Targeting and insertion of C-terminally anchored proteins to the mitochondrial outer membrane is specific and saturable but does not strictly require ATP or molecular chaperones

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A distinct class of proteins contain a C-terminal membrane anchor and a cytoplasmic functional domain. A subset of these proteins is targeted to the mitochondrial outer membrane. Here, to probe for the involvement of a saturable targeting mechanism for this class of proteins, and to elucidate the roles of chaperone proteins and ATP, we have utilized an *in vitro* targeting system consisting of *in vitro*-synthesized proteins and isolated mitochondria. To establish the specificity of targeting we have used a closely related protein pair. VAMP-1A and VAMP-1B are splice variants of the vesicle-associated membrane protein/synaptobrevin-1 (VAMP-1) gene. In intact cells VAMP-1B is targeted to mitochondria whereas VAMP-1A is targeted to membranes of the secretory pathway, yet these isoforms differ by only five amino acids at the extreme C-terminus. Here we demonstrate that, *in vitro*, VAMP-1B is imported into both intact mitochondria and mitochondrial outer-membrane vesicles with a 15-fold greater efficiency than VAMP-1A. We generated and purified bacterially expressed fusion proteins consisting of the C-terminal two-thirds of VAMP-1A or -1B proteins fused to glutathione S-transferase (GST). Using these fusion proteins we demonstrate that protein targeting and insertion is saturable and specific for the VAMP-1B membrane anchor. To elucidate the role of cytosolic chaperones on VAMP-1B targeting, we also used the purified, *Escherichia coli*-derived fusion proteins. ^{33}P-Labelled GST-VAMP-1B$_{61–116}$, but not GST-VAMP-1A$_{61–118}$, was efficiently targeted to mitochondria in a chaperone-free system. Thus the information required for targeting is contained within the targeted protein itself and not the chaperone or a chaperone–protein complex, although chaperones may be required to maintain a transport-competent conformation. Moreover, ATP was required for transport only in the presence of cytosolic chaperone proteins. Therefore the ATP requirement of transport appears to reflect the participation of chaperones and not any other ATP-dependent step. These data demonstrate that targeting of C-terminally anchored proteins to mitochondria is sequence specific and mediated by a saturable mechanism. Neither ATP nor chaperone proteins are strictly required for either specific targeting or membrane insertion.

Key words: import pathway, protein import, rat liver mitochondria, tail-anchored membrane protein, VAMP-1.

INTRODUCTION

C-terminally anchored proteins represent a unique subset of membrane proteins in the cell that play a number of critical roles [1]. Examples include the vesicle-associated membrane protein/synaptobrevin (VAMP) and syntaxin families of proteins, members of the soluble *N*-ethylmaleimide-sensitive-factor-attachment-protein receptor (SNARE) proteins, which are crucial elements of the vesicular trafficking system of the cell, and the Bcl-2 family of proteins, involved in apoptosis. These proteins share a domain structure consisting of an N-terminal cytosolic domain, the functional domain of the protein, and a stretch of hydrophobic residues at the extreme C-terminus of the protein that provides both the targeting signal and the membrane anchor. Because the membrane anchor is C-terminal the targeting of these proteins to their respective membranes must be post-translational rather than co-translational. The extent to which the targeting of these proteins is compartment-specific has been controversial. Early studies indicated that a prototypic member of this class of proteins, cytochrome b_5, could insert non-specifically into lipid membranes *in vitro* [2–4], suggesting that targeting would be relatively non-specific. More recently, however, it has been recognized that in some cases, but not all, localization is quite specific. For example, two closely related isoforms of the cytochrome b_5 gene have been found to have non-overlapping subcellular distributions. One isoform, rat outer mitochondrial membrane cytochrome b_5, is only present in mitochondria whereas the other, microsomal cytochrome b_5, is restricted to endoplasmic reticulum (ER) membranes [5]. Such restricted localization is not always observed for this class of proteins. Bcl-2 is found in the ER, the nuclear membrane and in mitochondria [6,7], bringing into question the specificity of the targeting apparatus. Yet even in the case of Bcl-2 the question remains whether the promiscuity of targeting is due to a lack of specificity in the targeting process, or the presence in one protein of multiple targeting signals. The bulk of evidence suggests that

Abbreviations used: VAMP, vesicle-associated membrane protein/synaptobrevin; SNARE, soluble *N*-ethylmaleimide-sensitive-factor-attachment-protein receptor; pSU9$_{1-69}$-DHFR, fusion protein between matrix-targeting signal of F_0-ATPase subunit 9 and mouse dihydrofolate reductase; ER, endoplasmic reticulum; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cpn$_{60}$, chaperonin heat-shock protein 60; Tom20, translocase of the outer membrane of mitochondria of 20 kDa; GST, glutathione S-transferase; SRP, signal-recognition particle; MSF, mitochondrial import stimulation factor; GRP$_{78}$, 78-kDa glucose-regulated protein.

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the only two membranes to which C-terminally anchored proteins can be targeted are the ER and the mitochondrial outer membrane [1,8,9]. Recently the identification of distinct targeting motifs of C-terminally anchored proteins for the ER [10] and mitochondria [11,12] has lent force to the notion that targeting is specific.

The targeting of mitochondrial matrix-targeted proteins has been well studied. Most of these proteins are nuclear-encoded, translated and transferred from the cytosol and imported into mitochondria by a post-translational mechanism [13]. The targeting of these proteins to mitochondria involves both mitochondrial membrane components and cytosolic factors [14]. About nine components of the mitochondrial import machinery have been identified in the mitochondrial outer membrane [14]. Cytosolic chaperone proteins, including mitochondrial import stimulation factor (MSF) and Hsp70 can interact with, and stimulate the import of, proteins destined for mitochondrial matrix [15,16]. It is unclear at present whether proteins targeted to the mitochondrial outer membrane by C-terminal signal/anchors utilize this same machinery, although there are some suggestions that at least some of the same components are employed [9]. The targeting signal for matrix-directed proteins is a cleavable N-terminal, amphipathic helix. This N-terminal targeting signal is not present in the C-terminally anchored proteins. Instead, the targeting signal is included in the membrane anchor itself. Mutagenesis studies suggest that this signal consists of a relatively short stretch of hydrophobic amino acids (less than 21), which is flanked at both ends by positively charged residues [11].

The mechanism of targeting C-terminally anchored protein to the outer membrane remains unclear. Based on several observations, a multi-step model for this mechanism has been proposed [9]. In this model the protein to be targeted associates with cytosolic chaperones during or shortly after translation. The chaperone-bound protein is then transferred to a hypothetical receptor protein on the mitochondrial surface. Finally, the receptor-bound protein is passed to an insertion complex that accomplishes integration of the protein into the bilayer. Here we focus on three major issues. First, we explore the specificity of targeting by testing for a saturable component of the targeting apparatus that is specific for the known targeting signal. The second issue is whether cytosolic chaperone proteins carry targeting information. As noted above, chaperones have been implicated in the delivery of matrix-directed proteins by preserving a conformation in the targeted protein, but the chaperones themselves do not contain targeting information. For C-terminal signal/anchor proteins the situation is somewhat different, as the targeting signal is hydrophobic, and a chaperone may conceivably have signalling function. This would be in analogy to the signal-recognition particle (SRP), which recognizes a hydrophobic signal peptide in targeting to the ER. In the case of SRP, which can loosely be considered a chaperone, the targeting receptor in the ER recognizes the SRP–signal-peptide complex, and therefore SRP is part of the recognition signal [17]. We wished to determine whether an SRP-like mechanism might be involved in targeting of C-terminally anchored proteins. A third issue is the requirement for ATP in protein import. Previous studies of C-terminal anchored protein targeting to mitochondria have demonstrated a requirement for ATP [9,18]. However, the import step that requires ATP has not been defined. ATP may well be required for the transfer of targeted proteins from chaperones to the mitochondrial receptor(s). The requirement for ATP in this manner for matrix-directed proteins has previously been identified [19–21]. However, it has also been suggested that ATP has a post-receptor role, perhaps in the insertion step of transport [9]. A direct test of the role of ATP in the import process is therefore called for.

To answer these questions an *in vitro* approach is the most direct and compelling avenue. A methodological problem with this approach is the demonstration of specificity. Isolated mitochondria are incubated with *in vitro*-translated protein and the association of the translated protein with mitochondria is taken to represent targeting. It is especially important to distinguish specific from non-specific targeting in such a system because some C-terminally anchored proteins are known to spontaneously insert into membranes *in vitro* [4].

