Biochemical Characterization of Transporter Associated with Antigen Processing (TAP)-Like (ABCB9) Expressed in Insect Cells

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The ATP-binding cassette (ABC) transporter, transporter associated with antigen processing (TAP)-like (TAPL) tagged with a histidine cluster was overexpressed, amounting to as much as 1—2% of total membrane proteins in Drosophila cell line S2. TAPL was effectively solubilized from membranes by Triton X-100, NP-40 and n-dodecyl-β-D-maltoside. Solubilized TAPL bound ATP-agarose and adenosine 5′-diphosphate (ADP)-agarose but not adenosine 5′-monophosphate (AMP)-agarose. The binding was competed for by excess free ATP, ADP, guanosine 5′-triphosphate (GTP) and dATP but not by AMP. Pyrimidine nucleotides such as uridine 5′-triphosphate (UTP) and cytidine 5′-triphosphate (CTP) were less effective competitors, suggesting that purine nucleotide triphosphates are substrates for TAPL. The ATP-binding of TAPL required Mg2+, and was observed at neutral pH. Chemical cross-linking experiments suggested that TAPL forms a homodimer in the membrane and under the solubilized conditions.

Key words ABCB9; nucleotide binding; transporter associated with antigen processing (TAP)-like; overexpression; solubilization

The ATP-binding cassette (ABC) transporters transport a variety of substrates across biomembranes using the energy of ATP hydrolysis. Human ABC transporters have been classified into seven subfamilies (A—G) based on the sequences and organizations of their ATP binding domains.1) Members of subfamily B transport a variety of cationic substrates of different sizes, from iron to antibiotics, anti-tumor compounds and antigenic polypeptides.1) This subfamily consists of half- and full-type transporters, well-characterized members being transporter associated with antigen processing (TAP).2,3) and multidrug resistance (MDR),4) respectively.

TAPL (TAP-like; ABCB9) is strongly homologous to TAP1 (ABCB2) and TAP2 (ABCB3).5,6) TAP plays an important role in major histocompatibility complex (MHC) class I antigen processing/presentation by transporting antigenic peptides from the cytosol into the endoplasmic reticulum (ER) lumen.7) Because of the strong similarity, it is expected that a potential function of TAPL could be associated with peptide transport as in the case of TAP.8) Furthermore, TAPL is highly conserved in rodents and man compared with TAP1 and TAP2, suggesting that the rate of evolution of TAPL was much slower than those of TAP1 and TAP2, although TAPL and TAP could have diverged from a common ancestor.6)

Most of the half-type ABC transporters are localized to intracellular organelle membranes and function as either homodimers or heterodimers.7,9,10) The TAPL overexpressed stably in a human ovarian carcinoma cell line was localized to lysosomes.11) In contrast to such cell biology,6,11,12) the biochemistry of TAPL has been elucidated very little.8) In this study, we expressed mammalian TAPL in invertebrate cells, and its biochemical properties such as nucleotide binding and dimer-formation were examined.

MATERIALS AND METHODS

Construction of Expression Plasmids for TAPL The EcoRI–Sall fragment encoding the full-length human TAPL (hTAPL) 12A isoform13) was inserted into Drosophila expression vector pAc5.1/V5-HisA (Invitrogen) using the EcoRI and XhoI sites. The stop codon of hTAPL was substituted with an Sall site.12) The rat TAPL (rTAPL) C-1 isoform cDNA fragment was similarly inserted into pAc5.1/V5-HisA. The two expression plasmids were named pAc5.1/V5-HisA–hTAPL and pAc5.1/V5-HisA–rTAPL, respectively.

