A mitochondrial membrane protein is required for translocation of phosphatidylserine from mitochondria-associated membranes to mitochondria

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The mechanism of import of phosphatidylserine (PtdSer) into mitochondria was investigated using a reconstituted system of isolated organelles *in vitro* in which PtdSer was translocated from donor membranes to mitochondria and was decarboxylated therein. Neither phosphatidylcholine nor phosphatidylethanolamine (PtdEtn) was translocated under the same conditions. Transfer of PtdSer from its site of synthesis on the endoplasmic reticulum and mitochondria-associated membranes [J. E. Vance (1990) J. Biol. Chem. **265**, 7248–7256] to its site of decarboxylation on mitochondrial inner membranes is predicted to be mediated by membrane contact. A mitochondrial membrane protein appears to be involved in the translocation event since proteolysis of proteins exposed on the mitochondrial surface potently inhibited PtdSer transfer, whereas proteolysis of surface proteins of mitochondria-associated membranes did not impair the transfer. The nature of the membranes that donate PtdSer to mitochondria *in vitro* is not crucial since PtdSer of mitochondria-associated membranes, endoplasmic reticulum and microsomes was decarboxylated to PtdEtn with approximately equal efficiency. The translocation of PtdSer to mitochondria was stimulated by magnesium and calcium ions and was inhibited by incubation of mitochondria with sulphydryl group-modifying reagents. Reconstitution of PtdSer translocation/decarboxylation using digitonin-solubilized mitochondria and PtdSer-donor membranes suggested that the putative PtdSer-translocation protein is primarily localized to contract sites between mitochondrial inner and outer membranes. These studies provide evidence for the involvement of a mitochondrial membrane protein in the import of newly-synthesized PtdSer into mitochondria.

INTRODUCTION

In animal cells, the formation of phosphatidylethanolamine (PtdEtn) from phosphatidylserine (PtdSer) requires both interorganelle and intramitochondrial translocation since PtdSer synthase, the enzyme that synthesizes PtdSer, is located on endoplasmic reticulum (ER) membranes [1–3], whereas the enzyme that decarboxylates PtdSer to PtdEtn (PtdSer decarboxylase) is located on the outer aspect of mitochondrial inner membranes [1,4,5]. Interestingly, in yeast, two PtdSer decarboxylases have been identified that are encoded by different genes and are located in different organelles. One protein, encoded by the *PSD1* gene, is mitochondrial whereas the other, encoded by the *PSD2* gene, is located in Golgi and vacuoles [6,7]. In animal cells, however, only a single PtdSer decarboxylase, which resides in mitochondria, has so far been described.

Some information on the mechanism by which PtdSer is imported into mitochondria has been provided by reconstitution of PtdSer transport in permeabilized cells and in systems of isolated organelles [8–11]. However, molecular details of the individual steps involved in the translocation event have not yet been elucidated. Movement of newly-synthesized PtdSer to mitochondria is believed to occur by a mechanism that does not require cytosolic proteins [8,11] but, most likely, involves a direct contact between mitochondria and the membranes that synthesize PtdSer [12]. The existence of zones of contact between the ER and mitochondria has been supported for many years on the basis of morphological evidence [13–17]. The concept that such regions of membrane contact exist was strengthened when mitochondria-associated, ER-like membranes were isolated from rat liver [18]. Mitochondria-associated membranes (MAM) are enriched, compared with the bulk of the ER, in several lipid-biosynthetic enzyme activities, particularly PtdSer synthase [18–20]. A specific marker protein for MAM in rat liver, PtdEtn *N*-methyltransferase-2, which is absent from other organellar membranes such as ER, Golgi, plasma membranes and mitochondria, has been identified [21].

Immunoelectron microscopy studies with rat-liver slices [21], and immunofluorescence localization studies with rat hepatocytes [19] using antibodies directed against PtdEtn methyltransferase-2, have revealed that membranes containing the methyltransferase are localized to defined clusters that do not correspond to the bulk of ER but are, for the most part, present in the vicinity of mitochondria. Membrane fractions similar to rat-liver MAM have also been isolated from mouse liver [22], the yeast *Saccharomyces cerevisiae* [23,24], rat brain [25] and Chinese hamster ovary cells [20]. Ardail et al. [22] have also demonstrated that MAM-like membranes from mouse liver are associated with contact sites between mitochondrial inner and outer membranes. Moreover, recent data support the idea that in intact cells the import of PtdSer into mitochondria from microsomes occurs via MAM [20].

PtdSer transfer to mitochondria is ATP-dependent in both intact and permeabilized cells [10,12,26]. However, neither ATP, nor any other nucleoside triphosphate, stimulates PtdSer transfer to mitochondria in cell-free systems [8,11]. Voelker [8] has suggested that the ATP-dependent step of PtdSer translocation *in vivo* precedes the events that are reconstituted with isolated

Abbreviations used: ER, endoplasmic reticulum; MAM, mitochondria-associated membranes; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; ERI, ER enriched in rough ER; ERII, ER enriched in smooth ER.

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organelles *in vitro*. For example, ATP might be required for a membrane-collision event in which the membranes that synthesize PtdSer become oriented in a position that facilitates PtdSer export to mitochondria. We now show that PtdSer import into mitochondria in a reconstituted system of isolated organelles *in vitro* requires a mitochondrial membrane protein.

