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Nucleotide binding of the C-terminal domains of the major histocompatibility complex-encoded transporter expressed in *Drosophila melanogaster* cells

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Abstract

The C-terminal domains of the mouse transporter associated with antigen processing (TAP) were expressed as soluble proteins in *Drosophila melanogaster* cells and labeled by [α-^{32}P]8-azido-ATP after UV-irradiation. The relative potencies of the nucleotides in preventing azido-ATP labeling were in the order of ATP > GTP > CTP > ITP > UTP for both the TAP1 and TAP2 C-terminal domains, suggesting ATP to be the natural substrate of the transporter. Our data provide the first evidence that the individual C-terminal domain of either TAP1 or TAP2 can be expressed as a functional ATP-binding protein.

Key words: Major histocompatibility complex; Antigen presentation; Transporter associated with antigen processing; Traffic ATPase; ATP-binding cassette transporter

1. Introduction

TAP1 and TAP2 are two subunits of the transporter associated with antigen processing (TAP). They are MHC-encoded and essential for the antigen presentation by class I MHC molecules [1–3]. Specifically, TAP transports antigenic peptides from the cytoplasm to the lumen of the endoplasmic reticulum [4,5], where the peptides assemble with the class I MHC heavy chain and β_2-microglobulin before being transported to the cell surface. The hydropathy profiles of TAP1 and TAP2 are similar, suggesting an N-terminal hydrophobic domain with possibly six membrane-spanning segments and a C-terminal hydrophilic domain. The C-terminal domains of TAP1 and TAP2 are highly conserved, with about 60% of their amino acid sequence identical [3]. In addition, the TAP C-terminal domains are highly homologous to the nucleotide-binding domains of traffic ATPase or ATP-binding cassette transporters [6–8]. Walker A and Walker B motifs [9], which are commonly found in the nucleotide-binding proteins, are also identified in the TAP1 and TAP2 C-terminal domains, suggesting that they may be able to bind nucleotides.

Experimentally, it has been shown that TAP-mediated peptide-transport is ATP-dependent [4,5]. However, questions remain whether ATP molecules interact with TAP directly. Discovery of the nucleotide-binding properties of the TAP proteins, localization of the nucleotide-binding sites along the TAP sequences, and illumination of the tertiary structures of the nucleotide-binding sites will certainly provide insight into the mechanism of the ATP-dependent peptide-transport by TAP. In order to facilitate the study, we expressed the C-terminal domains of the mouse TAP1 and TAP2 in *Drosophila melanogaster* Schneider cells. Since Schneider cells had been used to express various mammalian proteins quantitatively in their functional forms [10], it seemed likely that the C-terminal domains of TAP1 and TAP2 expressed in Schneider cells would be in their native configurations. The nucleotide-binding functions of the TAP C-terminal domains were investigated with photo affinity labeling of [^{32}P]8-azido-ATP. The experiments indicate that both the TAP1 and TAP2 C-terminal domains are capable of binding ATP with high affinities. The structures and functions of TAP will be discussed.

2. Materials and methods

2.1. Construction of the expression vectors

The cDNA fragments coding for the mouse TAP1 from Gln^{445} to Asp^{724} and the mouse TAP2 from Ser^{434} to Ala^{702} were isolated from their full-length cDNAs [3] using PCR (Polymerase Chain Reaction) with *Taq* polymerase (Perkin Elmer). Both TAP1 cDNA and TAP2 cDNA have been previously cloned into pRMHa3 [11], which will be described elsewhere. These plasmids were used as the templates in PCRs. For isolating TAP1 C-domain DNA, the primers used in the PCR were 5′-AAAGAGCTC ATG (CAC)$_6$ CAG AAG GCT GTG GGC TCC T-3′, which contains a *Sac*I site followed with the coding sequence for Met-(His)$_6$-Gln-Lys-Ala-Val-Gly-Ser, and 5′-GGA-GAAGAATGTGAGTGTGCATCGA-3′, which is the sequence of pRMHa3 downstream of the TAP1 insert. The primers for the TAP2 C-domain were 5′-TTTCC ATG GTG (CAC)$_6$ AGC AAC GTG GGC GCT GCT-3′, which contains an *Nco*I site and the sequence coding for Met-Val-(His)6-Ser-Asn-Val-Gly-Ala, and 5′-GTGCATCGAATTGC-ATGCCT-3′, the sequence of pRMHa3 downstream of the TAP2 insert. The TAP1 DNA fragment generated from PCR was cut with *Sac*I

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Abbreviations: TAP, transporter associated with antigen processing; MHC, major histocompatibility complex; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; ER, endoplasmic reticulum.