To address this issue we have utilized a novel pair of C-terminally anchored proteins, one of which is targeted to the ER/secretory pathway in cells, and the other of which is targeted to mitochondria. VAMP-1 was originally characterized as a SNARE protein involved in the regulated exocytosis of synaptic vesicles [22]. We recently identified a splice variant of VAMP-1, VAMP-1B, which differs by only five amino acids from the originally identified isoform, named VAMP-1A [11]. The sequence difference includes the truncation of the C-terminal hydrophobic anchor by four amino acids, and the addition of three charged amino acids at the extreme C-terminus. This small sequence change redirects VAMP-1B to the mitochondrial outer membrane rather than the ER. Here we use this pair to demonstrate that targeting of C-terminally anchored proteins to mitochondria *in vitro* is specific, involves a saturable process (presumably the targeting receptor), and that although chaperone proteins appear to assist transport and to confer ATP dependence to the process, the targeting apparatus recognizes the targeted protein directly rather than recognizing a chaperone–protein complex.

MATERIALS AND METHODS

General procedures

DNA manipulations were performed following standard protocols [23] and all plasmids were propagated in *Escherichia coli* strain DH5α except where indicated. SDS/PAGE was performed by standard procedures [24]. Western blots were performed using 1:10000 dilution of rabbit anti-cpn$_{60}$ (chaperonin heat-shock protein 60) antibody (a gift from Dr Nick Hoogenraad, La Trobe University, Melbourne, Australia) and 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Amrad Biotech, Melbourne, Australia). An ER-specific protein maker, the 78-kDa glucose-regulated protein (GRP$_{78}$), was detected using 2 μg/ml goat anti-GRP$_{78}$ antibody and 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Radioactivity analysis was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The treatment of reactions with apyrase, hexokinase and trypsin (Sigma, St. Louis, MO, U.S.A.) was as described in the Figure legends.

Plasmid constructions

The coding sequences for VAMP-1A and VAMP-1B were amplified separately by PCR from either rat brain or human HUVEC (human umbilical vein endothelial cell) cDNA libraries [11]. The PCR products were cut with *Eco*RI and ligated into the pGEM-4Z vector. The coding sequences for the C-termini of VAMP-1A (amino acids 61–118) and VAMP-1B (amino acids 61–116) were amplified by PCR using pGEM-4Z-VAMP-1A and pGEM-4Z-VAMP-1B as templates. The common upstream primer used for VAMP-1A and VAMP-1B was 5′-GATC-

GGATCCTTGCAGGCAGGAGCATCACA-3′, in which the restriction site for *Bam*HI is underlined. The downstream primers used for VAMP-1A and VAMP-1B were 5′-GACTGAATTC TTTCAAGTAAAAAAGTAGATTAC-3′ and 5′-GACTG-AATTCAATCAGTCCCGCCTTACAAT-3′, respectively, in which the *Eco*RI sites are underlined. For protein expression, the PCR fragments were digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI/*Eco*RI site of pGEX-2TK. DNA sequencing was performed to confirm that the VAMP-1A and VAMP-1B tails were fused to glutathione S-transferase (GST) in-frame.

Preparation of crude mitochondria, purified enriched mitochondria and mitochondrial outer-membrane vesicles

All of the following procedures were performed at 4 °C. Crude mitochondria were isolated from rat liver by the following method. Briefly, Dark-Agouti rat livers were cut up, homogenized in buffer A [20 mM Hepes, pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 % BSA and 1 mM pefabloc (Boehringer Mannheim Biochemica, Mannheim, Germany)], and centrifuged in a Beckman JA-10 rotor at 3000 ***g*** to remove nuclear debris. The post-nuclear supernatant was centrifuged in a Beckman JA-10 rotor at 16000 ***g*** for 15 min, yielding a crude mitochondrial pellet and a post-mitochondrial supernatant. The resulting crude mitochondrial pellet was resuspended with buffer B (buffer A minus 0.5 % BSA) and washed three times by centrifugation in a Beckman JA-10 rotor at 16000 ***g*** for 15 min. The resulting fraction is termed 'washed mitochondria'. For some experiments, these washed mitochondria were layered on a 30 % Percoll gradient in buffer B and centrifuged in a 55Ti rotor at 110000 ***g*** for 30 min. The resulting lower mitochondrial band (a light-brownish colour) was collected and precipitated by centrifugation in a JA-20 rotor at 9000 ***g*** for 10 min. Purified enriched mitochondria were obtained after washing the above pellet twice with buffer B to remove Percoll. To isolate mitochondrial outer membrane vesicles, purified enriched mitochondria were swollen by stirring in 10 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, for 30 min. The outer membrane was separated from mitoplasts by five strokes with a motor-driven Teflon/glass homogenizer. A post-mitoplast supernatant was obtained by centrifugation of the homogenate in a JA-20 rotor at 17500 ***g*** for 10 min. Mitochondrial outer-membrane vesicles were precipitated by centrifugation of the post-mitoplast supernatant in a 55Ti rotor at 250000 ***g*** for 1 h, resuspended in buffer B, aliquoted and stored at −80 °C. The protein concentrations of the above organelles and membrane preparations were determined using the bicinchoninic acid reagent kit (Pierce, Rockford, IL, U.S.A.) using BSA as a standard.

In vitro transcription, translation and mitochondrial import

VAMP-1A, VAMP-1B, Bcl-2 and $pSU9_{1-69}$-DHFR (the fusion protein between the matrix-targeting signal of F_0-ATPase subunit 9 and mouse dihydrofolate reductase) RNAs were synthesized by transcription *in vitro* (Promega, Madison, WI, U.S.A.) at 30 °C for 60 min. Translation of VAMP-1A and VAMP-1B was performed in either a nuclease-treated rabbit reticulocyte lysate system or a cell-free wheatgerm system (Promega), using [^{35}S]-methionine (ICN Pharmaceuticals, Irvine, CA, U.S.A.) as a radioactive label following the manufacturer's instructions. Translation products were analysed by SDS/PAGE (15 % gel) and quantification of the dried gel was performed by direct radioactive analysis using a PhosphorImager. All mitochondrial targeting was performed using washed mitochondria except where indicated. Part (10–20 %) of the translation reaction containing ^{35}S-labelled protein was incubated with 100 μg of intact rat liver mitochondria at 30 °C with import buffer (20 mM Hepes, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, 10 mM sodium succinate and 1 mM dithiothreitol) for the times indicated in the Figure legends. The import reaction was layered on the top of a 220-μl 220 mM sucrose cushion in buffer B and mitochondria were recovered by centrifugation in a Biofuge 13 at 13000 ***g*** for 10 min at 4 °C. Alkaline extraction was performed by incubation of the mitochondrial pellet with freshly prepared 0.1 M Na_2CO_3, pH 11.5, on ice for 30 min with periodic vortexing. The membrane pellets were collected by centrifugation in an airfuge (Beckman Instruments, Carlsbad, CA, U.S.A.) at 30 psi (207 kPa) for 10 min. The radioactive proteins were analysed by SDS/PAGE and quantified using a PhosphorImager. The relative insertion is represented as the percentage input (the ratio of mitochondrially incorporated protein to input translation product).

GST-fusion-protein preparation

E. coli BL21/DE3 cells were transformed with pGEX-2TK encoding either GST or GST fused in-frame to amino acids 61–118 of VAMP-1A (GST-VAMP-$1A_{61-118}$) and 61–116 of VAMP-1B (GST-VAMP-$1B_{61-116}$). A 2 litre culture in superbroth (50 μg/ml ampicillin) was seeded with 100 ml of an overnight culture and grown for 2–3 h at 30 °C. The cells were induced with 1 mM isopropyl β-D-thiogalactoside once they reached an attenuance of 1.0 (at 600 nm). After 5 h, the cells were collected by centrifugation and resuspended in 10 ml of 50 mM Tris/100 mM NaCl/5 mM EDTA/10 μg/ml leupeptin/1μM aprotinin/1 mM PMSF, pH 8.0. The cells were lysed by a probe sonicator (5 mm diameter) followed by the addition of Triton X-100 to 2 % and dithiothreitol to 5 mM. The supernatant was collected by centrifugation of the lysate at 12100 ***g*** for 10 min at 4 °C and then incubated with 2 ml of a 50 % slurry of glutathione-agarose beads (Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS buffer for 1.5 h at 4 °C with agitation. The beads were loaded into a 10-ml chromatography column (Bio-Rad, Hercules, CA, U.S.A.) and washed five times with 10 ml of ice-cold PBS/1 % Triton X-100, once with PBS/1 % Triton X-100/1 M NaCl, and once with PBS. The beads were resuspended with 1 ml of PBS/50 % glycerol, aliquoted and frozen in liquid nitrogen. The purity of the preparation and the yield of GST, GST-VAMP-$1A_{61-118}$ and GST-VAMP-$1B_{61-116}$ on the beads were assessed by SDS/PAGE and Coomassie Brilliant Blue staining. The full-length GST-fusion proteins presented as a major band in the gel were quantified by comparison with BSA. For competition experiments, GST-fusion proteins were eluted from the beads by incubation with the same volume of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0, and 7 M urea) at room temperature (22–25 °C) for 10 min. The eluate was collected and pooled following three repeated elution and centrifugation steps, aliquoted and assessed by SDS/PAGE.

