CMLS, Cell. Mol. Life Sci. 62 (2005) 1890–1899
1420-682X/05/161890-10
DOI 10.1007/s00018-005-5178-0

CMLS Cellular and Molecular Life Sciences

Research Article

Bacterial signal peptide recognizes HeLa cell mitochondrial import receptors and functions as a mitochondrial leader sequence

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Received 29 April 2005; received after revision 8 June 2005; accepted 17 June 2005
Online First 22 July 2005

Abstract. Phage display was used to identify new components of the mammalian mitochondrial receptor complex using Tom20 as a binding partner. Two peptides were identified. One had partial identity (SMLTVMA) with a bacterial signal peptide from Toho-1, a periplasmic protein. The other had partial identity with a mitochondrial inner membrane glutamate carrier. The bacterial signal peptide could carry a protein into mitochondria both in vivo and in vitro. The first six residues of the sequence, SMLTVM, were necessary for import but the two adjacent arginine residues in the 30-amino-acid leader were not critical for import. The signal peptides of *Escherichia coli* β-lactamase and *Bacillsus subtilis* lipase could not carry proteins into mitochondria. Presumably, the Toho-1 leader can adopt a structure compatible for recognition by the import apparatus.

Key words. Bacterial signal peptide; mitochondrial protein import; phage display; Toho-1; twin-arginine; Tom20; mitochondrial leader peptide.

A protein translocation apparatus is present in all organisms. A similarity exists between bacteria and higher organisms in that each has the ability to transport proteins across a membrane barrier. In bacteria, proteins are transported into the periplasmic space, while eukaryotes can transport proteins into different organelles. Since bacteria ultimately evolved to become mitochondria, bacterial remnants could be present in mitochondria. Therefore, some similarity might exist between mitochondrial import and bacterial protein secretion. The proteins that cross the membranes possess signal peptides either for secretion to the outside of the bacterial membrane or for import into mitochondria [1–3]. In either case, proteins are recognized by receptors present in the membrane [3–6]. However, major differences exist between secretory systems in bacteria and the import system in mitochondria. Bacteria have several types of secretory pathways including a general Sec pathway [7–10], a twin-arginine pathway (Tat) [11–13] and ATP-binding cassette (ABC) [14, 15] pathway. In contrast, mitochondria matrix space proteins use only Tom (translocase outer membrane) and Tim (translocase inner membrane) complexes for their translocation machinery.

Most mitochondrial proteins are synthesized on cytosolic ribosomes as preproteins with either N-terminal signal sequences or internal targeting signals [3, 6]. During or following synthesis, many preproteins are thought to associate with molecular chaperones that maintain the preproteins in a loosely folded translocation-competent conformation [16]. Preproteins are then targeted to the mitochondria and imported into the organelle. A protein complex, termed Tom, is responsible for recognizing and translocating preproteins into the organelle. In yeast, this complex includes the import receptors Tom20 and Tom70 and a general import pore that contains an import receptor (Tom22), a channel protein (Tom40) and small Tom pro-

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teins [3, 17, 18]. In mammals, this complex includes Tom20 [19–21], Tom70 [22], Tom22 [23], a channel protein Tom40 [24] and small Tom proteins [25]. Preproteins with N-terminal presequences are initially recognized by Tom20, whereas preproteins with internal targeting signals, such as inner membrane carrier proteins, are recognized preferentially by Tom70. Preproteins are then transferred to the general import pore and translocated into mitochondria [26, 27] in an energy-dependent process.

Mitochondrial import is to be generally hypothesized a posttranslational event, implying that proteins are completely synthesized before import starts [3]. However, some laboratories, including ours, have found that a co-translation import pathway can exist [28–30]. Recently, a co-translational pathway was presented based on a genome-wide analysis [31]. This report suggests that two classes of mRNA encode mitochondrial proteins. One class is associated with mitochondria-bound polysomes and the other is found with free polysomes. The mRNAs that bind to the mitochondria-associated ribosomes code for proteins that could be imported using a co-translational pathway. Interestingly, the authors reported that the mRNAs present in mitochondria-associated polysomes were transcribed from the genes that have been identified to be of bacterial origin. In contrast, the mRNAs associated with free ribosomes were transcribed from genes of eukaryotic origin. This observation supports the hypothesis of endosymbiosis, implying that bacteria were internalized by eukaryotes and finally converted into mitochondria [32, 33].

Here, we performed phage display using Tom20 as a binding partner to identify peptides that could be part of an unidentified protein involved in mitochondrial import. These could include Tom components or cytosolic ones that might be necessary for docking or allowing the newly synthesized protein to recognize mitochondria. We identified only two peptides based on their tight binding to human Tom20. Both peptides inhibited import of an authentic mitochondrial preprotein into mitochondria. One peptide had significant identity with a bacterial signal peptide. The other had partial identity with a mitochondrial glutamate carrier. In this paper, we show that the full-length bacterial signal peptide could act as a mitochondrial leader peptide that carries a protein into HeLa cell mitochondria. In addition, we report that the bacterial leader can function as a mitochondrial leader even when all the positive charges are removed.

