Pairing of the Nucleotide Binding Domains of the Transporter Associated with Antigen Processing*

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The transporter associated with antigen processing (TAP) comprises two structurally related subunits, TAP1 and TAP2, that form stable complexes in endoplasmic reticulum (ER) membranes. TAP complexes function in the translocation of peptides from the cytosol into the ER lumen for presentation by major histocompatibility complex class I molecules. Each TAP subunit contains an N-terminal membrane-spanning region with multiple membrane-spanning segments, and a C-terminal, cytosolic nucleotide binding region. To study the nature of the interactions occurring on the cytosolic face of TAP1/TAP2 complexes, we investigated quaternary associations mediated by two C-terminal fragments of human TAP1 (T1c, residues 452–748 and T1ctr, residues 472–748) and two C-terminal fragments of human TAP2 (T2c, residues 399–686 and T2ctr, residues 433–686). Each of these constructs contains the core nucleotide binding region as well as a long or short N-terminal extension. We show stable complex formation between T1c and T2c but not between T1ctr and T2ctr. The mechanistic implications of these results are discussed. We also show that each of the constructs except T1ctr interacts with wild type TAP1 and TAP2, indicating possibilities for homodimerization of TAP1 and TAP2, or of oligomerization of TAP1/TAP2 heterodimers on membranes.

The TAP2 transporter is an essential component of the major histocompatibility complex class I antigen presentation pathway (1, 2). The transporter comprises two subunits called TAP1 and TAP2. Each contains an N-terminal transmembrane region and C-terminal nucleotide binding domains (NBD) (Fig. 1A). TAP1 and TAP2 form a complex in endoplasmic reticulum (ER) membranes, with the NBD oriented in the cytosol (3). The complex functions to transport peptides from the cytosol into the ER, for presentation by major histocompatibility complex class I molecules. Functional forms of both TAP1 and TAP2 are required for peptide transport from the cytosol into the ER; defects in either TAP1 or TAP2 result in the significant loss of presentation of intracellular antigens (4, 5).

The TAP complex is a member of the ABC binding cassette (ABC) family of transmembrane transporters (6). Both prokaryotic and eukaryotic proteins constitute the ABC family, and these proteins function in the specific ATP-dependent transport of sugars, inorganic ions, amino acids, peptides, proteins, and a variety of other components across membranes. The mammalian members of the ABC family include the cystic fibrosis transmembrane conductance regulator that functions in the channeling of chloride ions (7), and the multidrug resistance proteins (MDR; for example P-glycoprotein) responsible for transporting hydrophobic drugs outside of cells and for conferring resistance of tumor cells to chemotherapeutic drugs (8, 9). ABC transporters share sequence similarities as well as a common structural organization. Although the overall domain organization is complex, all ABC transporters comprise two transmembrane regions with multiple membrane-spanning segments, and two NBD (6). ATP hydrolysis by the NBD appears to induce structural changes at the membrane which allows the passage of substrate across membranes. However, the precise molecular mechanisms that couple ATP hydrolysis to substrate translocation are poorly understood.

Electron microscopic investigations of P-glycoprotein have provided the first insights into a low resolution structure of an ABC transporter (10). Electron microscopic analysis of single particles and detergent-solubilized P-glycoprotein indicated a structure containing a large aqueous pore, open at the extracellular face of the membrane, and closed at the inner (cytosolic) face of the membrane by the nucleotide binding domains and/or cytoplasmic loops that separate the membrane-spanning segments (10). Such an architecture is likely to be conserved among other ABC transporters, with changes in the size of the substrate for different transporters being accommodated by changes in the nature of the cytosolic closure (gate).

For both P-glycoprotein and TAP, substrate binding sites have been identified in the membrane-proximal regions just N-terminal to the NBD. In the case of P-glycoprotein, the drug binding site was determined to be in the vicinity of predicted transmembrane helices 6 and 12, and this region has been suggested to undergo conformational changes upon ATP hydrolysis (11, 12). Using radiolabeled peptides that could be covalently cross-linked to TAP, the peptide binding sites on TAP1 and TAP2 were localized (13, 14). In a model for the transmembrane topology of TAP that was based upon homology with P-glycoprotein (15), the peptide binding sites of TAP are predicted to be in the membrane-cytosol boundary of the final predicted membrane-spanning helix, as well as in the cytosolic loops that connected the preceding two transmembrane helices (helices 4 and 5) (13). The characteristics of peptides translocated by TAP were studied using in vitro pep-
tide translocation assays (16–18). TAP preferentially translates peptides 9–15 amino acids in length and contains a fairly promiscuous peptide-binding site selecting peptides mainly based on the first three N-terminal and the C-terminal residues (19). Binding of peptides to TAP can occur in the absence of exogenous ATP (20, 21), but ATP hydrolysis is required for translocation of peptides across the ER membrane (16, 17).