GST-fusion-protein radiolabelling

The glutathione-agarose beads bound with GST-fusion proteins were washed with 10 volumes of HMK buffer (20 mM Tris, pH 7.5/0.1 M NaCl/12 mM $MgCl_2$). The sedimented beads were incubated with 0.3 volumes of protein kinase reaction mixture {HMK buffer, 0.3 units of bovine heart kinase (Sigma) and 10 mCi/ml [γ-^{33}P]ATP} at 4 °C for 30 min. Following the addition of 10 volumes of stop solution (10 mM sodium phosphate, pH 8.0, 10 mM sodium pyrophosphate, 10 mM EDTA and

1 mg/ml BSA), the beads were sedimented by centrifugation and washed with 10 volumes of PBS buffer. Finally, the radiolabelled fusion proteins were eluted as described above and assessed by SDS/PAGE and PhosphorImager.

Radiolabelled GST-fusion-protein targeting

^{33}P-Labelled GST fusion proteins (final concentration 500 nM, quantified according to molecular mass) in elution buffer containing 7 M urea were incubated with 100 μg of rat liver mitochondria at 30 °C for 30 min. The final concentration of urea in the import reaction was 0.2 M. The recovery of the mitochondrial pellet, alkaline extraction and analysis and quantification of imported signal were performed as described above.

RESULTS

Differential targeting of VAMP-1 isoforms is reproduced *in vitro*

We wished to determine whether *in vitro* the mitochondrial outer-membrane-targeting system could distinguish between a C-terminally anchored protein that is targeted *in vivo* to mitochondria (VAMP-1B [11]) and a splice isoform that is not mitochondrially targeted (VAMP-1A). As shown in Figure 1(A), VAMP-1B is identical in sequence with VAMP-1A from residues 1 to 113. However, their sequences diverge at the C-terminus, the predicted transmembrane segment. VAMP-1B has a shorter transmembrane segment by four amino acids compared with VAMP-1A, and ends with three charged amino acids. After incubation of reticulocyte lysate containing radiolabelled VAMP-1A and VAMP-1B with washed rat liver mitochondria, only VAMP-1B efficiently binds to mitochondria membranes (Figure 1B, lanes 3 and 4). The majority of the mitochondrially associated VAMP-1B is resistant to alkali extraction (Figure 1B, lanes 5 and 6). Quantification, accomplished by comparison of the total amount of VAMP-1 protein added to the reaction (Input, lanes 1 and 2) with the amount of protein imported to the mitochondria, indicated that VAMP-1B was imported with 15-fold higher efficiency than VAMP-1A. The recovery of an alkali-resistant form of VAMP-1B in the pelletable fraction depends on the presence of mitochondria (Figure 1C, lanes 4 and 6), indicating that VAMP-1B is integrated into the mitochondrial membrane lipid bilayer, and is not simply aggregating. The amount of VAMP-1B insertion increases with an increase in mitochondria

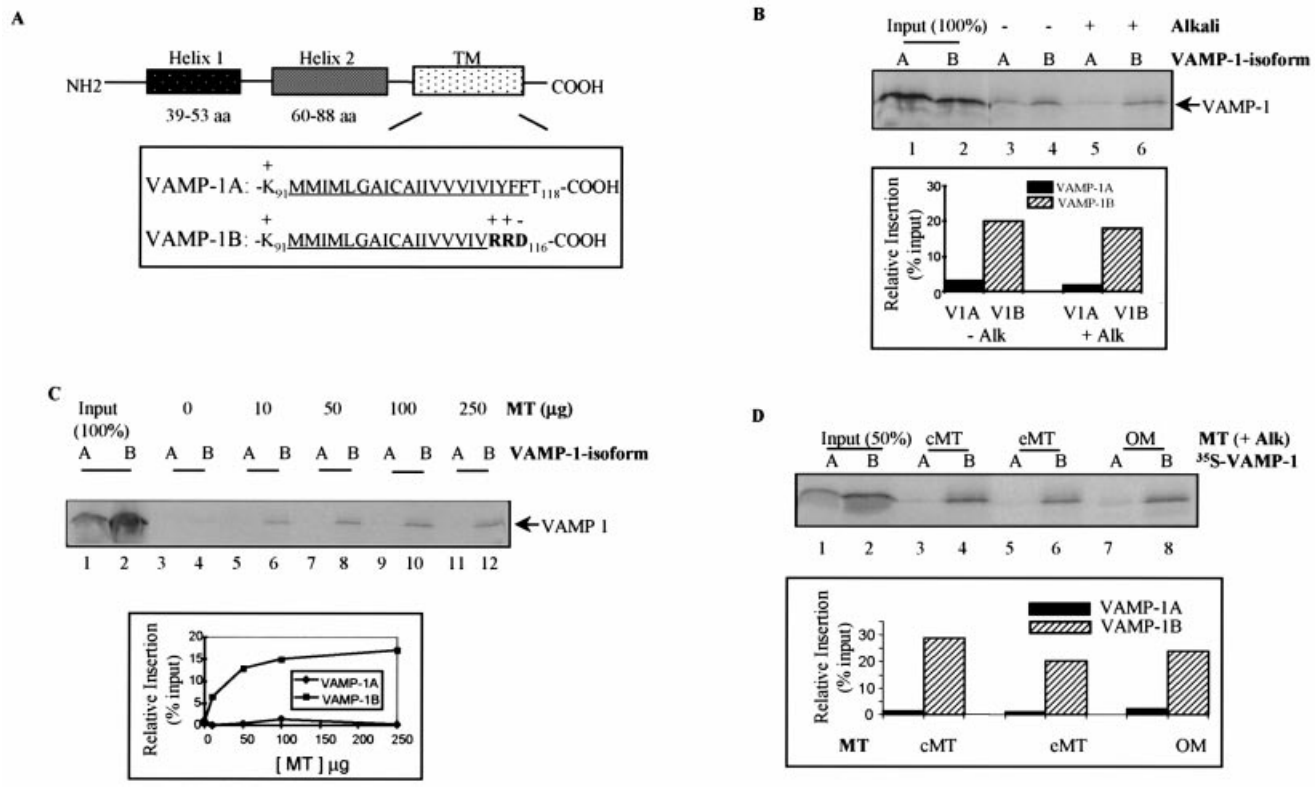

Figure 1 VAMP-1B is specifically targeted to mitochondria *in vitro*

(**A**) Scheme of VAMP-1 splice isoforms. VAMP-1 protein contains two putative helical domains and a transmembrane domain (TM). VAMP-1B is identical with VAMP-1A except for a slightly shorter hydrophobic domain and charged residues in its C-terminus. (**B**) *In vitro* import of VAMP-1 isoforms to mitochondria. ^{35}S-Labelled VAMP-1A and VAMP-1B were incubated with 100 μg of washed rat liver mitochondria at 30 °C for 30 min. Mitochondria were recovered and analysed by SDS/PAGE either directly or after extraction in 0.1 M Na_2CO_3, pH 11.5 ($\pm$alkali, Alk), as described in the Materials and methods section. The radioactivity of inserted VAMP-1 was quantified using a PhosphorImager. The relative insertion was calculated as the percentage of total radioactive protein added to each assay (Input) recovered with the mitochondrial pellet. Input represents the amount of translation product in each import assay. (**C**) Mitochondria are not limiting for the specific targeting of VAMP-1B. [^{35}S]Labelled VAMP-1A and VAMP-1B were incubated with intact mitochondria (MT; 0–250 μg), at 30 °C for 30 min. Mitochondria were recovered and analysed after alkaline extraction. For (**B**) and (**C**), lanes 1 and 2 represent 100% of the [^{35}S]protein added to each assay. (**D**) Crude mitochondria (cMT, 100 μg), purified enriched mitochondria (eMT, 25 μg) and mitochondrial outer-membrane vesicles (OM, 5 μg) were incubated with [^{35}S]VAMP-1A and [^{35}S]VAMP-1B at 30 °C for 30 min. Mitochondria and outer-membrane vesicles were pelleted by spinning in an airfuge at 30 psi (207 kPa) for 10 min. After alkaline treatment mitochondria and outer membranes were recovered and analysed. Lanes 1 and 2 represent 50% of the [^{35}S]protein added to each assay.

