Membrane Topology of the ATP-binding Cassette Transporter Associated with Antigen Presentation (Tap1) Expressed in Escherichia coli*

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The ATP-binding cassette transporters associated with antigen presentation (Tap1 and Tap2) mediate the transport of peptide fragments across the endoplasmic reticulum membrane of mammalian cells. Tap1 and Tap2 are closely related to one another and are believed to function as a heterodimer. Each protein possesses a hydrophobic domain predicted to span the membrane multiple times and a highly conserved nucleotide-binding domain. We have assessed the transmembrane topology of Tap1 by expressing a series of fusions to a reporter protein, the mature form of β-lactamase in Escherichia coli. From these data a topological model can be derived in which Tap1 spans the membrane eight times, with several large loops exposed in the lumen of the endoplasmic reticulum and with both the N and C termini (including the nucleotide-binding domain) residing in the cytoplasm.

Cytotoxic T lymphocytes (CTLs)1 continually survey cells for changes in cytosolic content. Antigens from cytoplasmic proteins are presented to the CTLs at the cell surface in the form of peptide fragments complexed with major histocompatibility complex I and β2-microglobulin molecules (1). These trimeric complexes are recognized by the T cell receptor on the CTLs. To assemble this trimeric complex, the peptide fragments which are normally generated by the proteasome in the cytoplasm, must be translocated into the lumen of the endoplasmic reticulum (ER). Two proteins, Tap1 and Tap2, are required for this transport process (2, 3). Tap1 and Tap2 each consist of a hydrophobic domain predicted to span the membrane multiple times, and an ATP-binding domain, which is believed to couple the energy of ATP hydrolysis to peptide transport. Tap1 and Tap2 function as a heteromer (4–6) and are members of the ATP-binding cassette (ABC) superfamily of transporters (7).

The transmembrane domains of ABC transporters typically (although there are exceptions; see below) consist of 12 clearly defined, putative membrane-spanning segments, which could, potentially, span the lipid bilayer. For a number of ABC transporters, both prokaryotic and eukaryotic, this predicted topology has been confirmed experimentally (8–10). The N-terminal

hydrophobic domains of Tap1 and Tap2 appear to differ from those of other ABC transporters in that the potential membrane-spanning segments are not clearly defined by conventional algorithms and appear to number more than 12 (for the Tap1-Tap2 complex). To clarify this situation we have analyzed the transmembrane topology of the human Tap1 protein using a genetic approach in which a reporter protein (the mature form of β-lactamase) was fused to a series of defined points along the length of the Tap1 protein. The orientation of the β-lactamase with respect to the membrane was assessed by its ability to confer ampicillin resistance when expressed in Escherichia coli. This approach, and the related phoA method, have been used, successfully, to study many other membrane proteins (11–13). The data generate a model in which Tap1 spans the membrane eight times with large extracellular (lumenal) hydrophilic loops and the N and C termini, including the nucleotide-binding domain, located in the cytoplasm. This predicted organization differs from that of many other ABC transporters, and its functional implications are considered.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strain DH5αF*: endE44 hsdR17 (rK- mK-) supE44 thi-1 recA1 gyrA96 relA1 ΔlacA1Z54 argF80151U169 fosB169 thi-1 recA1 gyrA96 relA1 ΔlacA1Z54 argF80151U169/ΔlacZΔM15) was used routinely. For certain studies the protease-deficient strain CH1790 (http://bissell-10.∆on it has supF strA ΔproC galOP:IS1 ΔbioA1 Bam-N1) (10) was used. Plasmid pYJ1 contains the tap1 cDNA (14) in the general cloning vector pKG18, pYZ4 and pYZ5 are two plasmids designed to facilitate the cloning and generation of terminal-β-lactamase fusions to eukaryotic genes.

Molecular Biology—Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs and used according to the manufacturer’s instructions. The oligonucleotides used in this study are listed in Table I.