Materials and methods

Construction of plasmid

To construct the glutathione S-transferase (GST)-peptide fusion proteins, the primers encoded for peptide 1, peptide 2 or leader sequence of rat liver mitochondrial aldehyde dehydrogenase (rat pALDH) with *Eco*RI and *Xho*I sites were annealed and digested with these two enzymes. The digested products were cloned into the *Eco*RI /*Xho*I double-digested pET-21a (+)-GST vector. To construct the Toho-1-EGFP (the first 30 amino acids of Toho-1 fused to enhanced green fluorescent protein) plasmid, primers for PCR were designed based on the known sequence of Toho-1 [34]. The plasmid that contained full-length Toho-1 provided by Prof Y. Ishii (Toho University School of Medicine) was used as a template for PCR. The PCR products were digested with *Bgl*II and *Eco*RI and were ligated to pEGFPN1 (Clontec) already digested with *Bgl*II and *Eco*RI. The mutant, Toho-1 (R7,8Q)-EGFP was made the same way except the 5′-primer contained the desired mutations. To construct the β-lactamase-EGFP (the signal peptide of β-lactamase fused to EGFP) construct, PCR primers were designed based on the published sequence of β-lactamase from *Escherichia coli* signal peptide. PCR was performed using a plasmid that contained the β-lactamase gene, as template. PCR products were digested with *Bgl*II and *Eco*RI and were ligated to pEGFPN1 already digested with *Bgl*II and *Eco*RI. To construct the lipase A-EGFP (the signal peptide of lipase fused to EGFP) construct (LipA-EGFP), primers were designed based on the published sequence. The construct was made as described for other EGFP constructs using *Bacillus subtilis* genomic DNA as template for PCR. Toho-1 (SML →AAA)-EGFP and Toho-1 (*Δ*SMLTVM)-EGFP were made by standard molecular biology techniques. To make the Toho-1-DHFR (the first 30 amino acids of Toho-1 fused to dihydrofolate reductase) construct, PCR was carried out using the plasmid containing the full-length Toho-1 as template. PCR products had two *Nde*I sites at each end. The PCR products were digested with *Nde*I and ligated to pT7-7 DHFR already digested with *Nde*I and dephosphorylated. All constructs were confirmed by sequencing at the Purdue University DNA sequencing center.

Expression and purification of tom20 Δ(2–28)

Tom20 Δ(2–28) (Tom20 missing residues 2–28) was expressed and purified to homogeneity as previously reported [35].

Screening for peptide ligands of tom20 Δ(2–28) with a phage display peptide library

The Ph.D.–12 phage display library (New England Biolab) was selected and screened (panning) for the ligands of Tom20 Δ(2–28) according to the manufacturer's instruction. The manufacturer informed us that the library is biased such that proline is over-represented while arginine and leucine tend to be under-represented. The purified Tom20 Δ(2–28) was dissolved at 100 µg/ml in 0.1 M $NaHCO_3$ (pH 8.6) and coated on a 60-mm petri dish by incubation overnight at 4°C with gentle agitation. The ex-

cess Tom20 Δ(2–28) was then removed and the dish was filled with blocking buffer and incubated for approximately 2 h at 4°C. After discarding the blocking buffer, the dish was washed six times with TBST, and a pool of phage (4×10^{10} phage in 1 ml of TBST) was added to the coated dish. After an approximately 30 min incubation, the non-bound phages were discarded and the dish was washed ten times with TBST. The bound phage was eluted with 1 ml of 100 µg/ml Tom20 Δ(2–28) in TBS. The eluted phage was titered and amplified according to the manufacturer's instruction. The amplified phage was used in the next round of panning. After the fourth round of panning, the eluted phage was amplified and the phage DNA was isolated and sequenced.

Binding of the peptides to tom20 Δ(2–28) by ELISA

ELISA was performed according to the manufacturer's (New England Biolab) instruction. The Tom20 Δ(2–28)-coated ELISA plates were incubated with a series of fourfold dilutions of the phages containing peptide 1 or 2 in 200 µl TBS, starting with 10^{12} phages and ending with 2×10^{5} phages. The HRP-conjugated anti-M13 antibody and HRP substrate solution were used to analyze phage binding to Tom20 Δ(2–28). For an inhibition assay, in addition to the phages, 10 or 100 mM of in vitro-synthesized peptide 1 or 2 was added to the Tom20 Δ(2–28)-coated plate and the same ELISA procedure was performed.

In vitro protein-protein interaction assay

GST, GST-peptide1 and GST-peptide 2 were expressed and purified according to the manufacturer's instruction. Purified Tom20 Δ(2–28) was incubated with an equal amount of glutathione-Sepharose-bound protein in the binding buffer [50 mM Tris at pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 (NP-40), 10% glycerol and protease inhibitor complex] at 4 °C for 1 h. After a brief centrifugation, the pellets were washed five times with binding buffer. Proteins were eluted in the SDS-PAGE sample buffer and separated on tricine SDS-PAGE. Tom20 Δ(2–28) was detected by Western blot with rabbit anti-Tom20 antibody and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody.

pALDH leader - MLRAALSTARRGPRLSRLL

Toho-1 leader- MMTQ**SIRRSMLTVMA**TLPLLFSSATLHAQANS

Peptide 1 - H**SLKNSMLTVMA**

Peptide 2 - SPH**TTPMQML**AH

Glutamate carrier --VIV**TTPM**E**ML**KI-------

Figure 1. Leader sequences and phage peptides. Leader peptides from rat liver pALDH, *E. coli* TUH12191 Toho-1, mouse glutamate carrier 1 along with peptide 1 and peptide 2. Identical amino acids are in bold type.

In vitro mitochondrial protein import and inhibition of import

The in vitro mitochondrial import assay was performed as described elsewhere [36]. For the inhibition assay, various concentrations of in vitro-synthesized peptide 1 or 2 or carbonyl cyanide m-chlorophenylhydrazone (CCCP, 25 µM) were added to the import assay before the addition of TNT (Promega) synthesized proteins. The import assay samples were analyzed on 10% SDS-PAGE and proteins were visualized by phosphor imaging.