DNA sequences were determined14) using an ABI PRISM TM310 with a Big Dye™ Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems) and M13F primer 5′-TTTCCAGTCAAGCAGC-3′. The molecular biological methods for DNA manipulations were based on standard procedures.15)

Transfection of Expression Plasmids into S2 Cells Drosophila Schneider 2 (S2) cells (3×10⁶ cells) were cultured in a 35 mm plate in Schneider Drosophila medium (GIBCO BRL) containing 10% fetal calf serum (GIBCO BRL), 50 units/ml penicillin G (Wako), and 50 mg/ml streptomycin sulfate (Meiji) at 22—25 °C the day before transfection. The expression plasmid encoding human or rat TAPL was transfected into S2 cells with pCoHygro (Invitrogen) by means of the calcium-phosphate method.16) The transfected cells were selected for three weeks using 300 μg/ml hygromycin B (Wako). The empty vector, pAc5.1/V-HisA, was introduced as a control (mock).

SDS-Polyacrylamide Gel-Electrophoresis and Western Blotting Protein samples were treated at 55 °C for 10 min and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel-electrophoresis [mini gel (12×10 cm), 1 mm thickness, consisting 3% (w/v) stacking gel (15 mA) and 7% (w/v) separation gel (30 mA)].17) Proteins were electro-blotted (200 mA, 1.5 h, ATTO Model-AE6675) onto an Immobilon™-P membrane (Millipore).18) The filter was rinsed with phosphate-buffered saline [10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl] (PBS) and then blocked for 1 h at room temperature with PBS containing 0.1% (w/v) Tween 20 (PBS-T) in the presence of 3% (w/v) bovine serum albumin (BSA). The filter was rinsed twice,
washed once (15 min) and twice (5 min) with PBS-T, and then reacted with mouse immunoglobulin (Ig) G_{i} anti-His6 peroxidase (Roche) \(\times 10000\) diluted with PBS-T) for 1 h. After rinsing (twice) and washing (once for 15 min and twice for 5 min) with PBS-T, chemiluminescence was detected with a Western blotting kit (Amersham Bioscience) using Hyperfilm-ECL (Amersham Bioscience).

**Preparation and Solubilization of Membranes** All the procedures were carried out at 4°C. Cells were harvested (2300\(\times g\), 5 min) and washed with ice-cold PBS. The cell pellet was suspended in homogenization buffer comprising 0.25 m sucrose, 25 mM KOAc, 5 mM Mg(OAc)\(_{2}\), 50 mM Tris–HCl (pH 7.5), and protease inhibitor mix \(3 \mu g/ml\) pepstatin A, 3 \(\mu g/ml\) leupeptin and 1 \(\mu M\) phenylmethylsulfonyl fluoride (PMSF), and then homogenized with a glass homogenizer with a Teflon pestle (60 strokes). Cell debris was precipitated (600\(\times g\), 5 min). The pellet was again suspended in the homogenization buffer, homogenized and centrifuged. The two supernatants were combined, and ultracentrifuged (80000\(\times g\), 1 h) (Beckman 50.3Ti rotor). The supernatant and precipitate were used as the cytoplasmic and membrane fractions, respectively. The membranes were suspended in storage buffer \(20\,mM\) (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH (pH 7.5) containing protease inhibitors and stored at -80°C until use. Protein concentrations were determined with a BIO-RAD Protein Assay \(19)\) using BSA (Fraction V, Sigma) as a standard.

Membranes (50 \(\mu g\) protein) were incubated on ice for 1 h in 50 \(\mu l\) of binding buffer \(20\,mM\) HEPES–NaOH (pH 7.5), 145 \(mM\) NaCl, 2 \(mM\) KCl, 1 \(mM\) dithiothreitol (DTT), 2 \(mM\) MgCl\(_{2}\), and protease inhibitor mix\(\) containing 1 or 2% of divalent cations and ethylenediaminetetraacetic acid (EDTA) were determined, the indicated concentrations of divalent cations and ethylenediaminetetraacetic acid (EDTA) were added to the binding buffer. 2-(Morpholino)ethanesulfonic acid (MES)–NaOH (pH 5.5) and HEPES–NaOH (pH 6.0, 7.0, 8.0, 8.5) buffers were also used. For the competition assay, a 5-fold excess of a nucleotide (0.2 \(\mu M\)) was added to the Triton X-100 solubilized membrane supernatant, followed by rotation for 1 h at 4°C. After centrifugation for 3 min at 8400\(\times g\), ATP-agarose was added.