Construction of β-Lactamase Fusions—A total of 39 in-frame Tap1-β-lactamase fusions were generated. Two approaches to generating these fusions were used, a random method and a directed method. Random fusions were generated as follows. The tap1 gene was cloned into plasmid pYZ4 (15) under control of the inducible E. coli lac UV5 promoter and a bacterial ribosome binding site, generating pYZtap1 (Fig. 1). pYZ tap1 was cleaved at its unique HindIII site (located near the C-terminal end of the postulated hydrophobic domain) and progressive deletions introduced into the tap1 gene with exonuclease Bal-31 (Fig. 2A). These randomly truncated derivatives of tap1 were fused to the coding sequence of the mature form of β-lactamase (Fig. 2A) and transformed into E. coli strain DH5αF, selecting for kanamycin resistance. The resultant colonies were screened for ampicillin resistance at high cell density (see below) to determine whether they expressed an in-frame Tap1-β-lactamase fusion. Plasmids that conferred resistance were sequenced to determine the Tap1-β-lactamase fusion junctions. This approach generated 29 independent, in-frame β-lactamase fusions between amino acids 11 and 342 of Tap1 (designated tap1 to tap342, respectively).

Directed Tap1-β-lactamase fusions were constructed as follows. Two fusions were constructed taking advantage of unique PvuII and HindIII restriction endonuclease sites within the tap1 gene. The coding se-
**Topology of Tap Transporter**

**TABLE I**

List of oligonucleotides and plasmids used in this study

| N-terminal primer | GTCGAAGACCCCATGGCTAGCTCTAGGTG | From Figs. 1 and 2B |
|-------------------|---------------------------------|---------------------|
| Reverse primers:  |                                 |                     |
| 122               | TTGGATCCGGCGGCTAGCCCTATGA       | See Fig. 1          |
| 40                | CAGCCGCTGCGGACGAG              | See Fig. 2          |
| 66                | GGCCCAGCGGGCTAGGC              | See Fig. 2B         |
| 88                | ACCCGCGTCCTGTCCTT             | See Fig. 2          |
| 220               | TGGACCATCTTTGATGATCC           | See Fig. 2          |
| 318               | CCCAGAGCATGATC             | See Fig. 2          |
| 323               | TCCCGAGAGCATGATCC           | See Fig. 2          |
| 492               | ACCACTGGGTCGCGAG              | See Fig. 2          |
| 748               | TTTCTGGACATGTCGAG             | See Fig. 2          |

**Plasmids**

- pYJ1
- pYZ4
- pY25
- pYZ 5tap1
- pYZ-tap1
- pYZ-tap15 to pYZ-tap748
- pYZtap1-PvuII

- pYZ4 with first 366 base pairs of tap1.
- pYZ4 with all of tap1.
- pYZ4 with all of tap1.
- pYZ-tap1 fusion where junction is at the PvuII site of tap1 (equivalent to ptap433).

**Fig. 1. Subcloning tap1 into the expression vector pYZ4.** The cloning was performed in two stages. First, a short DNA fragment from the 5’ end of the tap1 gene was amplified in a PCR using the tap1 plasmid pYJ1 as a template, and oligonucleotides “N-term” and “122” (reaction a). These primers introduced an NcoI site at the initiating ATG codon and a BamHI site immediately downstream of the Smal site of tap1. These sites were used to insert the PCR product between the NcoI and BamHI sites of pYJ4 (reaction c), placing the 5’ end of tap1 under the control of the inducible lac promoter (PlacUV5) and ribosome binding site (RBS). The remaining portion of the tap1 gene was excised from pYJ1 with XmaI and EcoRI (reaction d) and inserted between the XmaI and EcoRI sites of pYZ5-tap1 generating pYZtap1 (reaction e). N = NcoI; X = XmaI; B = BamHI; R = EcoRI.