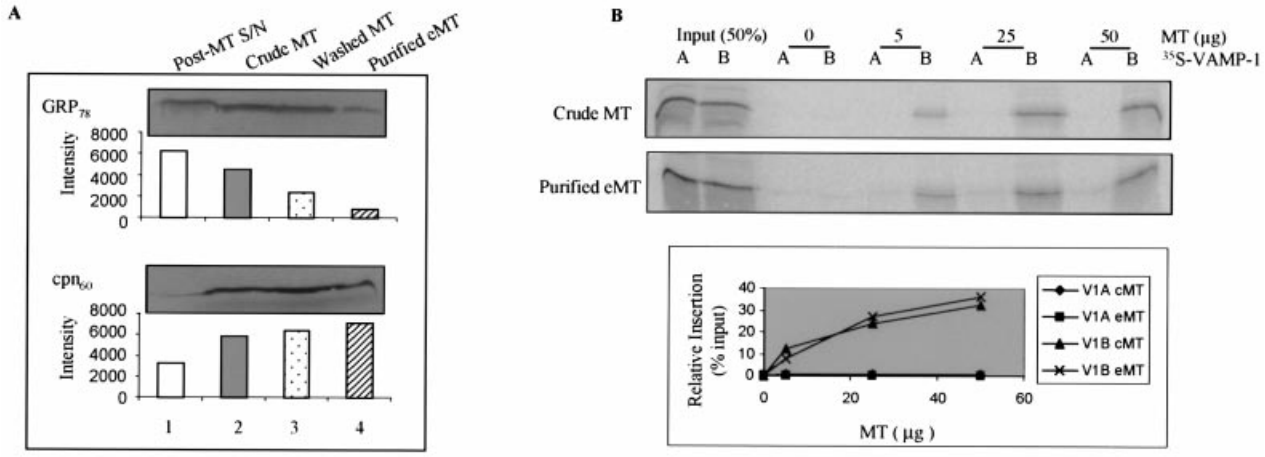

Figure 2 Insertion of VAMP-1B into mitochondria and highly purified mitochondria

(**A**) Comparison of the purity of mitochondrial preparations by Western blot (20 μg of protein/lane) using ER-specific marker (GRP_{78}) and mitochondrial matrix-protein marker (cpn_{60}). Microsome fractions isolated from rat liver post-mitochondrial supernatant (Post-MT S/N), crude mitochondria (MT), washed mitochondria and purified enriched mitochondria were normalized by protein concentration, analysed by SDS/PAGE and probed with anti-cpn_{60} or anti-GRP_{78} antibody. The intensity of the band detected by enhanced chemiluminescence was scanned and quantified by ImageQuant. (**B**) Comparison of relative insertion of VAMP-1B into crude mitochondria and purified mitochondria. [^{35}S]VAMP-1A and [^{35}S]VAMP-1B were incubated with 0, 5, 25 and 50 μg of crude mitochondria (cMT) or purified enriched mitochondria (eMT) at 30 °C for 30 min. The mitochondria were recovered and analysed after alkaline extraction as described in Figure 1. Lanes 1 and 2 represent 50% of the [^{35}S]protein added to each assay.

concentration and reaches a plateau (about 20 % of input) with 100 μg of mitochondria per reaction (Figure 1C, lower panel). This level of efficiency of import is similar to that measured previously for C-terminally anchored proteins [18,25]. It is unclear why in this and other studies the efficiency of transport is not higher. However, this experiment demonstrates that levels of mitochondria are not limiting. VAMP-1A insertion was low at all levels of mitochondria added, illustrating that the differential targeting was preserved even with high concentrations of mitochondria. Differential targeting was observed with gradient-purified mitochondria and outer-membrane vesicles as well as with the mitochondria derived from differential centrifugation used routinely. As shown in Figure 1(D), 25 μg of gradient-purified enriched mitochondria (lanes 5 and 6) and 5 μg of an outer-membrane preparation (lanes 7 and 8), prepared by a standard technique, exhibited similar activity for VAMP-1B insertion as 100 μg of crude mitochondria (lanes 3 and 4). The differential targeting was reproduced in both purified enriched mitochondria and outer-membrane vesicles. Quantification showed that the relative insertion of VAMP-1B into both purified enriched mitochondria and mitochondrial outer-membrane vesicles was 20 %, whereas that of VAMP-1A was only 1 %.

To confirm that the targeting of VAMP-1B observed in the import assay was not due to non-mitochondrial membranes contaminating our mitochondrial preparations the purity of membrane and organelle fractions was monitored using the ER marker GRP_{78} and the mitochondrial matrix protein cpn_{60}. Washing and gradient purification reduced the level of ER contamination to low levels (Figure 2A, upper panel) while, as expected, maintaining levels of the mitochondrial marker. Compared with cpn_{60} protein (Figure 2A, lower panel), GRP_{78} was reduced about 5-fold in purified enriched mitochondria (Figure 2A, compare lanes 4 in upper and lower panels). Whereas the ER contamination of the purified mitochondria was strongly reduced, the amount of VAMP-1B targeting was unaffected (Figure 2B). This indicates that membrane targeting detected here is indeed to mitochondrial membranes and not to contaminating ER membranes.

Characteristics of VAMP-1B targeting

The driving force for insertion of VAMP-1B into mitochondrial outer membrane remains unknown. To test the dependence of VAMP-1B targeting on inner-membrane potential, we used carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of inner-membrane electrochemical potential. ^{35}S-Labelled VAMP-1 and $pSU9_{1–69}$-DHFR [26] were incubated with intact washed mitochondria in either the absence or presence of 45 μM CCCP. Matrix targeting is marked by the proteolytic maturation of the protein by the matrix signal peptidase. While the import of the matrix-targeted protein $pSU_{1–69}$-DHFR was abolished by treatment with CCCP (Figure 3A, lanes 6 and 9), insertion of VAMP-1B into the mitochondrial outer membrane was unaffected by CCCP (Figure 3A, lanes 5 and 8). This demonstrates that, as has been shown previously for other outer-membrane-targeted proteins [18,25], the import of VAMP-1B (Figure 3A, lower panel) into mitochondria was independent of the mitochondrial electrochemical potential. To examine the topology of the inserted VAMP-1B, a proteolysis experiment was performed. Since the bulk of the protein is N-terminal to the membrane anchor, it is expected that if the protein is oriented with the N-terminus towards the cytosol the protein will be largely protease-sensitive when inserted into intact mitochondria, whereas if the N-terminus is oriented towards the inter-membrane space the protein will be protected from proteolysis by the mitochondrial outer membrane. After import, the bulk of the VAMP-1B protein was cleaved by exogenous trypsin (Figure 3B, lanes 4 and 6), indicating that VAMP-1B is anchored by the predicted transmembrane domain in the N_{cyto}–C_{in} orientation.