**Cross-Linking** T1 and T2 cells (CRL-1991 and CRL-1992, respectively, from ATCC\(\)\(^{20}\)) were cultured in Iscove’s modified Dulbecco’s medium (GIBCO BRL) supplemented with 20% (v/v) fetal bovine serum (JRH Biosci.). Membranes (20 \(\mu g\) protein) prepared as above were incubated in 20 \(\mu l\) of buffer \(50\,mM\) HEPES–NaOH (pH 7.0), 78 \(mM\) KCl, 1 \(mM\) DTT, 4 \(mM\) MgCl\(_{2}\), 8.4 \(mM\) CaCl\(_{2}\), 10 \(mM\) ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N’-tetraacetic acid (EGTA) and 1 \(mM\) PMSF for 30 min at 25°C in the presence of various concentrations of ethylene glycol bis(succinimidylyl propionate) (EGS), and then the cross-linking reaction was terminated by adding 1 \(m\) tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.5) to 50 mm. The reaction mixture was processed for 15 min at 25°C, and then a quarter volume of the sample buffer was added, followed by heating for 10 min at 55°C. The sample was analyzed by Western blotting after SDS-polyacrylamide gel-electrophoresis (7.5%, mini gel), with rabbit anti-mouse TAP1 antibodies as the first antibodies \((\times 5000\) diluted, STRESSGEN) and horseradish peroxidase conjugated donkey anti-rabbit IgG antibodies as the second ones \((\times 6000\) diluted, Amersham-Pharcma-Biotech).

Membranes of S2 cells expressing rTAPL (20 \(\mu g\) protein) were incubated in 20 \(\mu l\) of the binding buffer containing 1 \(mM\) PMSF (instead of the protease inhibitor mix) with various concentrations of EGS for 30 min at 25°C, and then further incubated after adding 1 \(m\) Tris–HCl (pH 7.5) to terminate the cross-linking reaction. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (7.5%, mini gel). m-Maleimidebenzoyl-N-hydroxysuccinimide ester (MBS), dithiobis(succinimidyl succinate) (DSP) and 3,3’-dithiobis(sulfosuccinimidylpropionate) (DTSSP) were reacted with 10 \(\mu g\) membrane. In the case of MBS, 10 \(mM\) EDTA was added to the binding buffer. All four cross-linking reagents (Pierce) were dissolved in dimethyl sulfoxide (DMSO) as 20 \(mM\) stock solutions.

Membranes \((0.25\,mg\,protein/125\,\mu l)\) were incubated for 20 min at 4°C in the presence of 0.4% (w/v) lecithin (Nippon Seiyaku) with either of the 2% detergents \([\text{Triton X-100, CHAPS, or n-dodecyl-\(\beta\)-d-maltoside}] in the solubilization buffer \(20\,mM\) HEPES–NaOH (pH 7.5), 150 \(mM\) NaCl, 20% (v/v) glycerol, 2 \(mM\) DTT, 1.5 \(mM\) MgCl\(_{2}\), and protease inhibitor mix]. After centrifugation (80000\(\times g\) for 1 h at 4°C, the supernatant (5 \(\mu l\)) was used for the cross-linking reaction and analyzed (7.5% mini gel).

**Chemicals** Restriction enzymes were purchased from NEB and MBI. T4 DNA ligase and Ampli-Taq were obtained from Promega and Perkin Elmer, respectively. The GENE CLEAN III KIT BIO101 was from BioRad. All other chemicals used were of the highest grade commercially available.