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and *Sal*I, and the TAP2 DNA fragment was cut with *Nco*I and *Sal*I. They were then ligated into pRMHa3 downstream of the metallothionein promoter. The complete DNA inserts were sequenced with dideoxynucleotide chain termination method on an ABI 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, CA). The recombinant plasmid for TAP1 C-domain expression was named pFH1A, and the plasmid for TAP2 C-domain was pFH2A.

2.2. Protein expression

22 μg of pFH1A, or pFH2A, was co-transfected with 2 μg phshsneo, a selection plasmid [12], into 12×10^6 Schneider cells by calcium phosphate method [10]. The transfected cells were cultured in Schneider medium (GIBCO/BRL) including 5% fetal bovine serum and 500 μg/ml of Geneticin (G418, GIBCO/BRL). The stable transfectants were selected and maintained in the presence of G418 at all time. Expression of the domain proteins was induced by adding $CuSO_4$ into the culture medium to the final concentration of 0.7 mM for 24 h.

2.3. Immuno-blot

After 24-h induction with $CuSO_4$, about 1000 Schneider cells were collected and lysed by suspending in 2 × SDS Sample buffer [13] followed by incubation at 95°C for 5 min. The prestained molecular weight markers (Bio-Rad) were used to calibrate the molecular masses of the proteins of interest. The proteins were separated on a 12.5% SDS-polyacrylamide gel and transferred electrophoretically to a BA-S 85 nitrocellulose filter (Schleicher and Schuell). The blotted filter was incubated either with the anti-TAP1 antiserum [3,14] or the anti-TAP2 antiserum [15,16]. The immunopositive proteins were visualized with an alkaline phosphatase-conjugated goat anti-rabbit Ig G (Calbiochem, La Jolla, CA).

2.4. Photo affinity labeling

After 24-h induction with $CuSO_4$, the Schneider cells were collected, washed twice with Dulbecco's phosphate-buffered Saline Solution (Irvine Scientific) and then suspended in the ice cold labeling buffer (100 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM Na_2EDTA, 200 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin). Thereafter, the process was carried out at below 4°C. The cells were homogenized and centrifuged at 100,000 × *g* for 30 min. The supernatant was equally aliquated, for each set of the labeling reactions, into the wells of a flat-bottom 24-well plate. Approximately, 5×10^6 cells were used for each labeling reaction. The final reaction volume was brought to 400 μl with the labeling buffer, including various concentration (0, 50, 200, 500, or 1000 μM) of ATP, GTP, ITP, UTP, or CTP, and 2, or 4 μM of [α-^{32}P]8-azido-ATP (ICN, specific activity 8.59 Ci/mmo). ATP, GTP, ITP, UTP and CTP were purchased from Sigma as sodium salts. The stock solutions of the nucleotides (100 mM) were all adjusted to pH 7.0 with NaOH and then diluted into the labeling mixtures to the desired concentrations. The reaction plate was kept shaking gently in the dark for 2 min. The labeling reactions were then started by irradiating the reaction mixtures with short-wavelength ultraviolet light from a hand-held UV lamp (254 nm, UVSL-25, Ultra-Violet Products, Inc., San Diego, CA), which was kept parallel with the plate at 2.5 cm distance. During the UV-irradiation, the reaction plate was kept gently rotating horizontally. 20 min later, the reactions were stopped by remove the UV light and adding 4 μl of 5 mM dithiothreitol into each reaction mixture. The domain proteins in the reaction mixtures were immuno-precipitated with either the anti-TAP1 antiserum or the anti-TAP2 antiserum. The precipitated proteins were dissolve in 2 × SDS sample buffer [13], heated at 95°C for 5 min and electrophoretically separated on 10–15% SDS-polyacrylamide gradient gels. The gels were fixed, dried and analyzed by autoradiography. The extent of radioactive labeling to the TAP C-domain proteins on the gels was also quantified by PhosphorImager Scanner (Molecular Dynamics, Inc. Sunnyvale, CA) and analyzed with software ImageQuant (Molecular Dynamics, Inc).