Direct observation of EGFP fluorescence in cultured cells

Microscopy analysis of HeLa cells was performed as described elsewhere [28]. When mitochondria were to be stained, 20 nM MitoTracker Red (Molecular Probes) was added to the medium and incubated for 20 min before fixation. When mitochondrial potential was examined in the presence of P1-EGFP, cells expressing P1-EGFP were stained with Mitotracker red dye. The cells were observed under an Olympus BX60 Fluorescence microscope.

Isolation of mitochondria from HeLa cells and submitochondrial fractionation

Mitochondria from HeLa cells was isolated as described elsewhere [28]. Sub-mitochondrial fractionation was carried out using different concentrations of digitonin (0.05–0.4%) as described previously [37]. Porin and succinate dehydrogenase were used as marker proteins. The succinate dehydrogenase assay was performed as described before [38]. Porin was detected by antibodies.

Immunoblot analysis

Culture and transfection of HeLa cells was performed as described previously [28]. The-whole cell extracts, or mitochondria or sub-mitochondrial fractions were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Monoclonal antibodies against *Aequoria victoria* GFP and porin (Molecular probes) were used as the primary antibodies; alkaline phosphatase-conjugated goat anti-mouse IgG was used as the second antibody.

Results

Identification of two peptides that interact with mammalian tom20 Δ(2–28)

Tom20, an outer membrane mitochondrial protein, plays an important role in protein import by interacting not only with leader peptides but also with other receptor proteins. We used phage display in an attempt to find a peptide that would correspond to an unidentified binding partner of

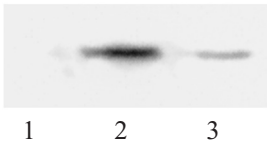

Figure 2. Binding of Tom20 to GST fusion peptides. Purified Tom20 Δ(2–28) was incubated with GST, GST-peptide 1 and GST-peptide 2 on a glutathione Sepharose column. After several washings with buffer, the proteins were eluted with SDS-PAGE buffer and separated on Tricine SDS-PAGE. The Tom20 Δ(2–28) was detected by anti-Tom20 antibody. Lane 1 was Tom20 Δ(2–28) with GST only, lane 2 was Tom20 Δ(2–28) with GST-peptide 1 and lane 3 was Tom20 Δ(2–28) with GST-peptide 2.

Tom20. Tom20 Δ(2–28) was chosen instead of full-length Tom20 because the truncated portion is soluble in aqueous media.

After four rounds of panning with the Ph.D.-12 phage display peptide library, 12 phage plaques were found and the phage DNA was purified to identify the sequence of the Tom20-binding peptides. The results showed that 10 of the 12 plaques contained the same 12-mer peptide sequence, which was HSLKNSMLTVMA (peptide 1), and the other two plaques contained another 12-mer peptide sequence, which was SPHTTPMQMLAH (peptide 2). Thus, only two unique peptides were identified from the library of 10^{12} independent peptides. The manufacturer's literature does not state that there is a bias against any amino acids in the library, but they verbally informed us that the library is biased such that proline is over-represented while arginine and leucine tend to be under represented. The bias against arginine and leucine is the most likely cause for our not finding many common mitochondrial leader peptides such as the one from aldehyde dehydrogenase that contains many of these residues. A Blast search was performed to determine whether or not these two peptides belonged to or shared significant

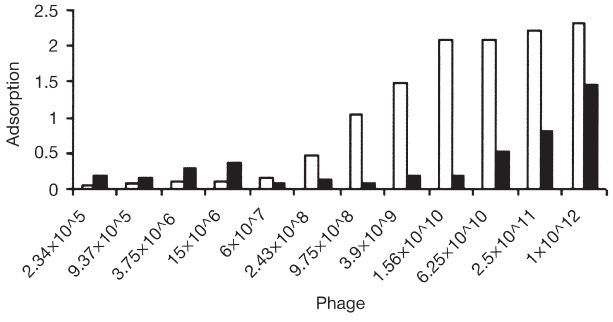

Figure 3. Binding of peptides 1 and 2 to Tom20 Δ(2–28) detected by ELISA. The Tom20 Δ(2–28)-coated plates were incubated with a series of fourfold dilutions of the phage containing either peptide 1 (white box) or 2 (black box) in 200 µl TBS. The phage concentrations ranged from 2.34×10^5 to 1×10^{12}. After washing the unbound phages, the bound phages were detected by adding HRP-conjugated anti-M13 antibody and HRP substrate. Plates were read using a microplate reader.