To characterize broadly the steps involved in transport, we wished to compare the targeting of VAMP-1B with that of another well-characterized C-terminally anchored mitochondrial

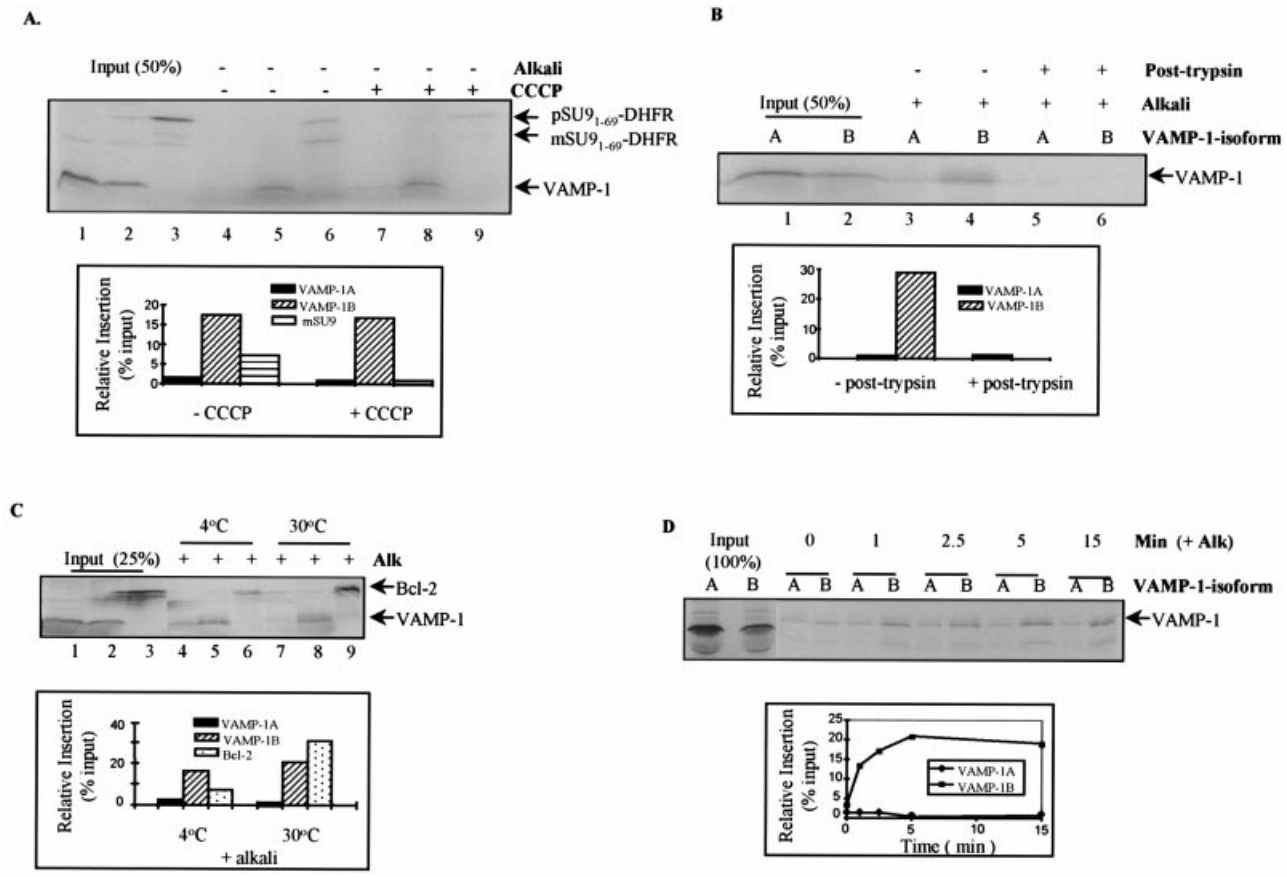

Figure 3 VAMP-1B insertion into mitochondria is independent of membrane potential, the protein is anchored in the outer membrane with its C-terminus and insertion is relatively temperature-insensitive and rapid

(**A**) CCCP inhibits pSU9$_{1-69}$-DHFR targeting but not VAMP-1B. ^{35}S-Labelled VAMP-1A, VAMP-1B and pSU9$_{1-69}$-DHFR were incubated with intact washed mitochondria at 30 °C, in the absence (lanes 4–6) or presence (lanes 7–9) of 45 μM CCCP. Mitochondria were recovered and analysed directly. m, mature-size protein. Lanes 1, 2 and 3 represent 50% of the [^{35}S]protein added to each assay. (**B**) VAMP-1B is anchored in the outer membrane. After import, mitochondria were treated with 30 μg/ml trypsin on ice for 30 min in the presence or absence of excess 100 μg/ml soya bean trypsin inhibitor ($\pm$ post-trypsin). Mitochondria were recovered and analysed after alkaline extraction. Lanes 1 and 2 represent 50% of the [^{35}S]protein added to each assay. (**C**) Effect of temperature on VAMP-1 import. ^{35}S-Labelled VAMP-1A, VAMP-1B and Bcl-2 were incubated with intact washed mitochondria at either 4 or 30 °C. Mitochondria were recovered and analysed after alkaline extraction (Alk, lanes 4–9). Lanes 1, 2 and 3 represent 25% of the [^{35}S]protein added to each assay. (**D**) Time course of mitochondrial targeting at 4 °C. ^{35}S-Labelled VAMP-1A and VAMP-1B were incubated with intact washed mitochondria for 0, 1, 2.5, 5 and 15 min. Mitochondria were recovered and analysed after alkaline extraction. The relative insertion was plotted as a function of time. Lanes 1 and 2 represent 100% of the [^{35}S]protein added to each assay.

protein, Bcl-2, and to determine the temperature dependency of the transport reaction. The recovery of the membrane-inserted alkali-resistant form of Bcl-2 was greater at 30 °C than at 4 °C (Figure 3C, lanes 6 and 9) but the insertion of VAMP-1B was only slightly increased at 30 °C when compared with 4 °C (Figure 3C, lanes 5 and 8). About 20% of the input VAMP-1B and 30% of the input Bcl-2 were inserted into membrane at 30 °C (Figure 3C, lower panel). In three additional experiments the efficiency of insertion of VAMP-1B and Bcl-2 was found to be similar to one another (results not shown). These data illustrate that the import of VAMP-1B into mitochondria is similar in efficiency to Bcl-2 but is less sensitive to temperature. This difference in temperature dependence could be at any one of several steps in transport (see Discussion).

The import of VAMP-1B at low temperature suggests that targeting is a high-affinity process. To examine this more closely we measured the time course of targeting of VAMP-1B to mitochondria at 4 °C. ^{35}S-Labelled VAMP-1A and VAMP-1B were incubated with intact washed mitochondria at 4 °C for 0, 1, 2.5, 5 and 15 min. Mitochondria were recovered and analysed after alkali treatment. Unlike VAMP-1A, the insertion of VAMP-1B into mitochondria increases with time (Figure 3D). The amount of insertion reaches a plateau at 5 min (Figure 3D, lower panel), indicating that the uptake of VAMP-1B to mitochondria is rapid even at reduced temperature.

VAMP-1B targeting into mitochondria is saturable

One characteristic of a receptor-mediated process is saturability. To test whether VAMP-1B targeting requires a mitochondrial receptor, we produced constructs for bacterial expression encoding the C-terminal half of VAMP-1A (residues 61–118) and VAMP-1B (residues 61–116) fused to the C-terminus of GST. The corresponding proteins were expressed in *E. coli*, and purified by affinity chromatography on glutathione-agarose. These purified, non-radioactive proteins were then added in mass amounts to transport assays to test for competition with the ^{35}S-labelled proteins produced by translation *in vitro*. To prevent aggregation, the GST-fusion proteins were eluted in a buffer containing 7 M urea. When diluted into the assay mix the final urea concentration was 0.7 M. Control experiments indicated that this level of urea had no effect on targeting (results not shown), as had been shown