**RESULTS**

**Stable Expression of Recombinant TAPL in Insect S2 Cells** To examine the biochemical properties of TAPL, we expressed human and rat TAPL (hTAPL and rTAPL, respectively) in *Drosophila* embryonic cell line S2. The recombinant hTAPL and rTAPL were detected in the membrane fraction but not in the soluble fraction (Fig. 1A). The apparent molecular size of recombinant TAPL was 72-kDa, i.e., slightly smaller than the calculated value (88-kDa). Such a
difference had been reported previously, possibly due to the nature of polytopic integral membrane proteins. Visual estimation suggested that the stable cell lines produced both hTAPL and rTAPL protein amounting up to 1—2% of the total membrane protein; using antibodies for the ABC region of TAPL, we compared the intensity of TAPL on the S2 cell membrane with that of the recombinant protein expressed in E. coli (not shown).

Solubilization of rTAPL from the Membranes

It seems likely that the relative higher expression of the recombinant TAPL protein in S2 cells is advantageous for biochemical study of this protein. Thus, we first determined whether or not rTAPL is effectively solubilized from membranes. Membranes were treated with various detergents [1 or 2% (w/v) of n-dodecyl-β-D-maltoside, n-octyl-β-D-glucoside and CHAPS, and 1 or 2% (v/v) of Triton X-100 and NP-40], and then a high-speed supernatant and precipitate were prepared. As shown in Fig. 1B, all the detergents at the different concentrations except octyl-β-D-glucoside solubilized rTAPL from the membranes. Three detergents, n-dodecyl-β-D-maltoside, Triton X-100 and NP-40, almost completely solubilized the rTAPL, while CHAPS was less effective.

Nucleotide Binding of rTAPL Solubilized from the Membranes

It was thought interesting to know whether or not the solubilized rTAPL retains the ability of ATP-binding since TAPL has a nucleotide binding domain characteristic of ABC transporters. Thus we solubilized rTAPL with 2% (v/v) Triton X-100, and the solubilized sample was mixed with agarose beads conjugated with either of the adenine nucleotides (ATP, ADP and AMP). As shown in Fig. 2A (upper), agarose beads conjugated with ATP and ADP bound rTAPL in a detergent solution. The rTAPL was recovered in the pellet fraction together with the beads. However, agarose beads with AMP did not bind rTAPL, the rTAPL remaining in the supernatant. These results clearly indicated that rTAPL binds to ATP and ADP but not AMP. The binding was competed for in the presence of excess amounts of free ATP, since rTAPL was recovered in the supernatant fraction (Fig. 2A, lower). Essentially the same results were obtained with rTAPL solubilized with n-dodecyl-β-D-maltoside and CHAPS (data not shown).

Specificity of Nucleotide Binding

To determine the nucleotide binding specificity in detail, either of the various nu-
cleotides was added in a 5-fold excess to the reaction mixture for binding of rTAPL to ATP-agarose. Figure 2B shows that free ATP, ADP, GTP or dATP but not AMP inhibited the binding. UTP or CTP was less effective. These results indicate that the nucleotide binding to rTAPL preferred to purine nucleotides with di- and tri-phosphates. The nucleotide binding effectively occurred at neutral pH (pH 6—8); the binding at pH 8.5 was less effective, and the efficiency of solubilization was lower at pH 5.5 (Fig. 2C). The ATP-binding to solubilized rTAPL required Mg²⁺, and the binding with Mg²⁺ was inhibited upon the addition of an excess amount of EDTA (Fig. 2D). In the presence of 20 mM EDTA, a higher amount of Mg²⁺ (40 mM) was required for the ATP binding, but Ca²⁺ or Zn²⁺ could not substitute for Mg²⁺ (Fig. 2E).