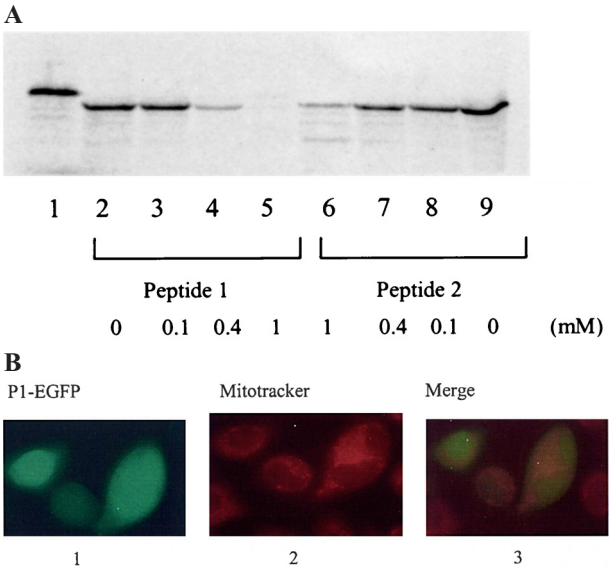

Figure 4. (*A*) In vitro import inhibition of pALDH by peptide 1 or 2. Import with isolated rat mitochondria was performed as described under Materials and methods. Various amounts of peptide 1 or 2 were added to the pALDH import assay. After import for 30 min at 30 °C, proteinase K was added to digest non imported proteins. The imported proteins were separated on 10% SDS-PAGE and visualized by PhosporImager. Lane 1 was TNT-synthesized pALDH, lanes 2 and 9 were import of pALDH without addition of peptide, lanes 3–5 were import in the presence of peptide 1, lane 3 was import in the presence of 0.1 mM, lane 4 was import in the presence of 0.4 mM and lane 5 was import in the presence of 1 mM peptide 1. Lanes 6–8 were import of pALDH in the presence of peptide 2, lane 6 was import of pALDH in the presence of 1 mM , lane 7 was import in the presence of 0.4 mM, lane 8 import in the presence of 0.1 mM peptide 2. (*B*) Fluorescent microscopy analysis of HeLa cells transiently expressing P1-EGFP fusion proteins. HeLa cells were cultured on a cover slip and transfected with 1–2 µg of plasmid DNA. Fluorescence microscopy was used to visualize the P1-EGFP construct. 1, images of HeLa cells transiently expressing P1-EGFP; 2, images of HeLa cells stained with Mitotracker red dye; 3, merging images 1 and 2

sequence similarity with known proteins. Peptide 1 was found to have substantial identity with the Toho-1 signal peptide from *E. coli* TUH12191 [34]. Toho-1 signal peptide is involved in translocation of carrier protein into the periplasmic space. The amino acid sequence SMLTVMA in peptide 1 is identical to a portion of the signal peptide of Toho-1 (fig. 1). Peptide 2 had partial identity with mouse mitochondrial glutamate carrier as indicated in figure 1.

Specific binding of peptides 1 and 2 to tom20 Δ(2–28)

An in vitro GST pull-down assay was performed to verify that peptide 1 or 2 could directly bind to Tom20 Δ(2–28). Peptide 1 or 2 was fused to GST and the purified GST or GST fusion proteins were incubated with purified Tom20 Δ(2–28) protein. After washing off the non-bound Tom20 Δ(2–28), the proteins left on the Sepharose beads were