Obtaining insights into the quaternary interactions that occur in the cytosolic domains and loops of ABC transporters will be important for understanding how ATP hydrolysis by the NBD translates to structural changes at the membrane. Dimerization of nucleotide binding domains has been suggested to occur between the nucleotide binding domains of bacterial ABC transporters (22), raising the possibility that ATP hydrolysis-induced alterations in the dimerization interface may in turn alter interactions at the membrane. Indeed, a crystallographic dimer was observed in the crystal structure of the nucleotide binding subunit of Salmonella typhimurium histidine permease, and has been suggested to be a functional dimer (23). Alternatively, when a substrate binding site is present on the cytosolic face of the membrane, ATP hydrolysis might induce structural changes that simultaneously reduce the affinity for substrate, and reorient the binding site, allowing substrate access to the pore. Such a mechanism seems indeed plausible, as it has been observed using vanadate trapping studies that ATP hydrolysis reduces affinity for P-glycoprotein substrates (24).

To determine whether the NBD of TAP1 and TAP2 heterodimerize, we investigated the nature of the quaternary interactions mediated by two C-terminal fragments of human TAP1 (T1c, residues 452–748 and T1ctr, residues 472–748) and two C-terminal fragments of human TAP2 (T2c, residues 399–686 and T2ctr, residues 433–686), expressed in insect cells (20). To determine TAP NBD interactions, we investigated the nature of the quaternary interactions mediated by two C-terminal fragments of human TAP1 (T1c, residues 452–748 and T1ctr, residues 472–748) and two C-terminal fragments of human TAP2 (T2c, residues 399–686 and T2ctr, residues 433–686), expressed in insect cells (Fig. 1A). All the truncated TAP constructs contain the core NBD (15), and in addition have long or short N-terminal extensions (Fig. 1A). T1c and T2c were previously expressed in Escherichia coli in order to study nucleotide binding to the NBD (25). The design of these constructs was based entirely upon hydrophobicity plots of TAP1 and TAP2, and each construct was initiated subsequent to the final distinctly hydrophobic stretch in the corresponding wild type protein sequence. Recent models for the topological organization of the TAP proteins (14, 15) (that are based upon sequence alignments with the MDR family and hydrophobicity plots), predict that the final transmembrane segments (transmembrane 6) of TAP1 and TAP2 are not very hydrophobic. Based upon these recent models, residues contained in the T1c, T1ctr, and T2ctr constructs would be entirely cytosolic in the context of the corresponding wild type TAP, whereas T2c would include approximately 10 residues that occur in the final transmembrane segment of wild type TAP2.

We show that all the truncated TAP constructs are functional for ATP binding, as assessed by 8-azido-ATP labeling. We show that T1c interacts with T2c as well as with wild type TAP2, and that both T2c and T2ctr interact with T1c as well as wild type TAP1. N-terminal residues of T1c are important for its interactions with TAP2, as T1ctr interactions with T2c as well as wild type TAP2 are impaired. In addition, we demonstrate interaction of T1c with wild type TAP1 and of T2c and T2ctr with wild type TAP2, indicating possibilities for homodimerization of TAP1 and TAP2, or of oligomerization of TAP1/TAP2 heterodimers on membranes.

**Experimental Procedures**

Construction of Baculoviruses Encoding TAP1 and TAP2 NBD (T1c, T2c, T1cT2c, T1ctr, and T2ctr)—The cDNA for human TAP1 and TAP2 was obtained from the laboratory of Dr. John Trowsdale and the following modifications introduced. For T1c and T1ctr, PCR was used to excise the nucleotides encoding residues 452–748 and 472–748, respectively, to introduce BamHI sites at the 5’ and 3’ ends of the truncated TAP1 genes, as well as to introduce DNA sequences encoding a hexa-histidine tail at the 3’ end of the TAP1 constructs. For T2c and T2ctr, PCR was used to excise nucleotides encoding residues 399–686 of TAP2 and 433–686, respectively, to add BglII sites at the 5’ and 3’ ends, and to add a DNA sequence encoding the Myc epitope (EQKLISEEDL) at the 3’ end of TAP2. The PCR products were ligated into the vector PCR2.1 (Invitrogen) or pRC/Script Amp SK+ (Stratagene) and sequenced. 5-Methyl-dCTP was used in the PCR reaction for T2ctr because internal SfiI site, which interferes with its ligation into the pRC/Script Amp SK+. The fusion constructs were individually subcloned into the BamHI or BglII sites of the baculovirus transfer vector pAcUW51 (PharMingen) to generate the viruses T1c, T2c, T1ctr, and T2ctr. For this purpose, the corresponding recombinant transfer vectors and BaculoGold DNA (PharMingen) were co-transfected into insect cells as described in the Pharmingen Baculovirus Expression Manual. Plaque assays were used to isolate pure viruses, which were used to re-infect cells for virus amplification. A fifth virus was also generated that had both the T1c and T2c constructs in single virus (T1cT2c) by sequentially subcloning DNA encoding T1c and T2c into the BamHI and BglII sites of the PAeUW51 transfer vector.

