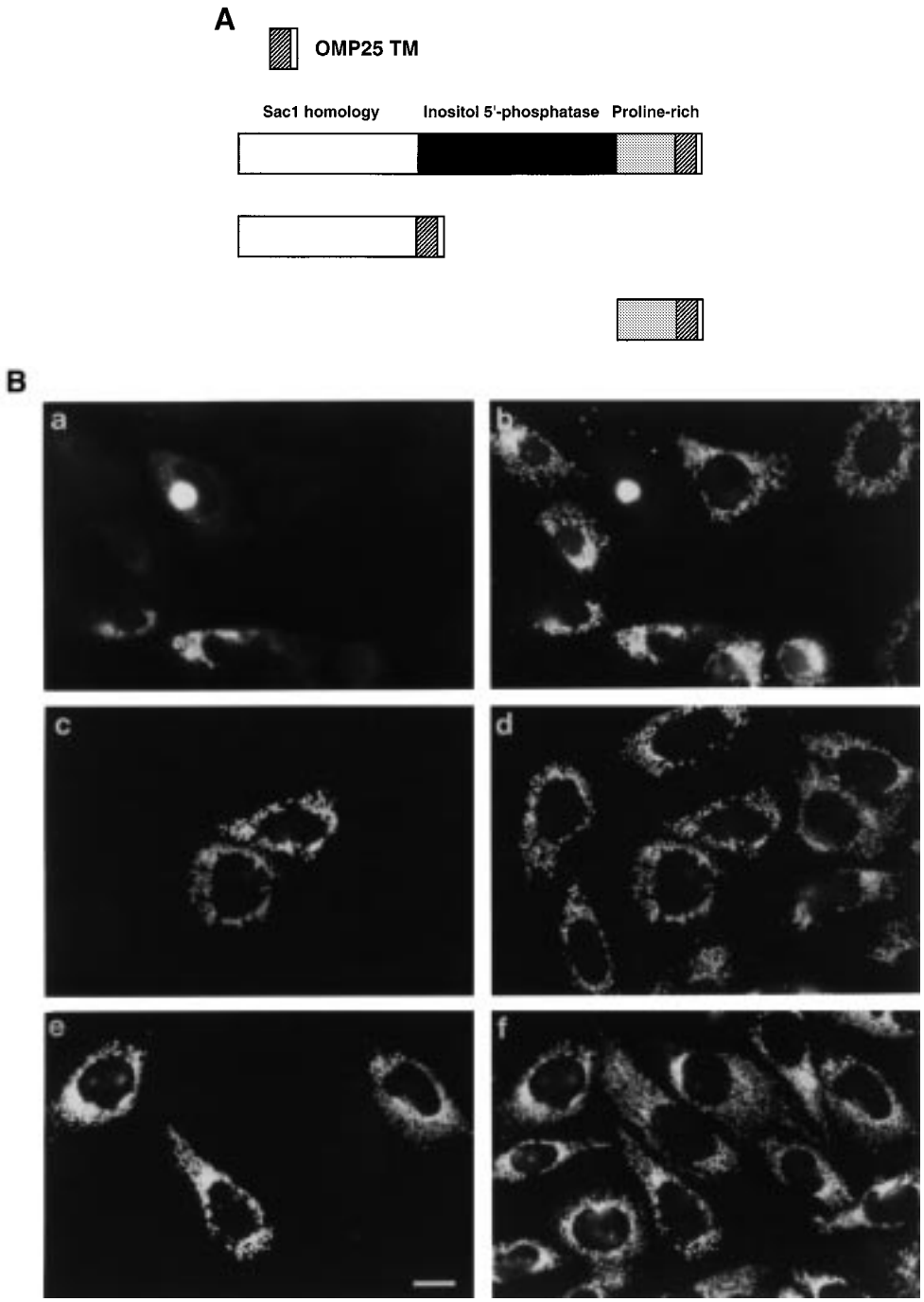


Fig. 9. Effect of expression of mitochondria-tagged synaptojanin 2A and its truncations on mitochondria in CHO cells. (**A**) Schematic representation of the structure of synaptojanin 2A constructs tagged with the C-terminal transmembrane region of OMP25. (**B**) Immunofluorescence of CHO cells transfected with the FLAG-tagged chimeric constructs for synaptojanin 2A (a and b), its Sac1 homology region (c and d) and its proline-rich region (e and f) fused to the transmembrane region of OMP25. The cells were double-stained with mouse monoclonal anti-FLAG antibody (a, c and e) and rabbit polyclonal antibody against cytochrome *c* oxidase (b, d and f). The scale bar corresponds to 10 μm.