**Results**

The Tap1 protein consists of an N-terminal hydrophobic domain and a C-terminal hydrophilic domain (the nucleotide-binding domain). A hydrophobicity plot of Tap1 predicts 10 transmembrane segments of Tap1. The precise fusion junctions, and their relation to the predicted transmembrane topology of Tap1, fusions between defined points in Tap1 and the mature form of β-lactamase were generated. When expressed in E. coli, β-lactamase acts as reporter of transmembrane topology. β-Lactamase breaks down ampicillin, an antibiotic whose target is extracellular. If β-lactamase is fused to a point in Tap1 which is periplasmic (equivalent to the lumen of the ER), ampicillin is hydrolyzed and the cells are ampicillin resistant. If β-lactamase is fused to a point in Tap1 which is intracellular (cytoplasmic), cells are ampicillin-sensitive.

Twenty-nine in-frame Tap1-β-lactamase fusions within the transmembrane domain of Tap1 were generated by random approaches. A directed approach was taken to generate an additional 10 fusions to defined points in the sequence to ensure complete coverage of the entire transmembrane domain (see “Experimental Procedures”). At least one fusion was generated to each of the hydrophilic loops separating the 10 predicted transmembrane segments of Tap1. The precision fusion junctions, and their relation to the predicted transmembrane...
FIG. 2. Fusion of the \(\beta\)-lactamase gene, encoding \(\beta\)-lactamase, to \(\text{tap}1\). A, random fusions. The endonuclease Bal-31 was used to generate progressive deletions from the unique HindIII site of \(\text{tap}1\). Plasmid \(\text{pYZ-tap}1\) was digested with HindIII and treated with Bal-31 at room temperature. Samples were removed every 2 min and the reaction stopped by addition of EGTA and transfer to 0 °C (reaction a). Overhanging DNA ends were filled in with the Klenow fragment of DNA polymerase and T4 DNA polymerase (reaction b). The remaining 3' sequences of \(\text{tap}1\) were removed by digestion with EcoRI (reaction c), and the coding sequence of the mature form of \(\beta\)-lactamase was inserted as a PstII-EcoRI fragment (reaction e). The resultant population of fused fragments was transformed into DH5\(\alpha\)F\(\epsilon\) selecting for kanamycin resistance. The DNA sequences of the fusion junctions were determined, identifying the amino acid residue of Tap1 at which \(\beta\)-lactamase was fused. B, directed fusions. A derivative of \(\text{pYZ-tap}1\) was constructed as a parental plasmid from which all the directed fusions were constructed. This was done by inserting the coding sequence of the mature form of \(\beta\)-lactamase into \(\text{pYZ-tap}1\) as a PstII-EcoRI fragment, replacing the 3' region of the \(\text{tap}1\) gene. The resultant plasmid was designated \(\text{pYZtap}1\). \(\text{pYZtap}1\) was then replaced (reaction d) with one of eight different PCR products using vent DNA polymerase (NEB) and \(\text{tap}1\) (in \(\text{pYJ1}\) as a template (reaction d)). These PCRs products contained the coding sequence of \(\text{tap}1\) from the NcoI or XmaI sites up to one of eight specific residues: alanine 40, leucine 66, glycines 88, 318, 323, 492, serine 220, and the C-terminal glutamate. The priming oligonucleotides were 122 and one of the following: 40, 66, 88, 318, 323, 492, 220, or 748 (reaction c). All the plasmids were transformed into \(\text{E. coli}\) strain DH5\(\alpha\)F\(\epsilon\), selecting for kanamycin resistance. All amplified regions and fusion junctions were sequenced to ensure no mutations had arisen.

segments, are shown in Fig. 3.

To assess the cellular location of the Tap1-\(\beta\)-lactamase fusion junctions, the maximum ampicillin resistance conferred by each of the fusions was assessed (Table II). Those fusions, which conferred resistance to ampicillin when plated at low density, were considered to be to fusions to an extracellular portion of Tap1 (equivalent to the ER lumen). The absolute level of ampicillin resistance differed considerably between fusions due to differences in levels of protein synthesis, stability, and/or folding. Nevertheless, any level of resistance implies an extracellular location. Those fusions, which conferred no ampicillin resistance when cells were plated at low density, were considered to place \(\beta\)-lactamase in a cytoplasmic location.