**Insect Cell Infection, Lysis, Immunoprecipitations, and 8-Azido (–)ATP Labeling of TAP Constructs—** Constructs were infected with T1c, T2c, T1ctr, or T2ctr, each at an m.o.i. of 5. After approximately 72 h, cells were lysed for 45 min on ice in 1 ml of lysis buffer (10 mM phosphate, 10 mM Tris, 130 mM NaCl, 1% Triton X-100, pH 7.5) containing 1 mM CaCl2 and 5 mM MgCl2 as well as a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 0.308 μM aprotinin, 10.5 μM leupeptin, 10 μM pepstatin, 1 mM benzamidine). The lysate was cleared of cellular debris by centrifuging at 40,000 × g for 45 min at 4 °C. 0.25 ml of the lysate was immunoprecipitated with 0.5 μl of a purified 9E10, an antibody that recognizes the Myc epitope tag (anti-Myc) contained in the T2c and T2ctr constructs. The samples were centrifuged again at 14,000 × g and supernatants next incubated for 1.5 h at 4 °C with an appropriate volume of protein A beads pre-washed in wash buffer (10 mM phosphate buffer, 10 mM Tris, 130 mM NaCl, 0.5% Triton X-100, pH 7.5) containing 1 mM CaCl2 and 5 mM MgCl2. Samples were centrifuged at 250 × g for 5 min and the supernatant discarded. The beads were washed three times with wash buffer containing 1 mM CaCl2 and 5 mM MgCl2, resuspended in 0.2 ml of the same buffer, and exposed to a 96-well plate. 10 μl of 100 mM ATP, pH 7.0, was added to samples with an appropriate volume of antibody A beads pre-washed with wash buffer but before adding SDS-PAGE sample buffer. Each TAP construct was confirmed by a 4% gel of the immunoprecipitates, followed by immunoblotting analysis with the anti-TAP1 antibody 148.3 (26) or the anti-TAP2 antibody (429.3) (27). For the immunoblotting analysis, proteins were transferred to nitrocellulose membranes by electrophoresis for 1 h at 100 V and the membranes probed with anti-TAP1 (0.15 ml of hybridoma supernatant) and anti-TAP2 (0.25 ml of hybridoma supernatant) to assay for expression of the TAP1 and TAP2 constructs, respectively. The membranes were then incubated for 2 h with 50 μg of a goat anti-mouse alkaline phosphatase (American Qualex) antibody. Blots were developed using the Bio-Rad alkaline phosphatase conjugate substrate kit. Dried blots were exposed directly on a PhosphorImager plate. Data were processed using a Molecular Dynamics PhosphorImager SI.

**Assessment of Complex Formation by Immunoprecipitation and Immunoblotting Analyses—** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experiments were performed with Sf21 insect cells infected with the appropriate viruses. An m.o.i. of 2.5–10 was used for each virus, depending upon the relative levels of expression of each protein, with the lower m.o.i. being used for the high expressors and the higher m.o.i. values for the low expressors. After approximately 72 h, cells were lysed in 1 ml of ice-cold lysis buffer containing the protease inhibitor mixture, and the lysate centrifuged at 40,000 × g for 45 min. 0.18 ml of the lysate (sufficient for a single lane on a SDS-PAGE gel) was immunoprecipitated as described above with (a) 0.75 μl of anti-His ascites, (b) 5 μg of purified 9E10 anti-Myc antibody, (c) 0.3 ml of the anti-TAP1 antibody, (d) 0.6 ml of the anti-TAP2 antibody, or (e) 50 μl of ascites fluid containing the irrelevant anti-class I major histocompatibility complex antibody (W6/32) (28). The wash buffer did not contain CaCl2.
or MgCl₂ except for the 8-azido-[α-³²P]ATP labeling experiment. At the end of the wash steps, the beads were resuspended in 6× SDS-PAGE sample buffer, and heated to 95 °C for 3 min. Proteins were separated using 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with 5 µl of the anti-His ascites, or 2.5 µg of 9E10, anti-
TAP1, or anti-TAP2 using the protocols described above.