mitochondria in the perinuclear region in the mutant extra-embryonic cells (Tanaka *et al*., 1998). Based on these observations, potential targets influenced by synaptojanin 2A-induced changes in phosphoinositide levels include microtubule-based motor proteins (for a recent review, see Hirokawa, 1998), motor-associated proteins such as dynactin (Gill *et al*., 1991), putative receptor(s) for the motor proteins on mitochondria (Vallee and Sheetz, 1996) and microtubule-associated proteins.

Although we have identified OMP25 homologs in other mammalian species (Figure 1D), we could not find a homologous gene product in the whole genome sequence of *S.cerevisiae*. Direct comparison with Mmm1p, Mdm10p and Mdm12p revealed no similarity. In addition, none of the products of three synaptojanin-like genes (*SJL1*, *2* and *3*) in *S.cerevisiae* bears the C-terminal motif for PDZ domain binding (Srinivasan *et al*., 1997). It therefore seems improbable that Sjl proteins are recruited to mitochondria in budding yeast in the same way as synaptojanin 2A is recruited by a PDZ domain-mediated interaction to

mitochondria in mammalian cells. The dependence of mitochondrial localization on different cytoskeletal elements in budding yeast and mammalian cells might be reflected in differences in other components of the machinery that controls their intracellular distribution. However, it should be pointed out that we did observe aberrant mitochondrial morphology, but not defective mitochondrial transport to buds, in a *Δsjl1 Δsjl2* mutant strain (Singer-Krüger *et al*., 1998), suggesting that Sjl proteins are implicated in proper mitochondrial organization in *S.cerevisiae*.

Alternative splicing and subcellular localization of synaptojanins

We and others have shown that several forms of synaptojanin 1 and 2 with distinct C-terminal regions are generated by alternative splicing in a tissue-specific and developmentally regulated manner (McPherson *et al.*, 1996; Ramjaun and McPherson, 1996; Nemoto *et al.*, 1997; Khvotchev and Südhof, 1998; Seet *et al*., 1998). The different C-terminal regions impart to them unique protein-binding properties, which may ensure their targeting and compartmentalization to specific cellular locations. Thus alternative splicing of synaptojanins provides a means of achieving tight control of inositol phospholipid levels in distinct microenvironments within cells. Here we have shown that the synaptojanin 2A-specific exon sequence at its C-terminal end mediates the interaction with OMP25, implicating synaptojanin 2A in regulation of the phosphoinositide pool on mitochondria.

Together with the recent identification of multiple alternatively spliced forms of dynamins and new dynamin-like proteins with distinct subcellular distributions (Shin *et al.*, 1997; Cao *et al.*, 1998; Imoto *et al.*, 1998; Kamimoto *et al*., 1998; Yoon *et al*., 1998), our studies support the notion that members of the synaptojanin family of inositol 5′-phosphatases and dynamin-like GTPases may cooperate functionally in various aspects of membrane dynamics. A dynamin or a dynamin-like protein associated with mitochondria in animal cells is yet to be identified, except for Fzo protein, with a GTPase domain remotely related to dynamin and implicated in mitochondrial fusion in *Drosophila melanogaster* (Hales and Fuller, 1997). However, Dnm1p and Mgm1p, *S.cerevisiae* dynamin-related proteins, associate with yeast mitochondria and are required for proper mitochondrial organization and functions (Otsuga *et al*., 1998; Shepard and Yaffe, 1999). It has been reported recently that overexpression of a mutant human dynamin-like protein results in perinuclear aggregation of mitochondria (Smirnova *et al*., 1998), raising the interesting possibility that dynamin-like protein and synaptojanin 2A may have a functional relationship in regulation of mitochondrial distribution in animal cells. These concepts will be tested by further characterization of synaptojanin family members and the cellular processes in which they participate.