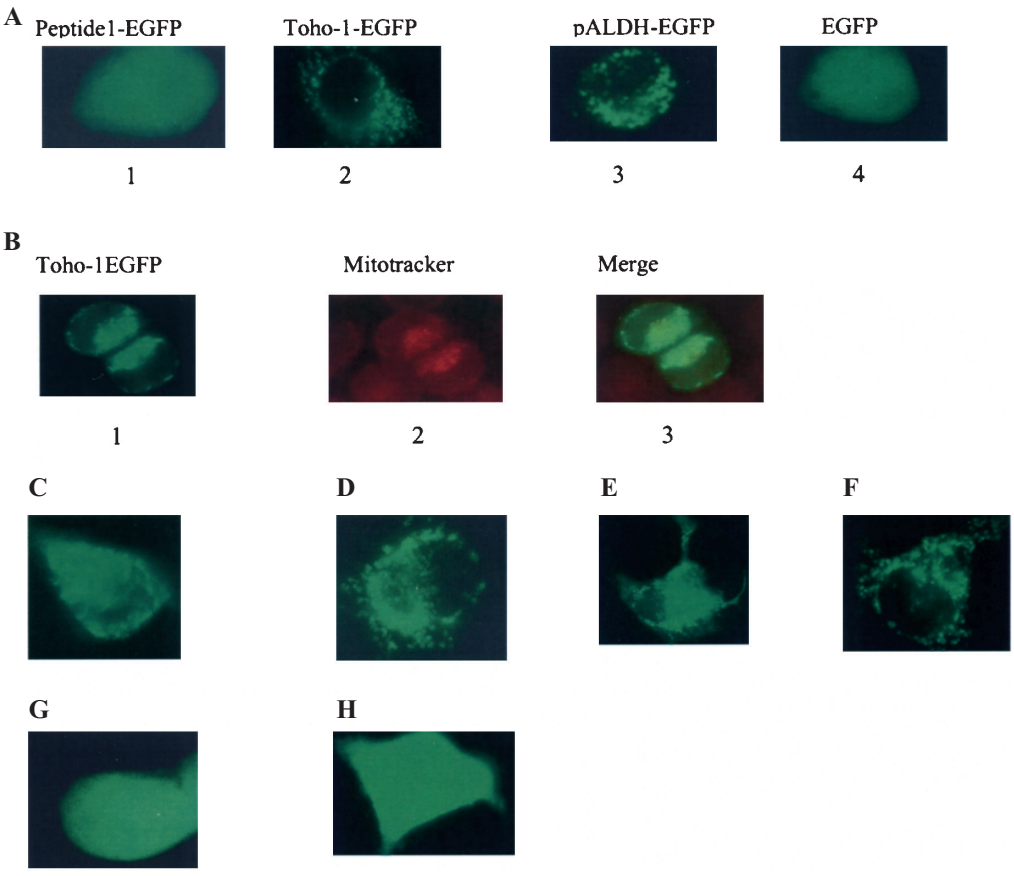

Figure 5. Fluorescent microscopy analysis of HeLa cells transiently expressing EGFP fusion proteins. HeLa cells were cultured on a cover slip and transfected with 1–2 µg of plasmid DNA. Fluorescence microscopy was used to visualize the EGFP constructs. The expressed proteins, without a mitochondrial signal, were distributed throughout the cell, including the nucleus. The proteins with mitochondrial signals were localized to mitochondria, which are seen either as small dots or long cylinders. The absence of fluorescence in the cytosol and nucleus is indicative of efficient mitochondrial import. (*A*) 1, peptide 1-EGFP; 2, Toho-1-EGFP; 3, pALDH-EGFP; 4, EGFP. (*B*) The cells were treated with Mitotracker red, and fluorescent images of EGFP (green) and Mitotracker red (red) were captured by an Olympus BX60 Fluorescence microscope. 1, images of HeLa cells transiently expressing Toho-1-EGFP; 2, images of HeLa cells stained with Mitotracker red dye; 3, merging images 1 and 2. (C–H) Fluorescent microscopy analysis of HeLa cells transiently expressing Toho-1(1–19)-EGFP (*C*), (R7,8Q)-EGFP (*D*), ΔSMLTVM (*E*), Toho-1 SML to AAA (*F*), β-lactamase-EGFP (G), LipA-EGFP (*H*).

eluted with SDS-PAGE sample buffer and separated on Tricine SDS-PAGE (fig. 2). Western blot using anti-Tom20 antibodies showed that Tom20 Δ(2–28) bound to both peptide 1 (fig. 2, lane 2) and peptide 2 (fig. 2, lane 3). GST alone did not interact with Tom20 Δ(2–28) (fig. 2, lane 1). An ELISA was performed to investigate the concentration-dependent binding of peptide 1 or 2 with Tom20 Δ(2–28). The results showed that each phage could bind to Tom20 Δ(2–28), with peptide 1 binding more strongly than did peptide 2 (fig. 3). Synthetic peptide 1 or 2 was added to the ELISA reaction and each peptide inhibited the binding of the respective phage to Tom20 Δ(2–28) (data not shown).

Inhibition of import of rat pALDH by peptide 1 and 2

Since both peptides bind Tom20 they were used in an in vitro assay to determine if they could inhibit the import of an authentic mitochondrial preprotein (pALDH). Different concentrations (0.1, 0.4 and 1 mM) of synthetic peptides were added to the in vitro import assay. The samples were analyzed on a 10% SDS-PAGE gel and proteins were visualized by phosphor imaging (fig. 4A). There was essentially no detectable inhibition by 0.1 mM peptide (fig. 4A, lanes 3, 8), but the mitochondrial import of pALDH was decreased to 30% and 70% when 0.4 mM peptide 1 or peptide 2 was added to the import reaction, respectively (fig. 4A, lanes 4, 7). The import was totally abolished when 1 mM peptide 1 was added to the import reaction (fig. 4, lane 5) and almost 70% inhibition (fig. 4A, lane 6) was found in the presence of 1 mM peptide 2. That peptide 1 could inhibit the import more efficiently than peptide 2, was not unexpected, since the results from the other binding assays indicated that peptide 1 bound to Tom20 more tightly than did peptide 2.

To rule out the possibility that the peptides inhibited import of pALDH by destroying the membrane potential, Mito-

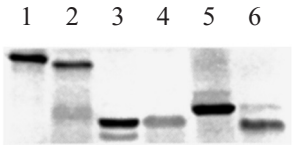

Figure 6. In vitro import of proteins into mitochondria. Import was performed as described under Materials and methods. Lanes 1 and 2 for pALDH, lanes 3 and 4 for Toho-1-DHFR, lanes 5 and 6 for pALDH-DHFR. Lanes 1, 3 and 5 were TNT synthesized proteins and lanes 2, 4 and 6 were those found after import into mitochondria representing imported protein. Proteins were visualized by Phospor Imager.

tracker red dye was employed. The dye accumulates in mitochondria only if they are functioning and have a high membrane potential. HeLa cells expressing P1-EGFP were stained with Mitotracker red dye (fig. 4B). Microscopic analysis showed the cells expressed P1-EGFP and mitochondria in that cell were stained with the dye. Thus, the expression of P1 in HeLa cells did not disrupt the membrane potential.

Mitochondrial protein import of Toho-1 signal peptide

Although both peptides interacted with Tom20 Δ(2–28), we used only peptide 1 in further studies as it bound Tom20 more tightly and had partial identity with a bacterial signal peptide. Peptide 1 was fused to EGFP and expressed in HeLa cells to investigate whether or not it could be used as a leader sequence when attached to a carrier protein. The green color was observed throughout the cell (fig. 5A). This showed that peptide 1 did not function as a mitochondrial leader peptide. However, as the sequence SMLTVMA was present in both peptide 1 and the signal peptide of Toho-1, the entire bacterial signal peptide was used to determine if it could function as a mitochondrial leader peptide. To test for this, Toho-1 signal peptide was fused to DHFR to make Toho-1-DHFR which was incubated with isolated HeLa cell mitochondria. After incubation and proteinase K digestion, the protein was found to have been imported into mitochondria (fig. 6, lane 4). As a control, pALDH and pALDH leader peptide fused to DHFR (pALDH-DHFR) were also used. Both were efficiently imported and processed into mitochondria (fig. 6, lanes 2, 6).