MATERIALS AND METHODS

Materials

[3-^{3}H]Serine, [*methyl*-^{3}H]choline and [1-^{3}H]ethanolamine were purchased from Amersham, Oakville, ON, Canada. Silica gel G TLC plates (0.25 mm thickness) were from BDH Chemicals. L-Serine, leupeptin, PMSF, dithiothreitol, *N*-ethylmaleimide, Mersalyl acid, diethylpyrocarbonate, dicyclohexylcarbodiimide, phenylisothiocyanate, *N*-acetylimidazole, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, digitonin and hydroxylamine were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. Phospholipid standards were from Avanti Polar Lipids, Birmingham, AL, U.S.A. All other reagents were from Fisher Scientific, Edmonton, AB, Canada.

Preparation of rat-liver subcellular fractions

Mitochondria, MAM and microsomes were freshly prepared from rat liver as described previously [11,18,19]. ER fractions were isolated from rat liver according to the procedure of Croze and Morré [27], modified as described [3]. ERI (enriched in rough ER) was obtained from the final sucrose gradient at the interface between sucrose solutions of 1.5 M and 2.0 M, whereas ERII (enriched in smooth ER) was isolated from the interface between 1.5 M and 1.3 M sucrose solutions [27].

Preparation of [^{3}H]PtdSer-labelled membranes

MAM, microsomes, ERI and ERII (5 mg of protein) were incubated for 1 h at 37 °C with 40 μCi of [3-^{3}H]serine (0.4 mM) in a total volume of 3 ml of buffer consisting of 25 mM Hepes (pH 7.4), 1 mM $CaCl_2$ and 2 mM $MgCl_2$. The mixture was centrifuged at 100000 $\boldsymbol{g}_{max}$ for 45 min in a Beckman Ti70.1 rotor. The resulting pellet (typically 8×10^4 c.p.m. in [^{3}H]PtdSer/mg of protein) was washed twice with isolation medium [250 mM mannitol/5 mM Hepes, pH 7.4/0.5 mM EGTA/0.1% BSA], then resuspended in isolation medium and set on ice.

Translocation *in vitro* of PtdSer from donor membranes to mitochondria

For reconstitution of PtdSer translocation using pre-labelled [^{3}H]PtdSer, ER, microsomes and MAM were labelled with [^{3}H]PtdSer (as described in the previous section), and each membrane fraction (500 μg of protein) was incubated separately at 37 °C for up to 4 h with mitochondria (500 μg of protein) in a total volume of 200 μl of 25 mM Hepes buffer (pH 7.4) containing 2 mM $MgCl_2$. In other experiments, the concerted synthesis, translocation and decarboxylation of PtdSer were reconstituted by incubation of MAM, microsomes or ER (500 μg of protein) with mitochondria (500 μg of protein) in a total volume of 200 μl of 25 mM Hepes buffer (pH 7.4) containing 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 4 μCi of [3-^{3}H]serine (0.4 mM) for up to 4 h at 37 °C.

Reactions were terminated by the addition of 6 ml of chloroform/methanol (2:1, v/v) and 1.8 ml of water. Phospholipids were extracted and PtdSer, PtdEtn and lysoPtdEtn were separated by TLC in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, by vol.). In this solvent system, lysoPtdEtn and PtdCho co-chromatograph. Therefore, in some experiments, lysoPtdEtn and PtdCho were separated by TLC in the solvent system chloroform/methanol/30% ammonia/water, 65:35:5:2.5 (by vol.). The concentrations of Ca^{2+}, Mg^{2+} and ATP of the translocation buffer were varied from 0 to 5 mM. The translocation-dependent decarboxylation of PtdSer was calculated as the % of d.p.m. in decarboxylated products (PtdEtn + lysoPtdEtn) compared with total incorporation of radioactivity into (PtdSer + PtdEtn + lysoPtdEtn).

Translocation *in vitro* of PtdSer from MAM to reagent-treated mitochondria or from proteolysed MAM to mitochondria

Mitochondria (500 μg of protein in 200 μl of isolation medium) were exposed to one of the following conditions: trypsin (25 μg/ml) at 0 °C for 45 min followed by addition of soya bean trypsin inhibitor (0.4 mg/ml); proteinase K (25 μg/ml) at 0 °C for 45 min; *N*-ethylmaleimide (5 mM), Mersalyl acid (1 mM), diethylpyrocarbonate (1 mM), dicyclohexylcarbodiimide (2.5 mM), phenylisothiocyanate (2 mM), *N*-acetylimidazole (33 mM), phenylglyoxal (2 mM) or 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (5 mM) at 30 °C for 30 min. For the experiment in which membranes were treated with KCl, mitochondria were incubated at 4 °C for 45 min in 10 mM Tris (pH 7.4) containing 125 mM KCl and 1 mM EDTA. For digitonin treatment, 20 mg of mitochondria were resuspended in 1 ml of buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4) and the indicated concentrations of digitonin (up to 4 mg/ml) at 0 °C for 15 min. All reactions were stopped by dilution with isolation medium. Mitochondria were re-isolated by centrifugation at 6300 $\boldsymbol{g}_{max}$ for 10 min then resuspended in 800 μl isolation medium. Reagent-treated mitochondria (500 μg of protein) were used in the translocation assay with [^{3}H]PtdSer-labelled MAM. In some experiments [^{3}H]PtdSer-labelled MAM were incubated with the reagents as described above then re-isolated by centrifugation at 100000 $\boldsymbol{g}_{max}$ for 1 h, resuspended in isolation medium and incubated with untreated mitochondria in the PtdSer-translocation assay.