3. Results

3.1. Expression of the C-terminal domains of TAP1 and TAP2

The C-terminal domains of TAP1 and TAP2 are defined based on the hydropathy analyses of the amino acid sequences [3]. For mouse TAP1, the C-terminal domain starts from Gln^{445}, and for mouse TAP2, at Ser^{434} (Fig. 1). These C-terminal domains have high degrees of sequence homology to the nucleotide-binding domains of traffic ATPase or ATP-binding cassette transporters [17] and contain the sequence of about 200 amino acid residues where the predicted secondary structures are typical of a nucleotide-binding fold [18]. In addition, Walker A and Walker B motifs [9], found in many nucleotide-binding proteins, were also identified in these domains (Fig. 1). It is, therefore, hypothesized that each C-terminal domain of TAP forms a nucleotide-binding domain. In order to test this hypothesis, the DNA fragments coding for the entire mouse TAP1 and TAP2 C-terminal domains (Fig. 1) were inserted into an expression plasmid, pRMHa3 [11] and transfected into *Drosophila melanogaster* cells. The expression of the TAP C-terminal domains was under the control of a metallothionein promoter and can be induced by Cu^{2+}.

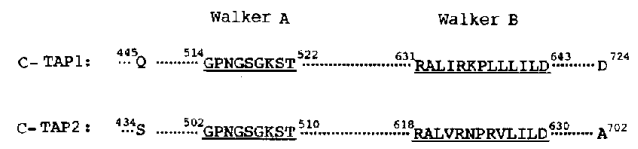

Fig. 1. Linear schemes of the C-terminal domains of mouse TAP1 (C-TAP1) and TAP2 (C-TAP2). The amino acid residues are presented with the single-letter amino acid codes. The numbering corresponds to the amino acid residues in the mouse full-length TAP1 and TAP2 sequences [3]. The Walker A and Walker B motifs [9] were indicated in both C-TAP1 and C-TAP2 by underlining the sequences. The recombinant C-TAP1 contains the mouse TAP1 sequence from Q445 to D724 and C-TAP2 contains the mouse TAP2 from S434 to A702. Secondary structure prediction with the Chou-Fasman algorithm suggests a typical nucleotide-binding folding, alternating β-strands and α-helices [18], located in TAP1 (between amino acids 490 and 690) and TAP2 (between amino acids 480 and 680).

The expression of the recombinant TAP1 and TAP2 C-terminal domains was detected with immuno-blotting. As shown in Fig. 2, two proteins from the cells transfected with the TAP1 C-terminal domain expression plasmid were found positive to the anti-TAP1 antiserum and were labeled as C-TAP1 (~31 kDa) and sC-TAP1 (~27 kDa). These two proteins were not found in the Schneider cells either not transfected or transfected only with the TAP2 C-terminal domain expression plasmid (data not shown). When the lysate prepared from the TAP1 C-terminal domain transfectants was applied on to a Ni-NTA agarose column [19], only the 31-kDa C-TAP1 protein was retained by the column, while the 27-kDa sC-TAP1 protein was washed out of the column without binding (data not shown). This suggests that the $(His)_6$-tag at the N-terminus of the recombinant TAP1 C-domain (see section 2) is intact in the 31-kDa C-TAP1 protein, but missed from the 27-kDa sC-TAP1 protein. By examining the TAP1 cDNA sequence, it is concluded

that both the proteins were derived from the TAP1 C-terminal domain DNA construct. The C-TAP1 protein was translated from the first initiation codon, while the sC-TAP1 protein was translated from an internal methionine codon, corresponding to Met^{475} of mouse TAP1 sequence [3]. Compared to the TAP1 C-terminal domain transfectants, only one immuno-positive protein, labeled as C-TAP2 in Fig. 2, was identified in the TAP2 C-terminal domain transfectants by the anti-TAP2 antiserum. This protein was not detected in the untransfected Schneider cells and the TAP1 C-terminal domain transfectants. The size of the protein is about 30 kDa on the immuno-blot, and comparable to the size predicted from the amino acid sequence of the recombinant TAP2 C-terminal domain protein. The recombinant C-terminal domain proteins of TAP1 and TAP2 were expressed in the cytoplasm of the Schneider cells, which was confirmed with immuno-fluorescent staining (data not shown). In agreement with the physical nature of their amino acid sequences, the recombinant C-terminal domain proteins were found to be water soluble. The yields of C-TAP1 and C-TAP2 proteins were estimated to be around 2 mg per liter.