**Metabolic Labeling of SF21 Cells for Assessment of the Formation of Various Complexes**—2 × 10⁶ SF21 cells were infected with the appropriate viruses at 27 °C for ~60 h. The supernatant was removed from the plates, and medium was changed to Grace’s insect medium without t-methionine (Life Technologies, Inc.). Plates were incubated for 1 h at 27 °C, and then the medium was removed and replaced with 3 ml of the same with 105 µCi of ³⁵S-labeled methionine/cysteine (ICN). After incubation at room temperature for 3 h with gentle rocking, cells were lysed in 1 ml of lysis buffer containing the protease inhibitor mixture as described above. 0.25–0.33 ml of lysate was immunoprecipitated with anti-His, anti-Myc, anti-TAP1, anti-TAP2, or W6/32 antibodies as described above. Samples were processed for immunoprecipitation as described above, separated on 10% polyacrylamide gels by SDS-PAGE, gels dried on a Bio-Rad gel, and exposed directly to a PhosphorImager plate. Data were processed using a Molecular Dynamics PhosphorImager SI. Alternatively, immunoprecipitated proteins were transferred from the gels to nitrocellulose membranes and analyzed by immunoblotting with anti-TAP1 and anti-TAP2 antibodies following the protocols described above.

**Quantitation of Metabolic Labeling Experiments**—Relative association levels of the truncated constructs were quantified by phosphorimaging analyses of radiolabeled immunoprecipitates (Figs. 3 and 5 and Table I). Proteins were labeled using Tran³⁵S-label, a labeling reagent that results in the incorporation of approximately 82% L-[³⁵S]methionine and 18% L-[³⁵S]cysteine, and immunoprecipitated with the appropriate antibody using the procedures described above. For quantifying relative association levels of the different interacting partners, the band intensities obtained for each protein from a given immunoprecipitation were first normalized with respect to each other to take into account the differing methionine/cysteine contents. T1c contains 4 methionines and 3 cysteines; T2c contains 6 methionines and 3 cysteines; T1ctr contains 4 methionines and 3 cysteines; T2ctr contains 4 methionines and 3 cysteines.

Using the T1c/T2c complex (Fig. 3A) as an example, the method used for quantifying relative association levels is indicated below. In the anti-His-based immunoprecipitation, co-precipitating T2c was first normalized for its differing methionine content relative to T1c: total intensity (T2c) = (82/100) × 6 (methionine component) + (18/100) × 3 (cysteine component); total intensity (T2c) = 90.1% (methionine component) + 9.9% (cysteine component).

Because T2c contains 6 methionines and 3 cysteines relative to 4 methionines and 3 cysteines in T1c, normalized T2c intensity = observed intensity × 0.901 + observed intensity × 0.099; ratio of intensities T2c/T1c (anti-His) = (normalized T2c intensity)/T1c intensity. A similar procedure was used to quantify the amounts of co-precipitating protein for each co-immunoprecipitation experiment shown in Figs. 3 and 5.

**RESULTS**

**Association of the NBD of TAP1 and TAP2**

We expressed residues 452–748 of human TAP1 and residues 399–686 of human TAP2 in insect cells. We refer to these constructs as T1c and T2c, respectively (C-terminal, cytosolic regions of TAP1 and TAP2). To facilitate assessment of the complex formation, a hexahistidine tag was engineered at the C-terminal end of T1c and a Myc tag was engineered at the C-terminal end of T2c. Recombinant baculoviruses were generated encoding T1ctr/T2c complex proteins. The expression of T1c could be demonstrated using a hexahistidine-specific antibody (anti-His) and a TAP1-specific antibody, 148.3 (26) (Fig. 1B, lanes 2 and 3). The expression of T2c could be demonstrated using the Myc-epitope-specific antibody (anti-Myc) and anti-TAP2 antibody, 429.3 (27) (Fig. 1B, lanes 5 and 6). The folding and functionality of T1c and T2c could be assessed using 8-azido-[α-³²P]ATP labeling (Fig. 1C, lanes 2 and 5), a photoactivatable ATP analog that can be covalently cross-linked to ATP-binding proteins. Photolabeling of both constructs could be competed with an excess of unlabelled ATP (Fig. 1C, lanes 3 and 6).

To establish whether the truncated TAP subunits associate, detergent (1% Triton X-100) lysates from cells infected with T1c/T2c or T1c+t/T2c were immunoprecipitated with antibodies directed against either T1c or T2c. These samples were subsequently separated by SDS-PAGE, transferred to nitrocellulose membranes, and the membranes immunostained with anti-His antibodies or anti-Myc antibodies, or with anti-TAP1 or anti-TAP2 antibodies. We observed that the truncated subunits associated. T2c co-precipitates in immunoprecipitations using the anti-His antibody, as demonstrated by immunoblotting analyses with either anti-TAP2 or anti-Myc antibodies (Fig. 2, B and D, lanes 1). Likewise, T2c co-precipitates in immunoprecipitations with anti-TAP1, as demonstrated by immunoblotting analyses with either anti-Myc or anti-TAP2 antibodies (data not shown). Conversely, T1c co-precipitates in immunoprecipitations with anti-Myc antibody, as demonstrated by immunoblotting with anti-TAP1 or anti-His antibodies (Fig. 2, A and C, lanes 2). Immunoprecipitations with anti-TAP2 also reveal the presence of T1c by immunostaining with anti-His or anti-TAP1 antibodies (data not shown). Bands corresponding to the molecular weights of T1c or T2c were not visualized using the irrelevant antibody W6/32 (28) (Fig. 2, A–D, lanes 3) or by immunoprecipitations of lysates from uninfected cells (Fig. 2, A–D, lanes 4 and 5).