Translocation *in vitro* of PtdCho and PtdEtn from MAM to mitochondria

MAM (500 μg of protein) were incubated for 1 h in 25 mM Hepes buffer (pH 7.4) containing 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 1 μCi of either [*methyl*-^{3}H]choline (0.4 mM) or [1-^{3}H]ethanolamine (0.4 mM) at 37 °C in a total volume of 200 μl. Presumably, PtdCho and PtdEtn were labelled via the base-exchange pathway, rather than by the CDP-choline or -ethanolamine pathways, since non-membrane-associated components are required for the latter. Radiolabelled MAM were washed then incubated in a volume of 200 μl at 37 °C for up to 4 h with mitochondria in translocation buffer. Mitochondria were subsequently re-isolated by Percoll gradient ultracentrifugation [18], or by centrifugation at 6300 $\boldsymbol{g}_{max}$ for 10 min and the amount of radiolabelled PtdCho and PtdEtn transferred was determined.

Membrane-fusion assay

Mitochondria (5 mg of protein) were incubated at 37 °C for 4 h with MAM (5 mg of protein) in 2 ml of 25 mM Hepes buffer (pH 7.4) containing various concentrations of $MgCl_2$ or $CaCl_2$, followed by addition of 10 mM EDTA. Mitochondria were re-isolated by centrifugation at 6300 $\boldsymbol{g}_{max}$ for 10 min and subsequent Percoll gradient centrifugation [18]. PtdSer synthase activity was

assayed [20] in isolated mitochondria and MAM to determine the extent of fusion of MAM with mitochondria.

Measurement of PtdSer decarboxylase activity

Both a $^{14}CO_2$-trapping method [26] and a [^{3}H]PtdSer-decarboxylation method were used with similar results. For the latter, the reaction mixture consisted of 0.1 M KH_2PO_4 (pH 6.8), 10 mM EDTA, 0.5 mg/ml Triton X-100, [^{3}H]PtdSer (90 μM, 2.4 μCi/μmol) and 1 mg of mitochondrial protein. The mixture was incubated in a final volume of 50 μl for 1 h at 37 °C. [^{3}H]PtdSer and [^{3}H]PtdEtn were extracted and separated by TLC. The extent of decarboxylation of PtdSer to PtdEtn was used to determine the specific activity of PtdSer decarboxylase.

Other methods

Protein content was determined according to the method of Lowry et al. [28] using BSA as a standard. Lipid phosphorus was analysed by the method of Rouser and Fleischer [29].

RESULTS

Reconstitution of the translocation-dependent decarboxylation of PtdSer

For reconstitution of PtdSer translocation, MAM and mitochondria were isolated from rat liver. Contamination of MAM by mitochondria was $< 20\%$, as estimated by comparison of the swpecific activity of the mitochondrial enzyme cytochrome *c* oxidase [30] in MAM and mitochondria. Analysis of PtdSer synthase activity [3,20] in mitochondria showed that only $\sim 5\%$ of mitochondrial proteins could be attributed to contamination by ER. When MAM were incubated with [3-^{3}H]serine, PtdSer was readily radiolabelled. After 4 h of incubation, less than 10% of radiolabelled lipids were PtdSer decarboxylation products (PtdEtn + lysoPtdEtn). Insignificant incorporation of [^{3}H]serine into lipids occurred when mitochondria were incubated without MAM, confirming that the mitochondria were minimally contaminated by PtdSer synthase. When MAM and mitochondria were co-incubated in the presence of 1 mM $CaCl_2$, 2 mM $MgCl_2$ and [3-^{3}H]serine, radioactivity was incorporated into PtdSer, then into PtdEtn and lysoPtdEtn in a time-dependent manner (Figure 1A). After 4 h of incubation the distribution of radioactivity between PtdEtn and lysoPtdEtn was $\sim 50\%$ in each lipid. The translocation-dependent decarboxylation of PtdSer, which we define as the percentage of d.p.m. in decarboxylation products (PtdEtn + lysoPtdEtn) compared with total incorporation of radioactivity into (PtdSer + PtdEtn + lysoPtdEtn), was $> 70\%$ after 4 h.

A second system for reconstitution of PtdSer translocation and decarboxylation, using pre-labelled PtdSer was also used. MAM were incubated with [3-^{3}H]serine for 1 h and [^{3}H]PtdSer-labelled MAM were re-isolated and incubated with mitochondria. The translocation-dependent decarboxylation of [^{3}H]PtdSer (as measured by production of [^{3}H]PtdEtn and [^{3}H]lysoPtdEtn) was monitored. After 4 h, 55% of [^{3}H]PtdSer had been translocated and decarboxylated (Figure 1B). Calculation of the percentage of translocation-dependent decarboxylation does not take into account any degradation of PtdSer, PtdEtn and/or lysoPtdEtn that presumably occurs, as indicated by Figure 1(B), which shows that the recovery of total d.p.m. in (PtdSer + PtdEtn + lysoPtdEtn) decreased by 17% during the 4 h incubation. These experiments demonstrate that co-incubation of MAM with mitochondria results in the translocation and decarboxylation of both newly synthesized (figure 1A) and pre-labelled (Figure 1B) PtdSer. In a control experiment, we investigated