3.2. *Azido-ATP labeling of the proteins*

It is very likely that these soluble C-terminal domain proteins retain their native tertiary structures, because Blue-sepharose, a resin that binds many nucleotide-binding proteins [20], retained the C-terminal domain proteins (data not shown), indicating possible nucleotide-binding functions of the recombinant proteins expressed in the Schneider cells. To investigate this possibility, photo affinity labeling with [^{32}P]8-azido-ATP, a method that has been used widely in studying various ATP-binding proteins, including many members in the traffic ATPase transporter superfamily [21–24], was used here.

Proteins in the supernatant fractions prepared from the transfectant homogenates were UV irradiated in the presence of 2 μM [^{32}P]8-azido-ATP and immuno-precipitated with either the anti-TAP1 antiserum or the anti-TAP2 antiserum. The immuno-precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and the labeling to the proteins was visualized by autoradiography. Two radioactive bands with the expected molecular masses for C-TAP1 and sC-TAP1 were detected in the C-TAP1 transfectants (Fig. 3, panel A, lane 1), and one with the size of C-TAP2 was detected in the C-TAP2 transfectants (Fig. 3, panel B, lane 1). These three bands were only found in the transfectants that had been induced with $CuSO_4$, but not in the non-induced transfectants (data not shown). The labeled proteins were further eluted from the dried gel slices and analyzed with immuno-blotting to confirm their identities. The two labeled proteins from the C-TAP1 transfectants were recognized by the anti-TAP1 antiserum, and the labeled 30-kDa protein from the C-TAP2 transfectants was rec-

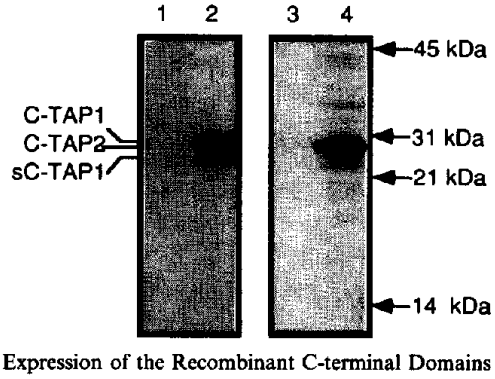

Fig. 2. Expression of the Recombinant C-terminal Domains of TAP1 and TAP2. Proteins from Schneider cells transfected with no plasmid (lane 1 and 3), or with plasmid pFH1A (lane 2), or pFH2A (lane 4), were immuno-blotted with either the anti-TAP1 antiserum (lane 1 and 2), or the anti-TAP2 antiserum (lane 3 and 4). The expressed recombinant proteins are indicated on the left side of the figure as C-TAP1, sC-TAP1 and C-TAP2. On the right side, protein standards are localized and expressed in kilodalton (kDa).