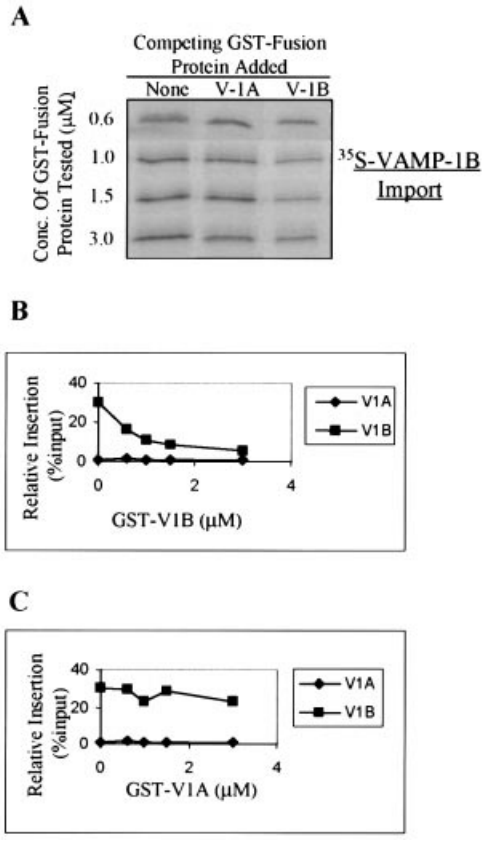

Figure 4 Inhibition of VAMP-1B insertion by purified GST-fused VAMP-1B

(**A**) In a series of import experiments purified GST-VAMP-1B$_{61–116}$ (V-1B) or GST-VAMP-1A$_{61–118}$ (V-1A; 0–3 μM) in 7 M urea were added to import mixtures containing 100 μg of intact washed mitochondria and ^{35}S-labelled VAMP-1B. For each experiment a no-peptide control, with just urea added, was included. The final concentration of urea in all reaction mixtures was 0.7 M. After 30 min of incubation at 30 °C, mitochondria were recovered and alkaline extracted. Recovered ^{35}S-labelled VAMP-1B and -1A (not shown in **A**) was analysed by SDS/PAGE and PhosphorImager analysis. Imported VAMP-1 proteins were quantified and compared with the input to the import reaction (not shown in **A**) as described in the Materials and methods section. (**B**) Quantification of inhibition of GST-VAMP-1B$_{61–116}$. (**C**) Quantification of inhibition of GST-VAMP-1A$_{61–118}$.

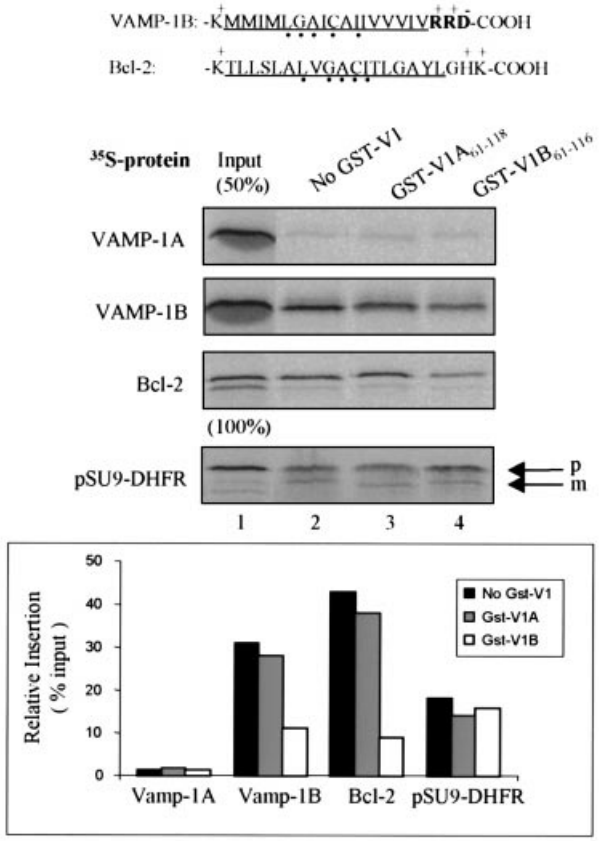

Figure 5 Competition of targeting of outer-membrane and matrix proteins to mitochondria by purified VAMP-1 protein chimaeras

VAMP-1B and Bcl-2 targeting into mitochondria is inhibited by GST-VAMP-1B$_{61–116}$. ^{35}S-Labelled VAMP-1A, VAMP-1B, Bcl-2 and pSU9$_{1–69}$-DHFR were incubated with 100 μg of washed mitochondria at 30 °C for 30 min in the absence of eluted GST-fusion protein (lanes 2), and in the presence of 3 μM GST-VAMP-1A$_{61–118}$ (lanes 3) or 3 μM GST-VAMP-1B$_{61–116}$, (lanes 4). The final concentration of urea in the import mixtures was 0.7 M. Mitochondria were recovered after alkaline extraction and analysed as described in Figure 1. Lane 1 for panels VAMP-1A, VAMP-1B and Bcl-2 represents 50% of the [^{35}S]protein added to each assay and lane 1 for panel pSU9$_{1–69}$-DHFR represents 100% of the [^{35}S]protein added to each assay. The dots below the VAMP-1B and Bcl-2 sequences indicate identical amino acids; p, precursor; m, mature.

previously for transport of proteins to the mitochondrial matrix [27]. The presence of GST-VAMP-1B$_{61–116}$ strongly inhibited the targeting and insertion of VAMP-1B into the mitochondrial outer membrane (Figure 4A). Inhibition was close to 80% at 3 μM GST-VAMP-1B$_{61–116}$, and was half-maximal at 1 μM. The corresponding protein derived from VAMP-1A had no significant effect on targeting and insertion at these levels (Figure 4B). These data demonstrate that VAMP-1B targeting to mitochondria is saturable and strongly implicate the involvement of a protein receptor in this process. Consistent with this concept, we found that pre-treatment of mitochondria with trypsin to remove surface proteins significantly inhibited VAMP-1B targeting (results not shown). This protease-sensitivity result mirrors previous reports for Bcl-2 [28]. These results suggest that the mitochondrial targeting of VAMP-1B depends on a saturable surface receptor.

Structural similarities between the VAMP-1B signal/anchor sequence and that of other C-terminally anchored outer-membrane proteins, such as Bcl-2, suggest that they may share a common import pathway. To test this directly the ability of GST-VAMP-1B to compete for the mitochondrial targeting of Bcl-2 was explored (Figure 5). In the presence of 3 μM GST-VAMP-1B$_{61–116}$, the insertion of both VAMP-1B and Bcl-2 were reduced by 65–75% (Figure 5, lanes 2–4). GST-VAMP-1A$_{61–118}$ had only a marginal effect. We have therefore established that VAMP-1B and Bcl-2 share a common uptake pathway that recognizes the structural similarities between the VAMP-1B and Bcl-2 targeting signals, but rejects the closely related VAMP-1A signal. The ability of GST-VAMP-1B to compete for targeting of a matrix-directed protein, pSU9$_{1–69}$-DHFR, was also tested. Neither GST-VAMP-1A$_{61–118}$ nor GST-VAMP-1B$_{61–116}$ had a significant effect on the import of pSU9$_{1–69}$-DHFR, as judged by the proteolytic conversion of precursor pSU9$_{1–69}$-DHFR into the mature form in the mitochondrial matrix. These results indicate that whereas VAMP-1B may share the same receptor as Bcl-2, and presumably other C-terminally anchored proteins, matrix-targeted proteins use a different receptor system or, alternatively, that a different step in transport is rate-limiting.

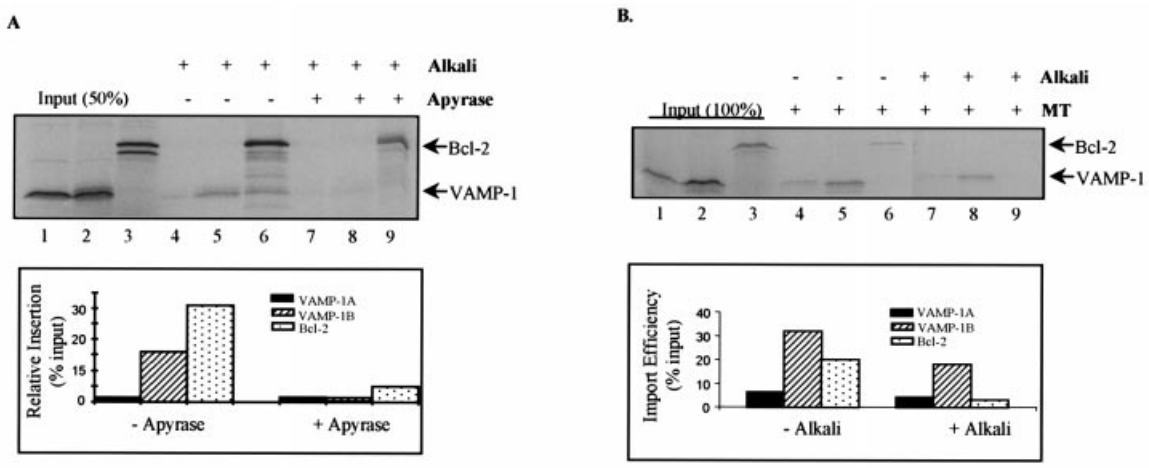

Figure 6 VAMP-1B targeting from reticulocyte lysates requires ATP

(**A**) Translation mixtures from reticulocyte lysate containing *in vitro*-translated VAMP-1A, VAMP-1B or Bcl-2, without (lanes 4–6) and with (lanes 7–9) 5 units of apyrase pretreatment at 24 °C for 20 min, were then incubated with 100 μg of washed mitochondria for 30 min at 30 °C. Mitochondria were recovered following alkaline extraction. Lanes 1 and 2 represent 50% of the [^{35}S]protein added to each assay. (**B**) Import of C-terminally anchored proteins from wheatgerm extract translations to mitochondria. ^{35}S-Labelled wheatgerm extract translated VAMP-1A, VAMP-1B and Bcl-2 were incubated with intact washed mitochondria (MT) at 30 °C for 30 min. Mitochondria were recovered and analysed directly (lanes 4–6) or after alkaline extraction (lanes 7–9). The relative insertion was calculated as a proportion of input. Lanes 1, 2 and 3 represent 100% of the [^{35}S]protein added to each assay.