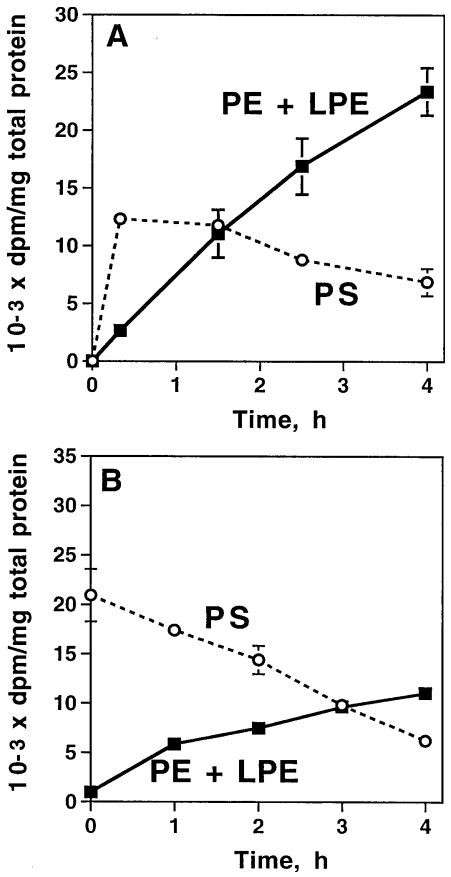

Figure 1 Translocation of PtdSer is reconstituted when MAM are incubated with mitochondria

(**A**) MAM and mitochondria were co-incubated with [3-^{3}H]serine for the indicated times, and then lipids were extracted. (**B**) [^{3}H]PtdSer-labelled MAM were incubated with mitochondria for the indicated times, and then lipids were extracted. For all incubations PtdSer (PS; ○) and its decarboxylation products (PtdEtn + lysoPtdEtn; PE + LPE; ■) were isolated by TLC and incorporation of radioactivity was measured. All data are means ± S.D. of three independent experiments. In some instances, error bars are too small to be visible.

whether or not MAM (which contain high PtdSer synthase activity) had fused with mitochondrial membranes during the incubation. Mitochondria were incubated for 4 h with MAM, after which the mitochondria were re-isolated by centrifugation on Percoll. The re-isolated mitochondria contained no measurable PtdSer synthase activity (results not shown) confirming that the reconstituted translocation and decarboxylation of PtdSer *in vitro* did not result from artifactual membrane fusion.

PtdCho and PtdEtn are not imported into mitochondria *in vitro*

We next determined whether phospholipid transfer to mitochondria in the reconstituted system was specific for PtdSer or whether PtdEtn and PtdCho could also be imported into mitochondria under the same conditions. MAM, radiolabelled with either PtdCho or PtdEtn, were incubated with mitochondria for up to 4 h after which the mitochondria were re-isolated and the amount of [^{3}H]PtdCho or [^{3}H]PtdEtn transferred to mitochondria was determined. No significant transfer of [^{3}H]PtdCho or [^{3}H]PtdEtn from MAM to mitochondria occurred, whereas under the same conditions PtdSer was efficiently transferred

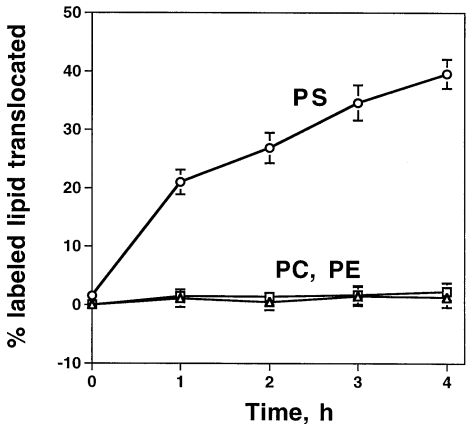

Figure 2 PtdCho and PtdEtn are not translocated to mitochondria

MAM, pre-labelled with either [^{3}H]PtdCho or [^{3}H]PtdEtn, were incubated with mitochondria for the indicated times after which mitochondria were re-isolated. The percentage of translocated [^{3}H]PtdCho (PC; △) or [^{3}H]PtdEtn (PE; □) was calculated as percentage of d.p.m. in lipids in re-isolated mitochondria relative to the initial d.p.m. in MAM. The percentage of translocated [^{3}H]PtdSer (PS; ○) was calculated from the data in Figure 1(B) as percentage of d.p.m. in decarboxylation products (PtdEtn + lysoPtdEtn) compared with d.p.m. in PtdSer in the starting preparation of MAM. All data are means ± S.D. of three independent experiments. In some instances, error bars are too small to be visible.

(Figure 2). These data indicate that the reconstituted import of phospholipids into mitochondria was specific for PtdSer.

PtdSer translocation to mitochondria is not donor-membrane specific

Microsomes, MAM and two ER fractions (ERI and ERII [27]) were labelled with [^{3}H]PtdSer and compared for their ability to donate PtdSer to mitochondria. All these membranes transferred PtdSer to mitochondria with approximately equal efficiency (the percentage of translocation-dependent decarboxylation was 65–77%), demonstrating that MAM were not the only possible PtdSer-donor membranes in the *in vitro* reconstitution assay. Moreover, in previous similar reconstitution experiments PtdSer liposomes were able to supply PtdSer to mitochondria for decarboxylation to PtdEtn [9].