Toho-1 signal peptide was fused to EGFP and expressed in HeLa cells to determine if the bacterial signal peptide could function *in vivo* as a mitochondrial leader peptide. The resulting protein was localized only in mitochondria (fig. 5A). As a positive control, pALDH-EGFP was expressed in HeLa cells and it too was localized to mitochondria (fig. 5A). Without a signal peptide, EGFP was not localized in mitochondria (fig. 5A). To further confirm the mitochondrial location, images from Mitotracker red-stained mitochondria were merged with images from mitochondria expressing Toho-1-EGFP (fig. 5B). Results from the merged images verified that the chimeric protein was located in HeLa cell mitochondria. Thus, the bacterial signal sequence could apparently carry a protein into HeLa cell mitochondria.

The first 19 residues of Toho-1 signal peptide were fused to EGFP and in vivo import was again performed. The first 19 amino acids were selected because rat pALDH possesses a 19-amino-acid leader peptide [28] and the 7-amino-acid sequence was present in the first 19 amino acid residues of Toho-1. The in vivo import of Toho-1 (1–19)-EGFP was also found to be poor suggesting that the C-terminal part of the Toho-1 signal peptide was necessary for import (fig. 5C).

The amino acid sequence of Toho-1 signal peptide had features that were typical for signal peptides that follow the Tat pathway for secretion into the bacterial periplasmic space. It possesses two adjacent arginine residues near the N terminus as well as a hydrophobic patch after the arginine residues. The arginine residues have been shown to be critical for signal peptides that follow the Tat pathway [9]. Replacement of these arginines by glutamine abolished periplasmic secretion [12]. Since positively charged amino acids are also essential for mitochondrial import, arginines 7 and 8 were mutated to glutamines in Toho-1 to test if this change could affect mitochondrial import. This construct was expressed in HeLa cells and found to be localized in mitochondria (fig. 5D). Finding import showed that the positive charges present in the native bacterial signal peptide are not necessary for mitochondrial import.

Lastly, to test for the importance of the peptide portion identified from the phage display experiment, the first six residues of the above sequence were deleted from Toho-1 to make Toho-1 (ΔSMLTVM)-EGFP. The import was found to be poor as cytosolic fluorescence was also observed showing that the sequence was critical for mitochondrial import (fig. 5E). As we have shown that all six

	Import
Toho-1 –MMTQ**SI**RR**SMLTVMA**TLPLLFSSATLHAQ-EGFP	++++
R7,8Q- MMTQ**SIQQSMLTVMA**TLPLLFSSATLHAQ-EGFP	++++
SML→ AAA- MMTQ**SI**RR**AAATVMA**TLPLLFSSATLHAQ-EGFP	++++
ΔSMLTVM- MMTQ**SI**RR**A**TLPLLFSSATLHAQ-EGFP	+
1-19-MMTQ**SI**RR**SMLTVMA**TLPL-EGFP	+

Figure 7. Manipulation of Toho-1 signal peptide in the Toho-1-EGFP construct. The signal peptide was manipulated and different constructs were employed for in vivo import in HeLa cell mitochondria. The number of plus signs refers to the relative degree of import. The twin-arginine residues are underlined.

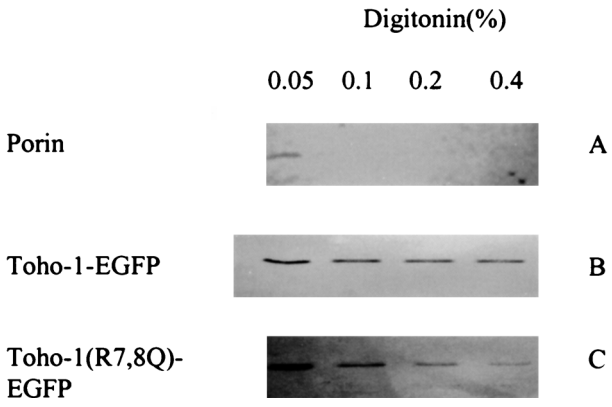

Figure 8. Sub-mitochondrial fractionation. HeLa cells were transfected with either Toho-1-EGFP or Toho-1 (R7,8Q)-EGFP and sub-mitochondrial fractionations were performed as described in Materials and methods. Mitochondria were treated with increasing concentrations of digitonin from 0.05 to 0.4%. After 5 min of incubation on ice, mitochondria were centrifuged. Supernatant and pellets were assayed for transfected proteins and markers. (*A*) Western blot analysis of pellet fraction with anti-porin antibody. (*B*) Western blot analysis for Toho-1-EGFP with anti-EGFP antibody. (*C*) Western blot analysis for Toho-1 (R7,8Q)-EGFP with anti-EGFP antibody.

residues cannot be deleted we changed the first three residues to alanine to make Toho-1 (SML→AAA)-EGFP and *in vivo* import was performed. The construct was imported to the same extent as Toho-1-EGFP into mitochondria (fig. 5F) showing that this change could be tolerated. Additional work will be necessary to understand the importance of individual residues of the bacterial leader for import and periplasmic transport. The import of the mutated signal peptides employed in this study is summarized in figure 7.