ATP-dependence of outer-mitochondrial membrane targeting

The involvement of ATP in VAMP-1B targeting was examined using apyrase to deplete ATP from the import reactions. When VAMP-1A, VAMP-1B and Bcl-2 were incubated with mitochondria, the targeting of VAMP-1B was completely abolished by ATP depletion (Figure 6A, lanes 5 and 8) and the targeting of Bcl-2 was significantly reduced (Figure 6A, lanes 6 and 9). Similar results were obtained when using hexokinase to deplete ATP (results not shown). These results are similar to those found previously for the ATP dependence of mitochondrial insertion of Bcl-2 [18].

Although the involvement of molecular chaperones in the import of C-terminally anchored proteins has not been demonstrated previously, molecular chaperones have been implicated in import of proteins to the mitochondrial matrix. Molecular chaperones require ATP for function, and the ATP dependence of import may therefore be due to the involvement of ATP-dependent chaperone proteins. An alternative role for ATP is in an ATP-dependent import or translocation event. These possibilities are explored below.

Import of VAMP-1B translated in a cell-free wheatgerm extract to mitochondria

Wheatgerm extract contains relatively few chaperones and does not contain the chaperone MSF that may have a specific interaction with proteins destined for import into mitochondria [15,29]. Therefore, to test the dependence of VAMP-1B targeting on MSF we measured the mitochondrial targeting of VAMP-1B translated in wheatgerm extract. VAMP-1B bound to mitochondria, as judged by the signal before alkaline extraction, and inserted into mitochondrial membrane with about 20% efficiency (Figure 6B, lane 5), as judged after alkaline treatment (Figure 6B, lane 8), similar to levels of insertion found when the protein was translated in reticulocyte lysate. No insertion of VAMP-1A was detected (Figure 6B, lanes 4 and 7). In contrast Bcl-2 was bound but not inserted into the membrane (Figure 6B, lanes 6 and 9). Adding reticulocyte lysate to the wheatgerm extract system did not increase import of VAMP-1B to mitochondria (results not shown). This indicates that wheatgerm extract contains sufficient levels of components required for VAMP-1B targeting and that MSF is not required for VAMP-1B targeting. Conversely, Bcl-2 insertion depends on a component of reticulocyte lysate not found in wheatgerm extract, possibly MSF. After depletion of ATP in the wheatgerm translation mixture with either apyrase or hexokinase, the import of VAMP-1B was reduced from 20% efficiency to 7%, but targeting was not completely abolished as it is in the reticulocyte lysate system (results not shown). This small, but significant, difference in the degree of ATP dependence between translation in wheatgerm and reticulocyte lysates suggests a relationship between the ATP dependence and cytosolic factors involved in the targeting process.

Cytosolic factor(s) are not required for selective VAMP-1B targeting

Are cytosolic chaperone proteins absolutely required for selective targeting of C-terminally anchored proteins? To test this we measured the import of VAMP-1A and VAMP-1B in the absence of any cytosolic proteins by utilizing the purified GST-fusion proteins GST-VAMP-1A$_{61–118}$ and GST-VAMP-1B$_{61–116}$. The fusion proteins were purified and maintained in 7 M urea to prevent aggregation. After purification, these proteins, which were engineered with an N-terminal protein kinase A phosphorylation site, were phosphorylated *in vitro* by incubation with protein kinase and [^{33}P]ATP. The ^{33}P-labelled proteins purified from *E. coli* extracts were diluted from the 7 M urea in which they were isolated and incubated in the absence or presence of intact mitochondria. [^{33}P]GST-VAMP-1B$_{61–116}$ efficiently targeted to and integrated into mitochondria whereas the corresponding VAMP-1A construct only slightly associated with mitochondria (Figure 7A, lanes 8 and 9). During GST-fusion-protein purification, both VAMP-1A and VAMP-1B were partially degraded (shown in Figure 7, lanes 2 and 3, lower bands). However, only the full-length VAMP-1B fusion protein was

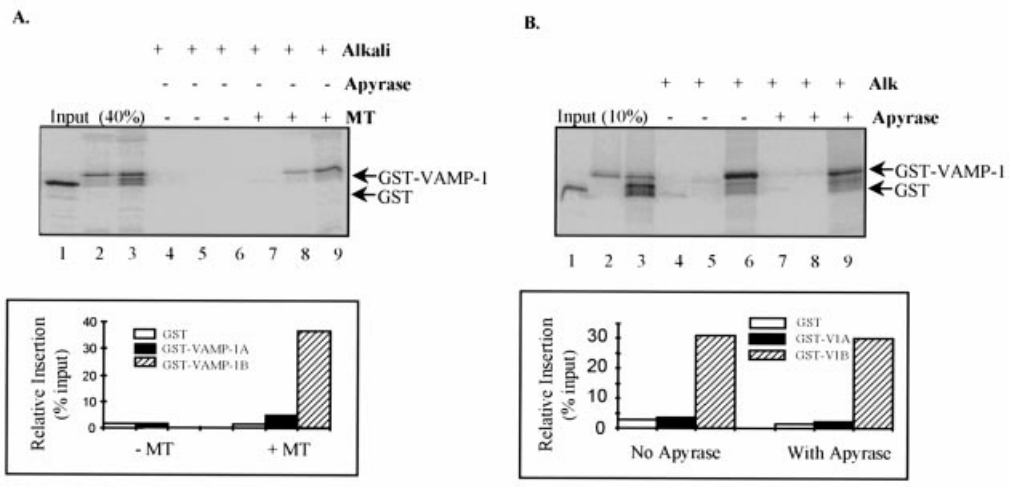

Figure 7 The VAMP-1B C-terminus mediates selective targeting to mitochondria in the absence of cytosolic proteins in an ATP-independent fashion

(**A**) Targeting of ^{33}P-labelled GST-fused VAMP-1 tails to mitochondria. Bacterially expressed GST-fusion proteins were purified and radiolabelled with [^{33}P]ATP by phosphorylation on beads and eluted with 10 mM reduced glutathione elution buffer containing 7 M urea. Radiolabelled GST (lanes 4 and 7), GST-VAMP-1A$_{61–118}$ (lanes 5 and 8) and GST-VAMP-1B$_{61–116}$ (lanes 6 and 9) in 7 M urea were incubated without (lanes 4–6) or with (lanes 7–9) washed mitochondria (MT) for 30 min at 30 °C. The final concentration of urea in the import mixture was 0.2 M. Mitochondria were recovered after alkaline extraction, and import efficiency was calculated as a proportion of input. Lanes 1, 2 and 3 represent 40% of the [^{35}S]protein added to each assay. (**B**) Mitochondrial uptake of the GST-VAMP-1B$_{61–116}$ does not require ATP. Radiolabelled GST (lanes 4 and 7), GST-VAMP-1A$_{61–118}$ (lanes 5 and 8) and GST-VAMP-1B$_{61–116}$ (lanes 6 and 9) were treated without (lanes 4–6) or with (lanes 7–9) 5 units of apyrase at 24 °C for 20 min before performing import. Mitochondria were recovered after alkaline extraction and analysed as described above. Lanes 1, 2 and 3 represent 10% of the [^{35}S]protein added to each assay.

substantially inserted into the membrane. Not surprisingly, GST alone was not incorporated into the mitochondrial membrane. The selective targeting of chaperone-free VAMP-1B conclusively demonstrates that chaperones do not have a direct role in the targeting process. It does not rule out, however, that chaperones may assist the process *in vivo*, or *in vitro* in the absence of urea, by maintaining proteins in a transport-competent conformation. Because this system lacks any lysate-derived cytosolic proteins, it was possible to test whether the ATP dependence of import measured previously with *in vitro*-translated proteins was due to the involvement of cytosolic components. The treatment of eluted ^{33}P-labelled fusion proteins with apyrase and inclusion of apyrase in the import reaction did not diminish the import of [^{33}P]GST-VAMP-1B$_{61–116}$ to mitochondria (Figure 7B, lanes 6 and 9). Similar results were obtained with hexokinase treatment (results not shown). These experiments establish two important aspects of the targeting process. First, cytosolic proteins are not absolutely required, so all the information required for targeting must reside in the targeted protein itself interacting with the mitochondrial receptor. Secondly, the import process is fundamentally ATP-independent. However, when cytosolic proteins assist the process an ATP requirement is imposed on the system.