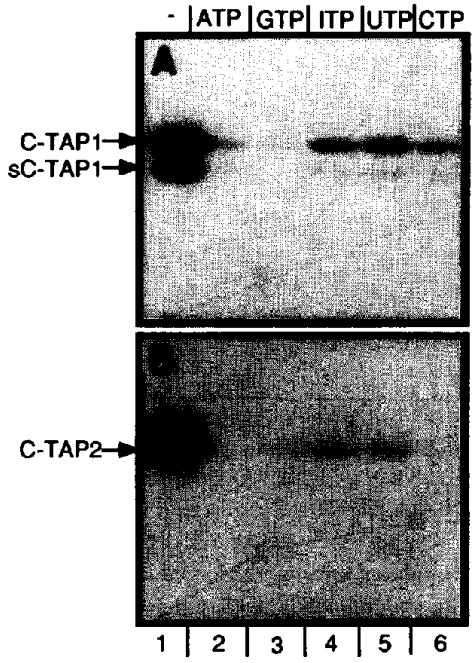

Fig. 3. Photo Affinity Labeling of the TAP C-terminal Domain Proteins with 8-Azido-ATP. Proteins from the Schneider cells transfected with pFH1A (A), or pFH2A (B) were labeled with [α-^{32}P]8-azido-ATP as described in section 2. The labeling reaction was carried out in the absence (lane 1), or presence of 1 mM ATP (lane 2), GTP (lane 3), ITP (lane 4), UTP (lane 5), or CTP (lane 6). The proteins were immuno-precipitated either with the anti-TAP1 antiserum (A) or anti-TAP2 antiserum (B). The radioactive bands corresponding to the recombinant TAP C-domain proteins were marked on the left side of the figure. It seems that in the presence of 1 mM CTP (lane 6), sC-TAP1 and C-TAP2 were not radioactively labeled. However, prolonged exposure of the gel revealed some labeling to sC-TAP1 and C-TAP2 in the presence of 1 mM CTP. A very low density band is visible in the lane 2 (A). It is believed that the radioactivity found in the lane 2 is from the lane 1, since in other experiments, the labeling to C-TAP1 was completely blocked by ATP at 500 mM, e.g. the experiment described in Fig. 4.

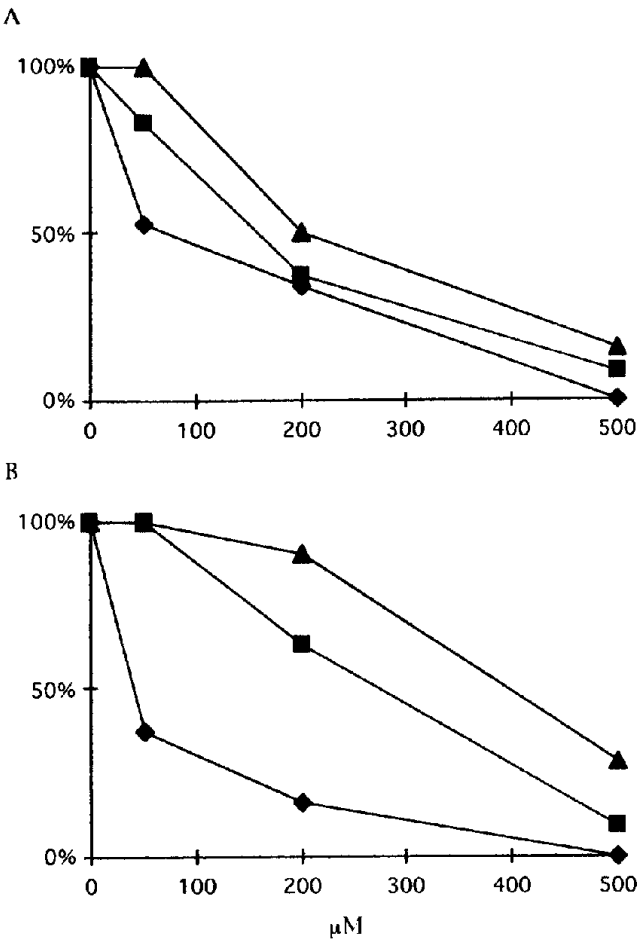

Fig. 4. Inhibition of the 8-Azido-ATP labeling to the C-TAP1 Protein (A) and the C-TAP2 Protein (B) by Nucleotides. Proteins were labeled with [α-^{32}P]8-azido-ATP (4 μM) in the presence of a various concentration (0, 50, 200, or 500 μM) of ATP (diamonds), GTP (squares), or ITP (triangles) as described in section 2. The radioactivity associated with the C-TAP1 and C-TAP2 on SDS-polyacrylamide gels was quantified by Phosphor-Imager Scanner and presented here on the Y-axes as the percentage labeling.

ognized by the anti-TAP2 antiserum (data not shown), which support that these three labeled proteins are indeed the recombinant TAP C-terminal domain proteins. Under the same experimental conditions, the intact TAP1 and TAP2 from RMA cells were also labeled by [^{32}P]8-azido-ATP (data not shown).