Radiolabeling and immunoprecipitation experiments were performed to directly observe T1c/T2c complexes. For this purpose, infected insect cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine 48–60 h after infection, lysates immunoprecipitated with anti-His, anti-Myc, anti-TAP1, anti-TAP2, or irrelevant antibodies and total proteins observed upon SDS-PAGE and phosphorimaging analyses (Fig. 3). Analysis of the images indicates the presence of a single major labeled band of molecular mass ~34 kDa, when cells were infected with either T1c or T2c (Fig. 3, A and B, lanes 1). By contrast, a doublet of bands was observed when cells were infected with both viruses (Fig. 3, A and B, lanes 2 and 3). The lower band of the doublet corresponds to T1c, and the upper band of the doublet to T2c, based upon comparisons of immunoprecipitations from cells that were infected with a single virus or both viruses (Fig. 3, A and B, compare lanes 1 (single infections) with lanes 2 and 3 (co-infections)). Anti-His- or anti-TAP1-based immunoprecipitations resulted in the recovery of significant amounts of T2c (Fig. 3A, lanes 2 and 3), whereas immunoprecipitations with anti-Myc or anti-TAP2 resulted in the recovery of low levels of T1c (Fig. 3B, lanes 2 and 3, and Table I). These results could be explained by the higher expression level of T2c relative to T1c, by interference of anti-Myc/anti-TAP2 and T1c/T2c combining sites, or other factors. The important point, however, is that each of the antibodies used allows for the observation of T1c/T2c complexes by co-immunoprecipitation analyses.

**Effect of N-terminal Truncations of T1c and T2c upon Association**

**T1ctr/T2c Complexes**—In infections of cells with T1c/T2c, immunoprecipitation/immunoblotting with anti-His or anti-TAP1 sometimes revealed the presence of a second band corresponding to a proteolytic fragment of T1c (for example Fig. 2, A and C, lanes 1). However, when immunoprecipitations were carried out with anti-Myc or anti-TAP2 antibodies, only the upper of the two T1c bands was observed to co-precipitate, as visualized by immunoblotting analysis with anti-His or anti-
FIG. 1. Summary of truncated TAP constructs that were generated. A, hydropathy plots (30) of TAP1 and TAP2, indicating the structural organization, location of the peptide binding sites (13), initiation sites for T1c, T1ctr, T2c and T2ctr, and the location of the core NBD as defined in Ref. 15. A window size of 18 residues was used to generate the hydropathy plots. The boundaries between the final membrane-spanning segment and the cytosolic regions of TAP1 and TAP2 have not been unambiguously defined. B and C, expression of T1c and T2c and labeling with 8-azido-[α-32P]ATP. Lysates from cells infected with T1c (lanes 2 and 3), T2c (lanes 5 and 6), or uninfected cells (lanes 1 and 4) were immunoprecipitated with the anti-His (lanes 1–3) or the anti-Myc (lanes 4–6) antibodies. Immunoprecipitated proteins were labeled with 8-azido-[α-32P]ATP. Proteins were separated by SDS-PAGE and transferred to membranes. B, immunoblotting analysis. Membranes were probed with anti-TAP1 (lanes 1–3) or anti-TAP2 (lanes 4–6) to confirm expression of T1c and T2c. Both proteins migrate at approximately 34 kDa, but can be resolved on 10% SDS-PAGE gels. The 60- and 24-kDa bands in all the immunoblots correspond to antibody heavy and light chains, respectively. C, phosphorimaging analysis. 8-Azido-[α-32P]ATP labeling had been carried out in the absence (lanes 1, 2, 4, and 5) or presence (lanes 3 and 6) of excess unlabeled ATP.
TAP1 (Fig. 2, A and C, lanes 2). We rationalized that the lower molecular weight T1c band observed corresponded to an N-terminal proteolytic fragment of the T1c construct, because the fragment was visualized by the anti-His antibody, which recognizes the polyhistidine epitope on the C terminus. This observation suggested that N-terminal regions of T1c were important for the T1c/T2c association.