Materials and methods

Yeast two-hybrid screening

The DNA fragment encoding the proline-rich region of rat synaptojanin 2A (amino acids 1030–1248) was amplified by PCR from the cloned cDNA (Nemoto *et al*., 1997) and subcloned into pBTM116 (a gift from Dr Stanley Hollenberg), which produces a LexA fusion and carries *Trp1*. A rat brain cDNA library constructed in pGAD10 vector (Clontech), which expresses a GAL4 activation domain fusion and contains *LEU2* as a selection marker, was screened by using the LexA–synaptojanin 2A proline-rich region bait construct and the yeast strain L40 [*MATa trp1-901 leu2-3,112 LYS2*:: $(lexAop)_4$-*HIS3 URA3*:: $(lexAop)_8$-*lacZ*]. Approximately 3×10^7 TRP^+LEU^+ transformants were selected on plates with supplemented minimum medium that lacked uracil, tryptophan, leucine, histidine and lysine in the primary screen and then tested for the β-galactosidase activity by the filter method in the secondary screen. One-hundred-and-thirty-five HIS^+LacZ^+ clones cured of the bait plasmid were tested for the specificity of the interaction by mating with the AMR70 strain transformed with the original LexA–synaptojanin 2A proline-rich region construct, the LexA–synaptojanin 1 proline-rich region construct expressing amino acids 1015–1317 of the major form of rat synaptojanin 1 (145 kDa form) or the lamin construct, as previously described (Vojtek *et al*., 1993; Ringstad *et al*., 1997). Thirty-six clones interacted specifically with the proline-rich region of synaptojanin 2A. By restriction mapping and partial sequence analysis, they were found to be independent isolates of four overlapping cDNAs encoding a novel protein with a PDZ domain, which we named OMP25.

Cloning of rat OMP25 cDNA

The cDNA fragment of a clone pGAD#1 was radiolabeled with [α-^{32}P]dCTP (Amersham) by the oligolabeling method and used as probe for screening of a rat brain λZAPII cDNA library (Stratagene) enriched in inserts >4 kb (Snutch *et al*., 1990) following standard techniques (Sambrook *et al*., 1989). The insert cDNA in a clone pBst/OMP25-8, with the largest ORF of 621 bp, did not contain an in-frame termination codon in the putative 5′-UTR. Complementary DNAs corresponding to the 5′-terminal region of rat OMP25 mRNA were obtained by two rounds of PCR on a rat brain λZAPII or pGAD10 cDNA library using the insert-specific primers corresponding to the complement of nucleotides 378–395 and 358–377 in OMP25 cDNA and the appropriate vector-specific primers. Sequence analysis of the PCR products indicated that they are identical except for length difference in their extreme 5′ ends. The consensus of the sequence of the four longest PCR products covers nucleotides 1–330, including an in-frame termination codon at nucleotides 312–314, in the composite cDNA sequence of OMP25 (Figure 1A).

Fusion protein production for protein-binding assays

The construct for production of GST fusion protein of the SH3 domain of rat SH3p4 has been described (Ringstad *et al*., 1997). The DNA fragment spanning the PDZ domain of OMP25 (amino acids 62–177) was generated by PCR and subcloned into pGEX4T-1 vector (Pharmacia) to produce the GST fusion protein. The DNA fragment for the three PDZ domains of rat DLG/SAP97 (amino acids 217–577) was amplified from rat brain total RNA by RT–PCR based on the published sequence (Müller *et al*, 1995). The GST fusion proteins were expressed in *E.coli* XL1 Blue and purified by gluthathione–Sepharose 4B column chromatography following the standard procedure. The proline-rich region of rat synaptojanin 2A was divided into four segments with 27–31 amino acids overlapping; segment I (amino acids 1030–1105), II (amino acids 1079–1158), III (amino acids 1128–1198) and IV (amino acids 1172–1248). The DNA fragments encoding these segments were generated by PCR. The last five amino acids of synaptojanin 2A in segment IV were replaced by alanine residues by PCR using the appropriate mutagenic primers. The MBP fusion proteins were produced by subcloning the PCR fragments into pMalC2 vector and purified by amylose resin according to the manufacturer's instructions (New England Biolab). Alternatively, the fragments were subcloned into pBTM116 to express the LexA fusion proteins for yeast two-hybrid assays.