When 1 mM ATP, or GTP, was included in the labeling reaction mixtures, the labeling to the recombinant C-TAP1, sC-TAP1 and C-TAP2 proteins was almost completely blocked (Fig. 3, lanes 2 and 3), whereas in the presence of 1 mM ITP, UTP, or CTP, the labeling to the proteins was only partially inhibited (lanes 4, 5 and 6 of Fig. 3). The findings that the labeling to the domain proteins occurred at a micromolar concentration of 8-azido-ATP (2 μM), and ATP and GTP can efficiently inhibit the labeling suggest that 8-azido-ATP was targeted to the nucleotide-binding sites of the proteins. It was concluded that the recombinant TAP1 and TAP2 C-terminal domain proteins expressed in *Drosophila* cells retain the nucleotide-binding sites and these sites have higher affinities for ATP and GTP than for ITP, CTP, and UTP.

3.3. *The nucleotide binding specificity of the TAP C-terminal domains*

A similar pattern of the labeling inhibition by the nucleotides was observed for the TAP1 and TAP2 C-terminal domains. It seems that ATP and GTP are the most potent inhibitors, CTP and ITP the second, and UTP the least potent inhibitor. However, it was not clear whether both the TAP1 and TAP2 nucleotide-binding sites prefer ATP over GTP, or GTP over ATP, or one site prefers ATP while the other prefers GTP. To further delineate the nucleotide specificity of the nucleotide-binding site within each TAP C-terminal domain, the labeling reaction was carried out in the presence of a various concentration (0, 50, 200, or 500 μM) of ATP, GTP, or ITP. The extent of the labeling to each domain protein was quantified and compared with the labeling of the same protein without any inhibitory nucleotide added to the reaction. The data were summarized in Fig. 4. For both TAP1 and TAP2 C-terminal domain proteins, ATP is the most potent inhibitor of the labeling among the nucleotides tested, with the 50% inhibition of the labeling at about 40 to 50 μM of ATP, whereas, the 50% inhibition was achieved by GTP at about 200 μM. In comparison with ATP and GTP, ITP is not efficient in preventing the domain proteins from labeling by 8-azido-ATP. In summary, the relative potencies of the nucleotides in blocking azido-ATP labeling to the recombinant TAP1 and TAP2 C-terminal domain proteins were in the order of ATP > GTP > CTP > ITP > UTP. Assuming that the inhibitory nucleotides compete with 8-azido-ATP at the same nucleotide-binding site within each domain protein, the potency of the nucleotides in preventing the labeling should correlate with their affinity for the nucleotide-binding site of the protein. The data indicate that ATP binds to both TAP1 and TAP2 nucleotide-binding sites with the highest affinity, suggesting that ATP is the natural ligand, or the substrate for TAP1 and TAP2.

4. Discussion

It is concluded from this study that both TAP1 and TAP2 contain an ATP-binding site within their C-terminal domains. Although their sequences are not identical, the two C-terminal domains were found to have a same nucleotide-binding specificity. This nucleotide specificity was also observed in other traffic ATPase transporters, such as MalK protein [21,23] and P glycoprotein [22], suggesting similarities in their tertiary structures. However, the TAP1 and TAP2 C-terminal domains might

have functional differences. They might alternately bind and hydrolyze ATP molecules in the catalytic cycle of the functional TAP1 and TAP2 heterodimer. Alternatively, the two C-terminal domains might regulate the peptide-transport function of TAP differently through their conformational changes during the ATP-binding and hydrolysis, the phosphorylation of their residues by protein kinases or by interacting with other ER membrane proteins and the proteins from the cytosol, such as chaperone proteins (Y.Y. and P.A.P., unpublished data).

A common tertiary structural model has been proposed for the ATP-binding domains in the traffic ATPase transporter superfamily [25]. However, crystal structural information has not been obtained for any of the ATP-binding domains of the traffic ATPase transporters. The intact TAP proteins expressed in Schneider cells were found to transport peptides in an ATP-dependent manner (data not shown), indicating the TAP1 and TAP2 are functional, but their expression levels were very low comparing to that of the C-terminal domain proteins. The low expression levels of intact TAP proteins are likely due to their ER membrane localization as indicated by immuno-fluorescent staining (data not shown). We have attempted to purify the intact TAP proteins from Schneider cells, but it has been difficult to purify the intact TAP proteins. In contrast, the TAP C-terminal domains were expressed as soluble proteins quantitatively (about 2 mg per liter) in Schneider cells and their purification would be much easier. Recently, the TAP C-terminal proteins have been purified by chromatography (data not shown) to the quantities sufficient for the X-ray crystallography and NMR study.

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