To directly investigate the involvement of N-terminal residues of T1c in the formation of T1c/T2c complexes, a baculovirus construct was engineered (T1ctr) encoding a truncated T1c that lacked 21 N-terminal amino acids of T1c (T1ctr; residues 472–748 of TAP1), but which contains the core NBD found in other ABC transporters, as well as a C-terminal hexahistidine tag as with the T1c construct (Fig. 1A). The expression of this truncated protein was verified using anti-His-based immunoprecipitations from lysates of cells infected with T1ctr (Fig. 4A, lanes 2), and the folding and functionality was established using 8-azido-ATP labeling (Fig. 4B, lanes 2 and 3). To assess T1ctr/T2c interaction, cells were co-infected with T1ctr+T2c, metabolically labeled, and analyzed by immunoprecipitation with anti-His and anti-Myc antibodies as described for T1c/T2c complexes. Visual inspections of the phosphorimages of anti-His and anti-Myc-based immunoprecipitations indicated that T2c or T1ctr, respectively, did not co-precipitate (Fig. 5A, lanes 3 and 4). Careful quantitation of band intensities after subtraction of appropriate controls verified that T1ctrT2c complexes were not being formed at significant levels (Table 1). Co-precipitation of T1ctr and T2c was also not observed using immunoblotting analyses such as those described in Fig. 2 (data not shown). Taken together with the analogous experiments described for T1c/T2c complexes (Figs. 2 and 3), these results indicated that N-terminal truncations of T1c impaired complex formation with T2c.

T1c/T2ctr Complexes—Because N-terminal truncations of T1c impaired T1c/T2c association, we next addressed whether deletion of the structurally analogous segment from T2c would also impair the formation of the TAP NBD complex. A baculovirus construct was engineered (T2ctr) encoding a truncated T2c, which lacked 34 N-terminal amino acids of T2c (T2ctr; residues 433–686 of TAP2), but which contains the C-terminal Myc epitope tag as in T2c (Fig. 1A). Protein expression was verified by anti-Myc-based immunoprecipitations of cells infected with T2ctr (Fig. 4A, lane 5). T2ctr also contains the core NBD found in other ABC transporters (Fig. 1A), and is capable of binding ATP, as established using 8-azido-ATP labeling (Fig. 4B, lanes 5 and 6). To assess T1c/T2ctr interaction, cells were infected with T1c+T2ctr, metabolically labeled, and analyzed as described for T1c/T2c complexes. Anti-His-based immunoprecipitations indeed showed the presence of a low intensity band (corresponding to T2ctr) that co-precipitated with T1c (Fig. 5B, lane 3; Table 1), and immunoblot analyses confirmed the presence of T2ctr (data not shown). Anti-Myc-based immunoprecipitations indicated only a slight enhancement of the T1c band relative to control (Fig. 5B, lane 4), correlating with the lower recovery of T1c/T2c complexes with the anti-Myc antibody compared with the anti-His antibody. These results indicated that, while N-terminal truncation of T2c may lower the affinity of the T1c/T2c interaction, the truncation does not destroy the ability to interact with T1c.

T1ctr/T2ctr Complexes—Using assays analogous to those described in Fig. 2 for observing T1c/T2c complexes, we were unable to demonstrate the formation of T1ctr/T2ctr complexes (data not shown). Metabolic labeling experiments were carried out to ask whether low levels of T1ctr/T2ctr association occurred. Cells were infected with T1ctr+T2ctr, metabolically labeled, and analyzed by immunoprecipitation with anti-His and anti-Myc antibodies as described for T1c/T2c complexes. On 10% SDS-PAGE gels, T2ctr migrates slightly slower than T1ctr, and the two proteins can be resolved. Visual inspections of the phosphorimages of anti-His-based immunoprecipitations indicated that T2ctr was not co-precipitating with T1ctr, as a single major labeled band was observed in immunoprecipitations from cells infected with T1ctr alone as well as in cells infected with T1ctr+T2ctr (Fig. 5C, lanes 2 and 3). Likewise, the anti-Myc-based immunoprecipitations did not indicate the presence of T1ctr (Fig. 5C, lane 4). Careful quantitation of band intensities after subtraction of appropriate controls verified that T1ctr/T2ctr complexes were not being formed at significant levels (Table 1).

Wild Type TAP2 Associates with T1c, and Wild Type TAP1 Associates with T2c and T2ctr

If the formation of T1c/T2c and T1c/T2ctr complexes parallels interactions that occur in wild type TAP1/TAP2 complexes, we would expect that T1c, T2c, and T2ctr would also associate with the partner wild type subunits. To investigate whether these interactions occurred, we used baculoviruses encoding wild type TAP1 (T1wt), and wild type TAP2 (T2wt), the construction of which has been previously described (26). Wild type TAP1 (T1wt) and wild type TAP2 (T2wt) are recognized by the anti-TAP1 and anti-TAP2 antibodies, respectively. However, neither protein should be recognized by the anti-His or anti-Myc antibodies, unless associated with the truncated TAP constructs. Co-infecting cells with wild type as well as the truncated constructs allowed for assessment of the pairing of each
truncated construct with the partner wild type subunit, using co-immunoprecipitation assays.