Construction of the plasmids for transient expression study

The constructs for expression of FLAG-tagged synaptojanin 1 and 2A, pcDNA3/FLAG SJ1 and pcDNA3/FLAG SJ2A, have been described (Nemoto *et al.*, 1997). The expression plasmids for OMP25 and its truncations encoding amino acids 62–206 (OMP25ΔN) or 1–177 (OMP25ΔC) were generated by PCR and subcloning into pHA, a modified pcDNA3 expression vector (Invitrogen) with an HA (hemagglutinin nonapeptide) epitope (Y.Nemoto, unpublished results). The C-terminal transmembrane region of OMP25 was amplified by PCR and subcloned into pEGFP C1 (Clontech) to generate pEGFP-OMP25C, which encodes the enhanced GFP fused with amino acids 170–206 of OMP25. The chimeric expression plasmids for synaptojanin 2A, the *Sac*1 homology region or the proline-rich region tagged with the transmembrane region

of OMP25 were constructed by PCR and subcloning into pFLAG, a modified pcDNA3 expression vector encoding a FLAG epitope (Y.Nemoto, unpublished results). The resultant plasmids, pFLAG/SJ2A-OMP25TM, pFLAG/SJ2ASac1-OMP25TM and pFLAG/SJ2APro-OMP25TM, encode FLAG-tagged amino acids 1–1218, 1–522 and 1030–1218 of rat synaptojanin 2A followed by amino acids 170–206 of OMP25, respectively.

Northern blot analysis

A Northern blot filter with 2 μg of poly(A)$^+$ RNA from various adult rat tissues (rat multiple tissue Northern blot, Clontech) was probed for OMP25 under stringent conditions. The DNA fragments used as probes were a PCR-amplified fragment spanning the PDZ domain (nucleotides 639–986) and a *Pst*I–*Xho*I fragment (nucleotides 3121–4329) and a *Xho*I–*Sty*I fragment (nucleotides 4329–4990) from the middle and distal parts of the 3′-UTR of OMP25 cDNA, respectively.

Yeast two-hybrid protein-binding assay

Yeast AMR70 strains were transformed with the indicated pBTM116 constructs and mated with the L40 strain harboring pGAD#1. The resulting diploid strains were selected on plates with supplemented minimum medium lacking uracil, tryptophan and leucine, and tested for growth on plates with supplemented minimum medium lacking uracil, tryptophan, leucine and histidine. For quantification of β-galactosidase activity, the diploid strains were grown in supplemented minimum medium lacking uracil, tryptophan and leucine at 30°C for 48 h. The yeast extracts were prepared from the culture and the β-galactosidase activities were assayed in triplicate using *o*-nitrophenyl-β-D-galactoside (Sigma) as substrate and expressed in Miller units (Miller, 1972).

In vitro solution protein-binding assay

The peptides were coupled via cysteine residues to SulfoLink coupling gel (Pierce) at 0.5 μmol of peptide/ml of gel following the manufacturer's instructions. The peptide-coupled beads were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST) and incubated with GST–PDZ fusion proteins in 1% BSA in PBST at 100 nM at 4°C for 2 h. The beads were washed three times with PBST. The bound fusion proteins were released by boiling in Laemmli sample buffer (Laemmli, 1970) and subjected to SDS–PAGE and immunoblotting with rabbit anti-GST antibody.

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Maxisorb F96, Nunc) were coated with the N- and C-terminal peptides of rat synaptojanin 2A by incubating with 50 μl/well of 1 μg/ml peptide solution in 50 mM $NaHCO_3$, pH 9.0, overnight at 4°C. Binding of serial dilutions of purified GST–PDZ fusion proteins or GST alone to the peptides was measured by ELISA essentially as described (Müller *et al*., 1996). The color reaction was developed in 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) in 50 mM phosphate–citrate buffer, pH 5.0, plus 0.02% (v/v) hydrogen peroxide for 10 min. The reaction was stopped with 3 M H_2SO_4, and absorbance at 492 nm was read by a microplate reader. Baseline absorbance from GST protein was subtracted from the values. Data were fit with a Hill equation ($Abs = Absmax/1 + [EC_{50}/[X]]^n$) (Abs = absorbance at 492 nm) using a non-linear least square algorithm.