DISCUSSION

The specificity of targeting of C-terminally anchored proteins to mitochondria

Current evidence indicates that the mitochondrial outer membrane and the ER are the only two sites in the cell competent for insertion of C-terminally anchored proteins. Here we have shown that whereas VAMP-1B is imported into the mitochondrial outer membrane efficiently, VAMP-1A is not. These experiments demonstrate that, *in vitro*, mitochondria could distinguish between two closely related proteins on the basis of the amino acid sequence in the C-terminal membrane anchor, mirroring results seen in intact cells [11]. The signal/anchor sequence of VAMP-1B closely matches in overall structure that of several other tail-anchored proteins of the mitochondrial outer membrane. These proteins all contain hydrophobic membrane-anchoring segments of around 17 residues, flanked by positively charged amino acids. Furthermore, mutagenesis of this region of VAMP-1B has identified these sequence characteristics as being crucial for a functional targeting signal. VAMP-1A and -1B are therefore a useful pair of proteins to establish the specificity of targeting to the outer membrane. This specificity is difficult to reconcile with a spontaneous insertion mechanism, given the small difference in sequence between VAMP-1A and -1B. To directly confirm that the targeting of C-terminally anchored proteins to mitochondria is specific, we demonstrate that the import pathway is saturable, but only by the VAMP-1B and not the VAMP-1A targeting sequence. As expected, the VAMP-1B targeting sequence also competes for uptake of Bcl-2, demonstrating that the mechanism being tested is general for C-terminally anchored proteins.

Like two other outer-membrane proteins, Bcl-2 [18] and the hybrid protein pOMD29-DHFR [30], VAMP-1B insertion is independent of membrane potential, indicating that the transport event is driven only by events in the outer membrane. The targeting of VAMP-1B is rapid and relatively temperature-insensitive. This is consistent with a transport mechanism that proceeds with high affinity and very high energy efficiency, much as has been observed for the targeting of the matrix proteins [26,31]. The difference in temperature dependence between VAMP-1B and Bcl-2 could conceivably be at the recognition step at the membrane surface or in a subsequent membrane-insertion step. We believe it is most likely that this difference reflects a distinction in how these proteins interact with cytosolic chaperones. As illustrated in Figure 6, VAMP-1B is transported efficiently when translated in wheatgerm lysates, whereas Bcl-2 is not. This is most probably because of a difference in chaperone interactions. Similarly the most temperature-sensitive step in

transport could involve the ATP-dependent transfer of these proteins from chaperones to the mitochondrial receptor. The difference in temperature dependence may therefore reflect a difference in the strength of interaction of VAMP-1B and Bcl-2 with chaperones, or a difference in the spectrum of chaperones that are bound by these proteins in lysates.

Evidence for a specific targeting receptor for C-terminally anchored membrane proteins

The specificity and saturability of C-terminally anchored protein targeting is strongly suggestive of the involvement of a targeting receptor. Limited proteolytic digestion of the mitochondrial surface inhibits targeting of Bcl-2 to mitochondria [9,18], consistent with our evidence for a saturable, protein-mediated uptake mechanism. Is the receptor one of the receptor complexes identified previously for import of matrix-targeted proteins? Antibodies to one component, Tom20 (translocase of the outer membrane of mitochondria of 20 kDa), have been reported to reduce, but not eliminate, targeting of Bcl-2 to mitochondria [9]. However, it has not been shown conclusively that Tom20 is itself the relevant receptor, or whether other components, such as an insertion pore, are required for targeting and/or insertion. Resolution of this question will likely require a more definitive biochemical approach. The ability to achieve import in a fully reconstituted system [32] coupled with our greater understanding of the structure of the targeting sequence for both mitochondria and the ER holds great promise in this regard.

The role of chaperone proteins and ATP

It has been shown that cytosolic chaperones bind to matrix-destined preproteins post-translationally and prevent them from misfolding or aggregating, and preserve their import competence [14]. For matrix-directed proteins the chaperones are not thought to impart targeting information themselves. However this question has remained open for tail-anchored proteins. We therefore asked whether a cytosolic factor is required for selective delivery of VAMP-1B to mitochondria. Given the hydrophobic nature of the C-terminal anchor/signal sequence it is not surprising that chaperones might be involved in preventing aggregation. But do chaperones have a role in the selectivity of targeting? The classical route of protein import into the ER provides an interesting paradigm for hydrophobic targeting sequences. In this system, soluble cytosolic SRP binds to a hydrophobic targeting signal peptide of the preprotein co-translationally, blocking translation, and transfers the translation apparatus to the ER by binding to the SRP receptor in the ER membrane [17]. In this case the targeting function is not provided directly by the signal peptide but indirectly by its interaction with SRP, which only targets to its receptor when bound to a signal peptide. To address the function of chaperones in specific mitochondrial targeting, it was essential to see whether mitochondrial import could be recreated in the absence of chaperones.

To accomplish this, transport of purified VAMP chimaeras, instead of VAMPs derived from *in vitro* translation, was tested. The chaperone-free VAMP-1B chimaera was indeed targeted efficiently to mitochondria. This demonstrates for the first time that targeting specificity does not reside in the interaction between the mitochondrial receptor and a chaperone, or chaperone–protein complex, but directly in an interaction with the targeted protein itself (Figure 7). It should be noted that these chimaeras were diluted from concentrated urea solutions to prevent aggregation of the hydrophobic membrane anchor. It seems likely that in the cell chaperones would function to prevent this type of aggregation, but these studies demonstrate that they do not have a direct role in specific targeting. The observation that ATP is required in the presence, but not the absence of cytosolic factors is most readily explained by a requirement for ATP in chaperone action. ATP is required for chaperones to release bound substrates and it is likely in this case that the release of the targeted protein from chaperones to the mitochondrial targeting receptor is the ATP-dependent step of transport. However, the participation of chaperones is only suggested indirectly by the present study. Which chaperones are involved and how they allow recognition of the targeting sequence by the mitochondrial receptor will require further investigation. The ATP-independent targeting of the chaperone-free VAMP-1B chimaera to mitochondria indicates that the interaction of the targeted protein with the mitochondrial outer membrane generates the driving force for translocation. The implication is that there is a gradient of affinity of the hydrophobic signal anchor sequence of tail-anchored proteins going from the targeting receptor to the insertion pore (if it is indeed involved) to the bilayer of the outer membrane. This gradient of affinity gives rise to an insertion mechanism that is independent of a chemical energy source.

Once the targeting receptor has been identified it will be of great interest to determine how the requirement to bind the signal/anchor sequence with adequate affinity to target the sequence from cytosol to mitochondria is balanced with the need to spontaneously release the same sequence to the insertion apparatus. The finding of mitochondrial targeting of [^{33}P]GST-VAMP-1B$_{61–116}$ also demonstrates that the VAMP-1B C-terminus (55 amino acids) contains sufficient information for selective targeting (including recognition by mitochondrial receptors and translocation into the membrane). Indeed we find that the sequence requirement for VAMP-1B-specific mitochondrial targeting resides solely in the transmembrane anchor sequence (L. Lan, S. Isenmann, O. Mckenzie and B. W. Wattenberg, unpublished work).

The data presented here demonstrate that mitochondrial surface proteins function as an apparatus for selective and direct interaction and translocation of tail-anchored proteins to mitochondria. Chaperones appear to assist but are not essential for selective targeting. Moreover, the insertion of C-terminally anchored proteins into the membrane does not require ATP. Further studies are ongoing to identify the components that recognize C-terminal mitochondrial targeting signals and insert them into the outer-mitochondrial membrane.

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