To exclude the possibility that such fusions failed to confer ampicillin resistance because no fusion protein was made, rather than because the fusion junction was intracellular, two tests were performed. First, their ability to confer ampicillin resistance at high cell density was assessed. If \(\beta\)-lactamase is synthesized but remains intracellular some cells lyse, releasing \(\beta\)-lactamase to hydrolyze ampicillin, which allows neighboring cells to grow when cells are plated at high density. All the fusions conferred resistance at high cell density, indicating that they do indeed express Tap1-\(\beta\)-lactamase fusion proteins. Second, the production of \(\beta\)-lactamase was examined by Western blotting (Fig. 4). All fusions were shown to produce \(\beta\)-lactamase fusion proteins, although the predicted full-length fusions could not always be detected due to proteolysis. Transferring selected fusions into a protease-deficient strain, CH1790, showed no difference in protein degradation (data not shown). Although degradation meant that full-length protein could not be detected, the full-length fusion protein must initially be synthesized in order for ampicillin resistance to be detected (as it was for all fusions). Furthermore, degradation cannot generate false positives (i.e., cannot indicate a fusion is to an extracellular segment when it is not) because by definition, high level ampicillin resistance conferred by extracellular Tap1-\(\beta\)-lactamase fusions demands that the \(\beta\)-lactamase moiety is synthesized and translocated.

Based on the above data the transmembrane topology of Tap1 expressed in \(\text{E. coli}\) can be determined. The predicted hydrophobic amino acids clusters (A to J in Fig. 3) were considered to be actual membrane-spanning segments when fusions upstream and downstream of the hydrophobic amino acid clusters located \(\beta\)-lactamase on opposite sides of the membrane. For example hydrophobic cluster B was designated as a membrane-spanning segment because an upstream \(\beta\)-lactamase fusion (to amino acid 40) was periplasmic, while a downstream \(\beta\)-lactamase fusion (to amino acid 66) was cytoplasmic. On this analysis, only 8 of the 10 predicted membrane-spanning segments traverse the membrane. These are indicated in Fig. 3. Clusters D and E do not appear to span the membrane. Some \(\beta\)-lactamase fusions were within potential membrane-spanning segments. These fusions conferred levels of ampicillin resistance consistent with the location of the preceding hydrophilic loop, presumably because the residual portion of the membrane-spanning segment present in the fusion was not sufficiently long to span the entire membrane or because topogenic information was present downstream of the actual membrane-spanning segment. For example, the fusion to amino acid 210 is located extracellularly, since it does not contain all the information required to transfer membrane-spanning segment F across the membrane: the downstream fusion, to amino acid 220, contains all the required topogenic elements and is located at the cytoplasmic face of the membrane. This additional topogenic information is presumably outside the hydrophilic membrane-spanning segments.

Fig. 5 (B and D) shows the topological model generated for Tap1 derived from these data. Tap1 expressed in \(\text{E. coli}\) spans the membrane eight times with several large extracellular loops.

DISCUSSION

Tap1 and Tap2 are related proteins which together form the Tap peptide transporter of the endoplasmic reticulum required for Class I-mediated antigen presentation. The transmem-
brane topology of Tap1 predicted from its primary sequence is, on first inspection, different from that of many other ABC transporters. To clarify the transmembrane topology of Tap1 we used an experimental approach, the topology reporter-protein system, which has been used extensively to monitor the transmembrane topology of membrane proteins (12), including many from eukaryotic cells (15, 17–22). Although this approach requires heterologous expression in *E. coli*, this species has previously been used to express functional eukaryotic transport proteins (23, 24), and several eukaryotic polytopic membrane proteins have been shown to fold correctly in the *E. coli* membrane (19, 22). However, it is possible that the topology of Tap1 expressed in *E. coli* differs from that in mammalian membranes.