**Cross-Linking of rTAPL** The T1 cells express cell surface MHC-class I molecules, while T2 cells do not. 20) We also confirmed the expression of mRNAs for TAP1 and TAP2 by means of polymerase chain reaction in T1 cells (not shown). These results suggest that T1 cells express functional TAP from TAP1 and TAP2. Membranes from T1 cells actually contained the TAP1 molecules while T2 cells without the TAP1 and TAP2 genes 20,21) did not express TAP1 (Fig. 3A). When the membranes from T1 cells were treated with cross-linking reagent EGS, the mobility of the TAP1 molecules shifted to a higher molecular weight position, 175-kDa (Fig. 3B); this 175-kDa band was suggested to represent a hetero-dimer of TAP1 and TAP2.21)

Then, the membranes from S2 cells containing rTAPL were treated with EGS. As shown in Fig. 4A, the 175-kDa product was detected concomitant with a decrease in the 72-kDa band for the rTAPL monomer. Such an observation suggested that rTAPL forms a dimer similar to the TAP1 and TAP2 hetero-dimer. Essentially the same results were obtained with MBS and DSP (Figs. 4B, C). However, membrane-impermeable reagent DTSSP did not give a 175-kDa band (Fig. 4D). These results suggest that cross-linking occurred between hydrophobic regions of the rTAPL molecules. Furthermore, the rTAPL solubilized with Triton X-100 and n-dodecyl-β-D-maltoside reacted with EGS and produced a shifted band corresponding to essentially the same size as that of the membrane bound form (Fig. 4E). Thus, these results strongly suggest that rTAPL could form a dimer on the membrane and in the detergent after solubilization. It must be noted that insect S2 cells do not express TAP1 and TAP2. Furthermore, we could detect TAPL homodimer by co-immunoprecipitation experiment using Myc- and His6-tagged TAPL (not shown).

**DISCUSSION**

In this study, we expressed mammalian TAPL (both human and rat TAPL) in cultured insect S2 cells. Significant amounts of both TAPL proteins were expressed on the intracellular membranes. Similarly we reported that the TAPL was expressed on the intracellular membranes of mammalian cultured cells. 12) rTAPL was solubilized from membranes easily in the presence of 1—2% of detergents such as Triton X-100, CHAPS, NP-40 and n-dodecyl-β-D-maltoside. The solubilized rTAPL retained the ability of nucleotide binding; the rTAPL bound to ATP and ADP, and showed a preference for purine nucleoside tri-phosphates and less affinity for pyrimidine nucleotides. Thus the membrane-bound TAPL and solubilized TAPL (this study) exhibited essentially the same nu-
cleotide specificity as to the nucleotide binding site, although we could not detect ATPase activity in the presence and absence of various peptides and ATPase inhibitors as determined by the colorimetric method (not shown). 23) Similar Mg$^{2+}$-dependent nucleotide binding shown for rTAPL has been reported for closely related TAP. 21) A half type ABC transporter is suggested to be functional in the dimer state in contrast to a full type ABC transporter. Closely related TAP is a hetero dimer of TAP1 and TAP2, since the function of TAP is decreased in the absence of either of the subunits. 23,24) Furthermore, the expression level of the TAP1 or TAP2 subunit without the counterpart was very low. Thus the stably expressed TAPL in insect cell membranes observed in this study must be a sign of formation of the homodimer of TAPL. The cross-linking patterns of TAPL on the membranes and in the detergent are closely similar to that of the TAP heterodimer, 21) supporting the homodimer of TAPL. The cross-linking patterns of TAPL comprising 6 to 59 residues are transported.8) Since cross-linking of TAP was stimulated in the presence of a transport peptide substrate, 21) we also determined the effects of various peptides on the cross-linking of rTAPL. However, our system did not demonstrate a positive or negative effect on the cross-linking of TAPL. The dihydrofolate reductase proteinfragment complementation assay 25) and the co-immunoprecipitation experiment 26) also suggested the homodimer of TAPL.

The physiological role(s) of TAPL has not been characterized although it has been reported that a variety of peptides comprising 6 to 59 residues are transported.8) Since cross-linking of TAP was stimulated in the presence of a transport peptide substrate, 21) we also determined the effects of various peptides on the cross-linking of rTAPL. However, our system did not demonstrate a positive or negative effect with peptides such as HLA-B27, HLA-A2, ICP47, amyloid-β and α-mating factor. 21,26—28) Nevertheless, our system will be helpful for further biochemical analysis of TAPL since at least nucleotide binding capability is preserved.

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