Translocation of PtdSer is stimulated by magnesium

The metal ion requirement for the mitochondrial import and decarboxylation of PtdSer *in vitro* was examined in mixtures containing various concentrations of $MgCl_2$ (Figure 3A) and $CaCl_2$ (Figure 3B). With the range of ion concentrations used, fusion of donor and acceptor membranes did not occur since essentially no PtdSer synthase activity was recovered in mitochondrial membranes re-isolated after the incubations (results not shown). The percentage of translocation-dependent decarboxylation of PtdSer increased from 33 to 76% as the Mg^{2+} ion concentration was increased from 0 to 5 mM (Figure 3A). In this reconstitution protocol, production of PtdEtn results from at least two processes, translocation of PtdSer from donor membranes to mitochondria, and decarboxylation of PtdSer to PtdEtn. To distinguish between the effect of Mg^{2+} on the translocation and decarboxylation steps, we showed that Mg^{2+} (at concentrations up to 5 mM) neither stimulated nor inhibited PtdSer decarboxylase (results not shown). Therefore, Mg^{2+} stimulates the translocation of PtdSer, but not its decarboxylation.

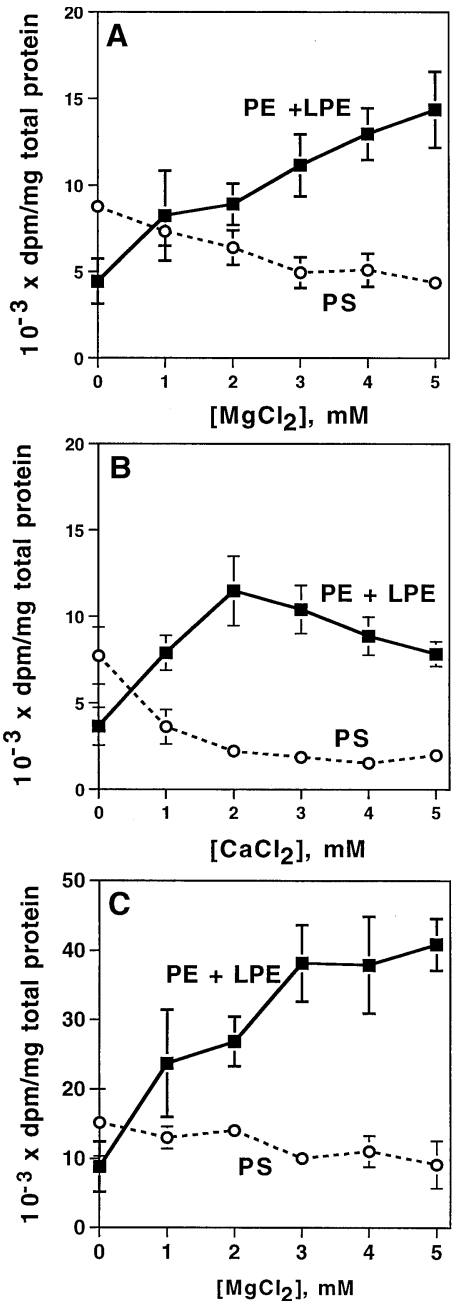

Figure 3 Mg^{2+} and Ca^{2+} ions stimulate PtdSer translocation

PtdSer in MAM was labelled with [^{3}H]serine, then the labelled MAM were incubated with mitochondria for 4 h at 37 °C in buffer containing either 1 mM $CaCl_2$ and the indicated concentrations of $MgCl_2$ (**A**), or 2 mM $MgCl_2$ and the indicated concentrations of $CaCl_2$ (**B**). Lipids were extracted and PtdSer (PS; ○) and its decarboxylated products (PtdEtn + lysoPtdEtn; PE + LPE; ■) were isolated. (**C**) Unlabelled MAM and mitochondria were co-incubated for 4 h at 37 °C with [^{3}H]serine in the presence of 1 mM $CaCl_2$ and the indicated concentrations of $MgCl_2$. Lipids were extracted and PtdSer (○) and its decarboxylation products (PtdEtn + lysoPtdEtn) (■) were isolated. All data are means ± S.D. of three independent experiments. In some instances, error bars are obscured by symbols.

Nor did the concentration of Ca^{2+} (0 to 5 mM) influence PtdSer decarboxylase activity (results not shown). In contrast, when the Ca^{2+} concentration of the incubation mixture was increased from 0 to 2 mM, the translocation-dependent decarboxylation of pre-labelled PtdSer increased from 33 to 62% (Figure 3B). However, when the Ca^{2+} concentration was in-

Table 1 Membrane protein requirement of MAM and mitochondria for PtdSer translocation/decarboxylation

Mitochondria or [^{3}H]PtdSer-labelled MAM were treated with trypsin, proteinase K, neuraminidase or KCl. Treated MAM were subsequently reconstituted with untreated mitochondria. In addition, treated mitochondria were incubated with untreated [^{3}H]PtdSer-labelled MAM. The translocation-dependent decarboxylation of [^{3}H]PtdSer was determined as the percentage of d.p.m. in decarboxylation products (PtdEtn + lysoPtdEtn) compared with total incorporation of radioactivity into (PtdSer + PtdEtn + lysoPtdEtn). PtdSer decarboxylase activity (nmol/h per mg of protein) of mitochondria was also measured. As a positive control for inhibition of PtdSer decarboxylase, some mitochondria were treated with hydroxylamine [12,42]. $^*P < 0.01$, significantly different from untreated control sample. ND, not determined. Data are means ± S.D. from three experiments using independent preparations of membranes.