Mitochondrial protein import

OMP25 was transcribed and translated from pBst/OMP25-8 *in vitro* by using T7 RNA polymerase and rabbit reticulocyte lysate (TNT-coupled reticulocyte system, Promega) in the presence of [^{35}S]methionine (Amersham) following the manufacturer's instructions. Radiolabeled human pre-OCT was prepared from a plamid encoding human OCT under the SP6 promotor (a gift from Dr Grazia Isaya, Yale University) in the same way, except for the use of SP6 RNA polymerase instead of T7 RNA polymerase.

Rat liver mitochondria were prepared as described by Mihara and Omura (1995). The *in vitro* mitochondria import reactions were carried out following the method of Shore and colleagues (Nguyen *et al*., 1986) either in the presence or absence of 1.0 μM CCCP (Sigma). The mitochondria were recovered by layering 50 μl aliquots over a 750 μl sucrose cushion (0.25 M sucrose, 10 mM HEPES–KOH, pH 7.5, 1.0 mM dithiothreitol) and centrifuging at 12 000 *g* for 5 min. Pellets were solubilized in Laemmli sample buffer either directly or after alkali extraction (Fujiki *et al*., 1982) or post-protease treatment. Alkali extraction was carried out by suspending in freshly prepared 0.1 M sodium carbonate, pH 11.5, at a concentration of 0.25 mg protein/ml and incubating on ice for 30 min with periodic vortexing. Membrane was collected by centrifugation at 70 000 r.p.m. for 30 min in a Beckman TL-100 ultracentrifuge. For post-proteinase treatment, the pelleted mitochondria were resuspended in the reaction mixture and incubated with 0.1 mg/ml proteinase K at a concentration of 0.25 mg protein/ml on ice for 20 min. The reaction was terminated by addition of phenylmethyl sulfonylfluoride to 2 mM, and mitochondria were immediately pelleted and solubilized in Laemmli sample buffer. The import products were resolved by SDS–PAGE and visualized by fluorography.

Antibodies

Rabbit polyclonal antibodies against cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) were generous gifts from Dr Cesare Montecucco (Università di Padova, Padova, Italy). A rabbit polyclonal antibody against the C-terminal peptide of rat synaptojanin 2A has been described (Nemoto *et al*., 1997). A rabbit polyclonal antibody against OMP25 was generated against an N-terminal 18 amino acid synthetic peptide coupled via cysteine at the C-terminal residue to keyhole limpet hemocyanin and affinity purified following the standard procedure (Harlow and Lane, 1988). Mouse monoclonal anti-FLAG antibody M5 was obtained from Eastman Kodak. Mouse monoclonal anti-HA antibody 12CA5 and rabbit polyclonal anti-HA antibody Y-11 were from BabCo and Santa Cruz Biotechnology, respectively.

Miscellaneous procedures

CHO cells were grown on coverslips in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected with expression plasmids using Lipofectamine (Gibco-BRL). Coverslips were fixed and processed for indirect immunofluorescence as described (Cameron *et al*., 1991). Where indicated, MitoTracker CMTMRos (Molecular Probes) was added to the culture medium at a concentration of 0.5 μM for 30 min to stain mitochondria before fixation. Coverslips were mounted in VectaShield (Vector Labolatories) and observed through an epifluorescent microscope Axiophot (Zeiss) or a confocal laser microscope MRC1024 (Bio-Rad). Procedures for SDS–PAGE, immunoblotting and subcellular fractionation of transfected cells were as described (Nemoto *et al*., 1997). Preparation of rat brain Triton X-100 extract and affinity chromatography onto GST fusion proteins pre-bound to a glutathione–Sepharose 4B column were performed as described (Ringstad *et al*., 1997). Protein concentrations were determined by the method of Bradford (1976) with BSA as standard. PCR was performed by using Vent polymerase (New England Biolab) following the manufacturer's instructions. The PCR-derived fragments were subcloned into pBluescript and the integrity was verified by nucleotide sequencing before introducing into the appropriate expression vectors.

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