**T1c/T2wt and T1ctr/T2wt Complexes**—To investigate the occurrence of T1c/T2wt association, cells co-infected with T1c (lanes 1 and 2) or T1c + T2c (lanes 2–4) were immunoprecipitated with anti-His (lanes 1 and 2), anti-TAP1 (lane 3), or irrelevant (lane 4) antibodies. B, cells infected with T2c (lane 1) or T1c + T2c (lanes 2–4) were immunoprecipitated with anti-Myc (lanes 1 and 2), anti-TAP2 (lane 3), or irrelevant (lane 4) antibodies.

**FIG. 3.** Direct observation of T1c/T2c complexes by metabolic labeling. SDS-PAGE and phosphorimaging analyses of immunoprecipitates from metabolically labeled cells. A, cells infected with T1c (lane 1) or T1c + T2c (lanes 2–4) were immunoprecipitated with anti-His (lanes 1 and 2), anti-TAP1 (lane 3), or irrelevant (lane 4) antibodies. B, cells infected with T2c (lane 1) or T1c + T2c (lanes 2–4) were immunoprecipitated with anti-Myc (lanes 1 and 2), anti-TAP2 (lane 3), or irrelevant (lane 4) antibodies.

**TABLE I**

Association levels between different truncated TAP NBD pairs

| Complex         | Association range |
|-----------------|-------------------|
| T2c/T1c (anti-His) | 0.4–0.85          |
| T2c/T1ctr (anti-His) | 0.02–0.04        |
| T2ctr/T1c (anti-His) | 0.13–0.21        |
| T2ctr/T1ctr (anti-His) | 0–0.04           |
| T1c/T2c (anti-Myc) | 0.1–0.35          |
| T1ctr/T2c (anti-Myc) | 0.02–0.04        |
| T1c/T2ctr (anti-Myc) | 0–0.04           |
| T1ctr/T2ctr (anti-Myc) | 0–0.01           |

**FIG. 4.** Expression of T1ctr and T2ctr and labeling with of 8-azido-[α-32P]ATP. Lysates from cells infected with T1ctr (lanes 2 and 3), T2ctr (lanes 5 and 6), or uninfected cells (lanes 1 and 4) were immunoprecipitated with the anti-His (lanes 1–3) or the anti-Myc (lanes 4–6) antibodies. Immunoprecipitated proteins were labeled with 8-azido-[α-32P]ATP. Proteins were separated by SDS-PAGE and transferred to membranes. A, immunoblotting analysis. Membranes were probed with anti-TAP1 (lanes 1–3) or anti-TAP2 (lanes 4–6) to confirm expression of T1ctr and T2ctr. B, phosphorimaging analysis of membranes. 8-azido-[α-32P]ATP labeling had been carried out in the absence (lanes 1, 2, 4, and 5) or presence (lanes 3 and 6) of excess unlabeled ATP.

8-azido-ATP labeled}

regions of T2c and T2ctr that are involved in interacting with T1c are also accessible for interaction in the context of the full-length TAP2 subunit.

As expected based upon the inability to observe significant
levels of T1ctr/T2c or T1ctr/T2ctr complexes, T1ctr association with T2wt was impaired (Fig. 6A, lanes 4 and 5). Quantitation of the phosphorimages after subtractions of appropriate controls indicated that T1ctr/T2wt complexes were not formed, as detected by either the anti-His or the anti-TAP2 antibody.

**T1wt/T2c and T1wt/T2ctr Complexes**—To investigate the association of T2c and T2ctr with wild type TAP1 (T1wt/T2c and T1wt/T2ctr complexes), cells were co-infected with T1wt+T2c or T1wt+T2ctr, metabolically labeled, and analyzed by immunoprecipitation with anti-TAP1 and anti-Myc antibodies. Anti-Myc-based immunoprecipitations indicated that wild type TAP1 co-precipitated with both T2c and T2ctr (Fig. 6B, lanes 2 and 4), although at low levels. These results were confirmed by immunoblotting analyses with anti-TAP1 (data not shown). The T1wt/T2c association was also visualized by immunoprecipitations with the anti-TAP1 antibody when the T2c expression level was high. Taken together, these results indicated that regions of T1c that are involved in interacting with T2c and T2ctr are also accessible for interaction in the context of the full-length TAP1 subunit. The ability to visualize the formation of T1c/T2wt as well as T1wt/T2c complexes in assays with at least one of the antibodies used indicates that the interactions we observe between T1c and T2c are also likely to occur in the context of the wild type subunits.

**Self-association Mediated by Truncated TAP Constructs**

Since the NBD of TAP1 and TAP2 share approximately 60% sequence identity, and the question of whether either TAP subunit can homodimerize remains unresolved, we next investigated whether the truncated TAP constructs self-associated with the corresponding wild type subunits. Self-association could only be assessed using the anti-His and anti-Myc antibodies, since the anti-TAP1 and anti-TAP2 antibodies directly recognized both the truncated constructs as well as the wild type constructs.