Sub-mitochondrial fractionations

To determine where in mitochondria Toho-1-EGFP was located, sub-mitochondrial fractionation of HeLa cells transfected with Toho-1-EGFP was performed. Mitochondria, isolated from HeLa cells, were treated with different concentrations of digitonin ranging from 0.05 to 0.4%. Two mitochondrial marker proteins were used to verify the location; one was porin, a mitochondrial outer membrane protein, the other, succinate dehydrogeanse, an inner membrane protein. At 0.1% of digitonin, porin was almost completely removed from mitochondria as verified by Western blot using anti-porin antibody (fig. 8A). Traces of succinate dehydrogenase activity appeared after treating mitochondria with 0.1% digitonin, but it was mostly found in the supernatant after treating mitochondria with 0.2% digitonin. Toho-1-EGFP was found in the mitochondrial pellet even after incubation of mitochondria with 0.2% of digitonin suggesting that the protein was located in the matrix space (fig. 8B). Following the same digitonin treatment, the mutant Toho-1 (R7,8Q)-EGFP was also found in the same fraction suggesting that the neutral leader was also in the matrix space (fig. 8C).

β-Lactamase and lipase A were not imported into mitochondria

Since Toho-1-EGFP was imported into mitochondria, the leaders from two other bacterial secreted proteins were investigated for their ability to function in HeLa cell mitochondrial import. β-Lactamase was selected because although it is an efficient Sec pathway signal it cannot form an amphiphilic helix, as explained in the Discussion and presented in figure 9. This amphiphilic helix is a prerequisite for many leaders that function in mitochondrial import so lactamase served as a negative control. β-Lactamase from *E. coli* was fused to EGFP and expressed in HeLa cells. The green color of EGFP was observed throughout the cell showing that this signal peptide did not bring a carrier protein into mitochondria (fig. 5G). Lipase A from *B. subtilis* was selected as a possible leader as it too has twin-arginine residues like Toho-1 and it can form an amphiphilic helix as will be discussed. When the signal peptide of LipA was fused to EGFP and expressed in HeLa cells, the green color was found throughout the cell showing that it did not function as a mitochondrial leader (fig. 5H). **A**pparently, therefore, an amphiphilic helix and positive charges are not the only determining factors for a leader to function in mitochondrial import.

Effect of CCCP on protein import

Most mitochondrial matrix protein import depends upon the inner-membrane potential so import is abolished if the membrane potential is destroyed with ionophores like CCCP [30]. Here, we investigated the need for membrane potential on the import of Toho-1-DHFR into HeLa cell mitochondria. pALDH-DHFR was used as a control protein. In the presence of 25 µM CCCP, neither Toho-1-DHFR nor pALDH-DHFR were imported into mitochondria, showing that the bacterial signal peptide also needed the membrane potential to function (data not shown).

Discussion

Finding that just two peptides interacted with Tom20 Δ(2–28) from the library of 10^{10} independent peptides was unexpected even though there was some bias against two amino acids. This may be explained by the fact that in screening the selection of positives is based on finding those with the tightest binding. More important than the peptides that were not found was the fact that two unexpected ones were found. Both were peptides that proved to bind to pure Tom20 and to inhibit import of pALDH in an in vitro assay. The import inhibition of pALDH in the presence of peptides was most likely due to their binding to the receptor and competing for pALDH binding to Tom

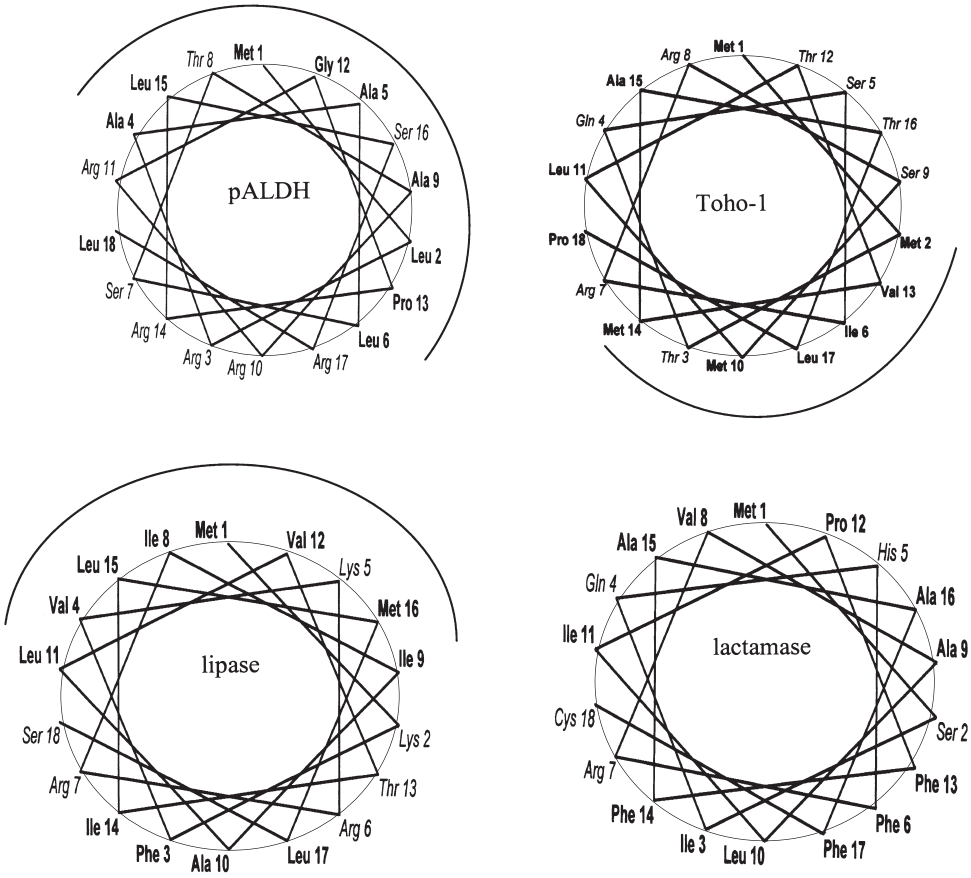

Figure 9. Helical wheel plots for the leaders. The first 18 residues of each leader used in the study are shown along with that of pALDH. For both Toho-1 and lipase, amphiphilicity can be found, as with pALDH. Lactamase does not appear to form an amphiphilic structure. Hydrophobic residues appear in bold print and the hydrophobic portions are indicated by a line.