		Treated mitochondria	
Treatment	Treated MAM, translocation-dependent decarboxylation of PtdSer (%)	Translocation-dependent decarboxylation of PtdSer (%)	PtdSer decarboxylase activity (nmol/h per mg of protein)
Untreated	50 ± 2	65 ± 15	34 ± 2
Trypsin	42 ± 11	15 ± 14*	31 ± 3
Proteinase K	44 ± 8	24 ± 12*	31 ± 3
Neuraminidase	57 ± 14	62 ± 7	35 ± 2
KCl, 125 mM	49 ± 4	60 ± 10	41 ± 5
NH_2OH	ND	10 ± 8*	5 ± 4*

Table 2 Specific chemical modification of mitochondrial proteins impairs PtdSer translocation

Mitochondria were treated with the indicated reagents and subsequently incubated with [^{3}H]PtdSer-labelled MAM. The translocation-dependent decarboxylation of [^{3}H]PtdSer [i.e. the percentage of d.p.m. in (PtdEtn + lysoPtdEtn) compared with total incorporation of radioactivity into (PtdSer + PtdEtn + lysoPtdEtn)] was measured. PtdSer decarboxylase activity (nmol/h per mg of protein) of treated mitochondria was also determined. $^*P < 0.001$, significantly different from untreated mitochondria. ND, not determined. Data are means ± S.D. of three independent experiments. Abbreviations used: NEM, *N*-ethylmaleimide; MSL, Mersalyl acid; DEPC, diethylpyrocarbonate; DCCD, dicyclohexylcarbodiimide; PITC, phenylisothiocyanate; NAID, *N*-acetylimidazole; PGO, phenylglyoxal; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid.

Treatment	Modified residue or group	Translocation-dependent decarboxylation (%)	PtdSer decarboxylase activity (nmol/h per mg of protein)
Untreated		59 ± 10	34 ± 5
NEM (5 mM)	Cys	28 ± 5*	37 ± 7
MSL (0.5 mM)	Cys	21 ± 6*	28 ± 16
DEPC (1 mM)	His	51 ± 5	30 ± 6
DCCD (2.5 mM)	-COOH	52 ± 4	ND
PITC (2 mM)	$-NH_2$	50 ± 8	ND
NAID (33 mM)	Tyr	52 ± 9	ND
PGO (2 mM)	Arg	49 ± 3	ND
DIDS (5 mM)	Lys	32 ± 1*	18 ± 6*

creased from 2 to 5 mM the incorporation of [^{3}H]serine into (PtdEtn + lysoPtdEtn) gradually declined, possibly reflecting the stimulation of PtdEtn degradation by Ca^{2+}-dependent phospholipase(s) [31].

In the alternative translocation assay in which the concerted synthesis, translocation and decarboxylation of PtdSer were reconstituted at various concentrations of $MgCl_2$, the percentage of the translocation-dependent decarboxylation of PtdSer increased from 36 to 82 % as the Mg^{2+} concentration was increased from 0 to 5 mM (Figure 3C). The total d.p.m. in (PtdSer + PtdEtn + lysoPtdEtn) also increased when the concentration of Mg^{2+} was increased, presumably as a result of the continued synthesis of [^{3}H]PtdSer from [^{3}H]serine and/or stimulation of PtdSer synthesis by Mg^{2+} [32]. The activity of PtdSer decarboxylase was not affected by increasing the Mg^{2+} concentration from 0 to 5 mM. Figures 3(A) and 3(C) indicate, therefore, that Mg^{2+} stimulates the translocation of PtdSer to mitochondria. Since Ca^{2+} (at concentrations of up to at least 10 mM) stimulates PtdSer synthesis [32], we were unable to determine from this type of experiment whether or not increasing the Ca^{2+} concentration increased the translocation of newly made PtdSer to mitochondria. As predicted from other studies [10,11], ATP (2 mM) did not stimulate either PtdSer translocation or PtdSer decarboxylation (results not shown).

Role of a mitochondrial membrane protein in PtdSer translocation

We hypothesized that PtdSer import into mitochondria would require specific membrane proteins of PtdSer-donor membranes and/or mitochondrial membranes. To test this hypothesis, the reconstitution assay was performed under two different conditions: (i) [^{3}H]PtdSer-labelled MAM were incubated with mitochondrial membranes that had been treated with protease (trypsin or proteinase K) to inactivate surface proteins; and (ii) protease-treated, [^{3}H]PtdSer-labelled MAM were incubated with untreated mitochondria. In other experiments, MAM and/or mitochondria were treated with 125 mM KCl to strip peripheral proteins from the membrane surface. As shown in Table 1, neither salt treatment nor proteolysis of MAM affected the translocation-dependent decarboxylation of PtdSer. In a control experiment we confirmed that under the conditions used the trypsin was proteolytically active on membrane proteins, since treatment of MAM with trypsin resulted in a 70–95 % decrease in the specific activity of two membrane proteins; PtdEtn *N*-methyltransferase and NADPH:cytochrome c reductase (results not shown). Therefore, membrane-surface proteins of MAM appear not to be required for translocation of PtdSer.

In contrast, when mitochondria were treated with trypsin or proteinase K, the translocation-dependent decarboxylation of PtdSer was markedly decreased, whereas PtdSer decarboxylase activity was unaffected under the same conditions (Table 1). Treatment of mitochondria with neuraminidase or KCl did not affect the translocation-dependent decarboxylation of PtdSer, suggesting that proteins required for translocation are neither sialylated glycoproteins nor peripheral membrane proteins. These data imply that import of PtdSer into mitochondria is mediated by a protein that is tightly bound to the mitochondrial membrane and is exposed on the membrane surface.