A topological map of Tap1 was generated, with eight membrane-spanning segments and both the N and C termini located in the cytoplasm, in agreement with the location of this domain.
predicted by \textit{in situ} antibody labeling experiments (5). The distribution of positive charges around the first membrane-spanning segment (three arginines preceding it and two succeeding it before the next membrane-spanning segment see Fig. 3) is consistent with the positive inside rule (25), while the distribution of positive charges around subsequent membrane-spanning segments is less adherent to this rule as is the case with other eukaryotic polytopic membrane proteins (26).

Although the \(\beta\)-lactamase and the related \textit{phoA} methods for mapping transmembrane topology have been informative for membrane proteins, they involve fusing truncated versions of Tap1 to \(\beta\)-lactamase and any model generated must be considered within this limitation. For example, if Tap1 has C-terminal topological determinants these would be deleted in fusion proteins and may influence the folding observed. Nevertheless, this has not proved a problem in determining the folding of other polytopic membrane proteins using this method where data have been confirmed by other, biochemical approaches (9, 17, 27). The topological model presented here is consistent with other available data. Perhaps significantly, the topology of Tap1 is very similar to that determined for the MalF protein, an \textit{E. coli} ABC transporter for maltose. MalF has eight membrane-spanning segments arranged along the polypeptide in a similar 3:2:2:1 order (Fig. 5) with similar large extracellular (lumenal) loops (8).

The transmembrane topology of Tap differs from the paradigm for ABC transporters, although several other exceptions have been reported (e.g. Refs. 8, 28, and 29). More importantly, the model places several large loops in the lumen of the endoplasmic reticulum. This is unusual for ABC transporters, where the large loops are generally cytoplasmic, but may reflect the fact the Tap interacts with the major histocompatibility class I molecule in the ER lumen (30–33). One of these loops contains two hydrophobic segments (clusters D and E), which were initially predicted to span the membrane. However since the experimental data suggest they do not traverse the membrane they may associate with the lumenal face of the membrane or be buried within the tertiary or quaternary structure of a protein complex in the ER.

Tap1 and Tap2 are closely related in primary sequence. However, Tap2 is slightly shorter than Tap1 (by 40 amino acids). Both \(\text{tap}1\) and \(\text{tap}2\) genes are organized in 11 exons. Comparison of the length of coding sequence within each exon shows that difference in length between the hydrophobic domains of the two proteins is mainly due to differences within the first exon. An optimal alignment of the Tap1 and Tap2 sequences (Fig. 6) indicates that hydrophobic cluster E is absent in Tap2 and that there is also considerable divergence in the region around and including hydrophobic cluster D. These
are the two hydrophobic clusters of Tap1 which do not appear to span the membrane. Thus, it seems likely that Tap2 has the same transmembrane topology as Tap1 but that the large luminal loop containing hydrophobic clusters D and E is much reduced in size. As Tap1, but not Tap2, interacts with the major histocompatibility class I molecule in the ER (30, 32, 33), it is tempting to speculate that this large hydrophobic loop of Tap1 plays a role in this interaction. The topology for Tap1 determined here provides a working model to facilitate further structure-function analysis.

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Note Added in Proof—Since submitting this work, studies have appeared indicating the peptide binding site of Tap is in a region that our structure-function analysis.

FIG. 6. Sequence alignments of Tap1 with Tap2. Tap1 and Tap2 were aligned using the Bestfit program of the Wisconsin University Genetics Computer Group (WUGCG) package version 8.1 with a gap weight of 10 and length weight of 0.1 (percent identity was 36.6 and similarity 58.5). Black boxes above the alignment indicate the hydrophobic clusters of Tap1 shown experimentally in this paper to span the membrane. Open boxes above the alignment indicate the predicted membrane-spanning segments, which on the basis of data in this study appear not to span the membrane. The nucleotide binding motifs Walker A and B (35), which form part of the ABC domain, are indicated for orientation. The boundaries of sequences encoded by different exons are indicated below the protein sequence. In this alignment these boundaries are identical for Tap1 and Tap2 with the exception of the boundary between exons 10 and 11.

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