20. Disruption of membrane potentials by the peptides was eliminated by the Mitotracker data shown in figure 4B.

Peptide 2 had partial identity with glutamate carrier but it was not studied further. Peptide 1 had significant sequence identity with the Toho-1 leader peptide, a bacterial signal peptide. The N-terminal 30 amino acids of Toho-1 were fused to EGFP and the construct was expressed in HeLa cells. Surprisingly, fluorescence microscopy showed that the construct was localized in HeLa cell mitochondria. For the first time, a bacterial leader has been shown to bring protein into mammalian mitochondria presumably using the Tom20-containing complex. Apparently, peptide 1 alone could not function as a mitochondrial leader. Most probably, the length of the 12-mer peptide was not sufficient for mitochondrial import [3] although it could bind Tom20.

In an in vitro study using isolated HeLa cell mitochondria, the extent of mitochondrial import of the bacterial signal peptide was compared with a natural mitochondrial leader peptide. Although the bacterial leader peptide brought carrier proteins into isolated mitochondria, it was less efficient than the mitochondrial leader. However, from the in vivo study, that bacterial peptide was apparently imported efficiently since no cytosolic accumulation of fluorescence was observed.

Mutational studies of Toho-1 signal peptide were performed to determine if the seven amino acids in the peptide were essential for the bacterial leader to function as a mitochondrial import leader. The first 19 amino acids of Toho-1 could function as a mitochondrial leader but not nearly as well as the entire signal peptide of Toho-1. This result showed that the C terminus part of Toho-1 signal peptide was also needed for proper translocation to mitochondria. Deletion of the portion corresponding to the peptide identified by phage display produced a non-functional leader suggesting that these residues were indeed essential.

Toho-1 signal peptide possesses two adjacent arginine residues, a signature sequence for the Tat pathway. These arginine residues have been found critical for Tat-predicted signal peptides for periplasmic translocation in bacteria [12, 39]. Import was not affected by replacement of these residues by glutamine suggesting that positive charges are not essential for mitochondrial import of Toho-1.

The structure of Tom20 bound with pALDH leader showed that an amphiphilic helix of the leader is a necessary conformation for Tom20 recognition during import [40]**.** The helical wheel plots presented in figure 9 show that the leaders from both Toho-1 and lipase, like pALDH, could in theory form an amphiphilic helix while the leader from lactamase could not. Thus, finding that the latter was not a functional leader for mitochondrial import was not unexpected. What was unexpected, though, was that the leader from lipase did not function in import. Schatz and others have shown that random peptides could act as a mitochondria leader and the proposal has been made that many bacterial proteins could be imported into mitochondria [41]. Those observations and calculations were based on the traditional amphiphilic helix prediction using peptides that possess positive charges. Finding that lipase did not bring a protein into mitochondria and that Toho-1 without a positive charge could function as a leader shows that the factors that make a mitochondrial leader sequence are not totally understood.

Lucattini et al., [42] recently reported that YhaR, a bacterial protein, could penetrate through the yeast mitochondrial outer membrane. They predicted, based on computer simulation, that as many as 5% of the proteins encoded by *E. coli* have features that can serve as a mitochondrial leader. Consistent with this prediction was the finding that enteropathogenic *E. coli* produce the effecter molecule EspF that was targeted to mitochondria and this target was essential for pathogenesis [43]. A helical wheel plot showed that this leader could form an amphiphilic helix, a prerequisite conformation. Mutation of its two arginines destroyed the ability of the leader to work properly. In contrast, Toho-1 (R7,8Q)-EGFP was imported in vivo as well as native mitochondrial leader peptide despite having no positive charges.

We have shown in many studies that as long as the leader could maintain a stable helical structure it would be functional even if positive charges were removed and negative ones introduced [44]. Presumably, the Toho-1 leader without its two arginines remained a viable leader because of structural considerations, indicating that the acid chain hypothesis might not be totally valid as has already been proposed [45].

Here, we show for the first time that a bacterial leader with twin-arginine residues can function as a mitochondrial import signal and that the charges are not essential. Though the peptide binds to Tom20, whether it needs to use this Tom component for its actual translocation to the matrix space is not known. The generality of the findings reported here will depend upon future studies with other bacterial leaders. Our finding only one bacteria leader from the phage display approach might be an indication that this was an isolated event in which the peptide properties mimic those required by the receptor for binding and translocation. The small bias of the library may also have prevented us from finding other peptides of interest. Even if no other peptides are found, having a leader that is very different from a typical mitochondrial leader might allow us to use it to learn more about the interaction between natural leaders and Tom components.

Acknowledgements. This work was supported in part by National Institute of Health grants AA10795 and GM 53269. This is journal paper number 2005–17695 from the Purdue University Agriculture Experiment Station. We thank Prof. Y. Ishii (Toho University) for the Toho-1 plasmid and Prof. S. Broyles (Purdue University) for giving us HeLa cells. H. W. thanks the Institute for Advanced Study at La Trobe University for his appointment and for providing him office space during the writing of this manuscript.

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