Sulphydryl-reactive reagents inhibit PtdSer translocation

We hypothesized that specific amino acid residues of the putative PtdSer-translocation protein might be required for PtdSer-translocation activity. To probe the nature of amino acid residues required for PtdSer translocation, we examined the impact of several amino acid-modifying reagents on this process. The effects of most of these reagents on translocation were distinct from those on PtdSer decarboxylase activity. Table 2 shows that two thiol-modifying reagents, *N*-ethylmaleimide and Mersalyl acid, inhibited the translocation-dependent decarboxylation of PtdSer by 53 and 64 %, respectively. However, neither agent significantly inhibited PtdSer decarboxylase. Other amino acid-modifying reagents – diethylpyrocarbonate (reactive with histidine residues), dicyclohexylcarbodiimide (reactive with carboxyl groups), phenylisothiocyanate (reactive with amino groups), *N*-acetylimidazole (reactive with tyrosine residues) and phenyl-

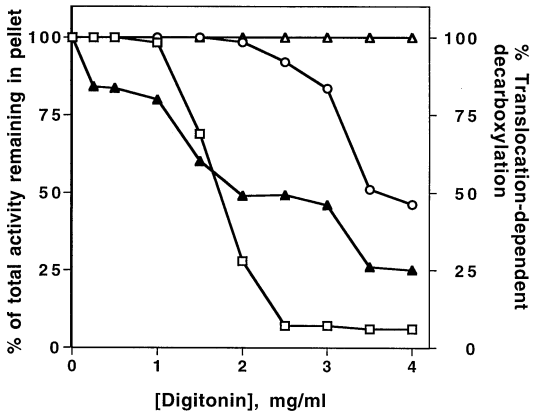

Figure 4 PtdSer translocation to, and decarboxylation by, digitonin-treated mitochondria

Mitochondrial outer membranes were partially solubilized using the indicated concentrations of digitonin. Solubilized membranes were separated from particulate membranes by centrifugation. Mitochondrial membranes (pellet) were incubated for 4 h at 37 °C with [^{3}H]PtdSer-labelled MAM. Lipids were extracted and PtdSer and its decarboxylation products (PtdEtn + lysoPtdEtn) were isolated by TLC. The translocation-dependent decarboxylation of PtdSer (▲) was calculated as the percentage of d.p.m. in (PtdEtn + lysoPtdEtn) compared with total radioactivity in (PtdSer + PtdEtn + lysoPtdEtn). Specific activities of PtdSer decarboxylase (△), monoamine oxidase (□) and succinate:cytochrome *c* reductase (○) were determined in the mitochondrial pellet and supernatant (outer membranes). The percentage of enzyme activity remaining in the pellet after digitonin treatment was calculated. The experiment was repeated three times with similar results.

glyoxal (reactive with arginine residues) – had negligible effects on the translocation-dependent decarboxylation of PtdSer (Table 2). Diisothiocyanatostilbene-2,2′-disulphonic acid (reactive with lysine residues) inactivated the translocation-dependent decarboxylation of PtdSer (Table 2), but also inhibited PtdSer decarboxylase activity to a similar extent. These data suggest that a mitochondrial protein that requires a thiol group for activity is involved in PtdSer import into mitochondria.

Sub-mitochondrial location of the protein mediating PtdSer translocation

Since the putative mitochondrial PtdSer-translocation protein was inactivated by exogenously added protease, we postulated that the protein was exposed either on the cytosolic surface of mitochondrial outer membranes or at contact sites between mitochondrial outer and inner membranes. Hovius et al. [33] have reported that a low concentration of digitonin solubilizes mitochondrial outer membranes leaving inner membranes and contact sites intact. Therefore, as a means of defining the sub-mitochondrial location of the putative translocation protein, mitochondria were treated with various concentratins of digitonin, after which solubilized outer membranes and the remaining particulate membranes (i.e. inner membranes and contact sites) were separated by centrifugation. The translocation-dependent decarboxylation of PtdSer was assessed, as were the specific activities of monoamine oxidase (a marker of mitochondrial outer membranes), and succinate:cytochrome c reductase and PtdSer decarboxylase (markers of mitochondrial inner membranes). Figure 4 shows that mitochondrial outer membranes were partially solubilized at digitonin concentrations above 1.5 mg/ml, as indicated by the decrease in activity of monoamine oxidase, and were completely solubilized with 2.5 mg/ml digitonin. The amount of protein remaining in the pellet was gradually depleted as the digitonin concentration was increased. For example, with 2.5 mg/ml digitonin, the amount of mitochondrial protein in the pellet was $\sim 50\,\%$ of that of mitochondria not treated with detergent. In contrast, at all digitonin concentrations up to 3 mg/ml, mitochondrial inner membranes remained intact as determined by little or no reduction in the activities of succinate:cytochrome *c* reductase and PtdSer decarboxylase (Figure 4). Moreover, in the presence of 2.5 mg/ml digitonin (at which concentration the outer membranes were completely solubilized), $\sim 50\,\%$ of the translocation-dependent decarboxylation activity remained (Figure 4). These data indicate that the majority of the protein mediating PtdSer translocation between MAM and mitochondria is most likely concentrated in contact sites between mitochondrial outer and inner membranes.

DISCUSSION

These studies provide data showing that in a reconstituted system *in vitro* PtdSer translocation to mitochondria is mediated by a mitochondrial membrane protein, domains of which are exposed on the mitochondrial surface. Proteolysis of mitochondria with trypsin inhibited the translocation, providing evidence that translocation of PtdSer requires a mitochondrial protein. In contrast, the translocation was unaffected by proteolysis of MAM. Therefore, proteins on the surface of PtdSer-donor membranes appear not to be required for PtdSer translocation. The translocation of PtdSer to mitochondria was stimulated by Mg^{2+} and Ca^{2+} and was inhibited by sulphydryl group-reactive reagents. In contrast with the present observations, the translocation-linked decarboxylation of PtdSer has been previously reported not to be inhibited by protease treatment of mitochondria in a reconstituted system *in vitro* consisting of mitochondria and microsomes [9]. Although we cannot fully explain this apparent discrepancy, the mitochondria used previously were possibly associated with MAM, which might have shielded the mitochondrial PtdSer-translocation protein from proteolysis. Our studies indicate that the putative mitochondrial PtdSer-translocation protein has regions that are exposed on the cytosolic surface of the mitochondrion and suggest that the protein is concentrated at contact sites between mitochondrial outer and inner membranes.

An obligatory sequence of events must occur during PtdSer import into mitochondria. First, PtdSer must be transferred from its site of biosynthesis to mitochondrial outer membranes or contact sites. From there, the PtdSer presumably undergoes trans-bilayer movement to the inner aspect of the outer membrane, although little is known about the trans-bilayer movement of PtdSer in mitochondrial membranes. Dolis et al. [34] recently showed that the $t_{1/2}$ for trans-bilayer movement of PtdCho across the mitochondrial outer membrane was 2 min or less; however, the movement of PtdSer was not examined. The trans-bilayer movement of PtdSer across the plasma membrane has, however, been extensively studied. A candidate aminophospholipid translocase, which is a Mg^{2+}-dependent ATPase of the P-type ATPase family, has been purified from erythrocytes [35], chromaffin granules [36] and synaptic vesicles [37], and the cDNA encoding this protein has been cloned [38]. Another protein, called 'scramblase', which mediates the trans-bilayer movement of phospholipids in the plasma membrane has also recently been purified from erythrocytes [39] and its cDNA cloned [40]. However, it is not yet clear whether similar proteins are present in mitochondrial membranes. Once the PtdSer has been transferred to the inner leaflet of the outer membrane the PtdSer must then either cross the intermembrane space to the outer aspect of the inner membrane for decarboxylation, or the decarboxylase on the inner membrane must have direct access to PtdSer on the

inner leaflet of the outer membrane. One way in which this sequence of events might be readily accomplished would be if all these events occurred at contact sites between mitochondrial outer and inner membranes. Interestingly, MAM-like membranes have been reported to be associated with mitochondria at the same sites at which outer and inner membranes are in contact [22]. It is noteworthy, however, that Hovius et al. [41] have reported that mitochondrial contact sites are not enriched in PtdSer decarboxylase activity.

Although MAM have been implicated as the membranes that donate PtdSer to mitochondria in an intact cell [20], proteins on the surface of MAM do not appear to be required for PtdSer translocation *in vitro*. In agreement with these observations, mitochondria can import PtdSer *in vitro* from a variety of PtdSer biosynthetic membranes such as MAM, microsomes or ER, or even from liposomes [9]. Our data suggest that the PtdSer-translocation protein is a mitochondrial membrane protein that binds PtdSer. In the intact cell PtdSer is synthesized in both MAM and ER. Therefore, why do MAM, rather than ER membranes, preferentially associate with mitochondria and mediate PtdSer import into mitochondria *in vivo*? One possibility is that the specific contact that occurs *in vivo* between MAM and mitochondria was not reconstituted in the *in vitro* assay. Alternatively, in the reconstituted translocations *in vitro* the donor membrane concentrations were very high. Thus, PtdSer might be transferred *in vitro* to mitochondria upon collision with an excess of any PtdSer-containing membranes. We have previously shown that the specific activity of PtdSer synthase is two–four-fold higher in MAM than in ER or microsomes [18,20]. Therefore, membrane domains that are enriched in newly made PtdSer might be generated in MAM. We suggest that, *in vivo*, the PtdSer-binding protein on the mitochondrial surface preferentially binds these PtdSer-enriched regions of MAM and thus mediates lipid transfer.

In reconstituted systems with isolated organelles *in vitro*, PtdSer translocation is ATP-independent [9,11], whereas in intact [26] and permeabilized cells [10] the translocation requires ATP. A scenario that would be consistent with these experimental observations is that MAM *in vivo* would become juxtaposed to mitochondria in an ATP-requiring step. PtdSer would then be transferred to mitochondrial outer membranes in an event not requiring ATP. In the isolated organellar-reconstitution system, the endogenous juxtaposition of MAM and mitochondria would have been disrupted, but mitochondria would be able to contact any PtdSer-containing membrane with which they collided *in vitro*, and thus import of PtdSer would proceed.

The experimental findings reported in this paper support the hypothesis that transfer of PtdSer to mitochondria is mediated by a membrane contact event and suggest that a mitochondrial protein plays a central role in PtdSer translocation. The data indicate that the mitochondrial protein involved in PtdSer translocation is partially exposed on the mitochondrial surface. In addition, the PtdSer-translocation protein contains a sulphydryl group that is necessary for mediating PtdSer import into mitochondria.

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