Anti-His-based immunoprecipitation of lysates from cells co-infected with the indicated viruses were immunoprecipitated with anti-His (lanes 1–3) or anti-Myc antibodies (lanes 4–6) and labeled proteins visualized by SDS-PAGE and phosphorimaging analysis. A, T1ctr/T2c and T1ctr/T2ctr interactions. Infections were with T1c (lanes 2 and 6), T1c+T2c (lanes 3 and 4), or T2c (lanes 1 and 5). B, T1c/T2ctr interaction. Infections were with T1c (lanes 2 and 6), T1c+T2ctr (lanes 3 and 4), or T2ctr (lanes 1 and 5). C, T1ctr/T2ctr interaction. Infections were with T1ctr (lanes 2 and 6), T1ctr+T2ctr (lanes 3 and 4), or T2ctr (lanes 1 and 5).
infected with T1c+T1wt revealed the presence of T1wt in the immunoprecipitations (Fig. 7A, lane 2), a result that was confirmed by immunoblotting analyses with the anti-TAP1 antibody (data not shown). By contrast, in anti-His-based immunoprecipitations of lysates from cells co-infected with T1ctr+T1wt, the T1wt band was not significantly enhanced relative to immunoprecipitations with the irrelevant antibody (Fig. 7A, compare lane 4 with lane 6). Because T1ctr was also shown to be impaired in its associations with T2c, T2ctr, and T2wt, these results suggest the possibility that T2c, T2ctr, wild type TAP2, and wild type TAP1 all bind to the same site on T1c.

Anti-Myc-based immunoprecipitation of cells co-infected with T2c+T2wt or T2ctr+T2wt, both revealed the presence of significant amounts of wild type TAP2 (Fig. 7B, lanes 2 and 4), results confirmed by immunoblotting analyses with anti-TAP2 antibody (data not shown). Thus, our results indicate that T1c associates with wild type TAP1, and that T2c and T2ctr both associate with wild type TAP2.

**DISCUSSION**

NBD-mediated homodimerization or heterodimerization may represent a general mechanistic theme in ABC transporter function. In the *S. typhimurium* histidine permease system, which comprises two membrane-spanning subunits (HisQ and HisM) and two copies of the ATP-binding subunit HisP, the active form of HisP has been suggested to be dimeric (22). The $K_D$ value for HisP dimerization was estimated to be 100 μM, indicating that monomer-monomer interactions are very low affinity (22). The crystal structure of HisP which has recently been solved showed the presence of a dimer (consisting of two monomers related by a crystallographic two-fold axis), which was suggested to correspond to the structure of HisP within the HisQMP2 transport complex (23).

To investigate NBD associations within the TAP complex, we used a baculovirus-based expression system to generate (in insect cells) four different constructs containing TAP1 and TAP2 NBD: T1c, T2c, T1ctr, and T2ctr. Previous studies showed that T1c was functional for ATP binding, whereas T2c did not bind ATP when both constructs were separately expressed in *E. coli*, and purified and refolded from inclusion bodies (25). Here we show that T2c binds ATP when expressed in insect cells, and that T1ctr and T2ctr are also functional for ATP binding. Thus, the location of the truncation site does not appear to interfere with the folding of the core NBD.

T1c, T2c, T1ctr, and T2ctr contain N-terminal extensions of approximately 34, 54, 14, and 20 residues, respectively, relative to the core NBD contained in other ABC transporters. In order to estimate the relative interaction propensities for the binding of each of these constructs to a partner TAP NBD, association levels were quantitated from the immunoprecipitation assays described in Figs. 3 and 5 (Table I).

For each interaction, the protein directly recognized by the antibody was quantitated by phosphorimaging analyses, and the intensity of the associated protein was also quantitated and normalized to take into account any differences in methionine/cysteine contents (see “Experimental Procedures”). The ratio of intensities (association level) was computed from multiple independent experiments, and the observed ranges tabulated (Table I). The association level for each interaction is expected to vary as a function of the expression level of the different proteins, the particular viral clone used, the m.o.i. of infection, as well as other factors such as the extent of degradation in a given cell lysate preparation; these factors account for the broad range of association levels observed for a given interaction, summarized in Table I. Nevertheless, the observation of low, near-zero (<0.1) association levels in multiple independent experiments indicates an impairment in the particular set of interactions relative to other complexes.

For each interaction, complexes were less efficiently isolated with the anti-Myc antibody than with the anti-His antibody (Table I). Results from the anti-Myc-based immunoprecipitations indicated a significant level of complex formation only for the T1c/T2c interaction (Table I). Anti-His-based immunoprecipitations correlated with these results, and additionally indicated that low but significant levels of complex formation also occurred between T1c and T2ctr. However, for the T1ctr and T2c interaction, and for the T1ctr and T2ctr interaction, association levels were in the range of 0–0.04, suggesting, at best, a very low level association. Thus, contacts mediated by the N-terminal residues contained in T1c may stabilize and enhance the affinity of the T1c/T2c complex. It is possible that T1ctr interacts with T2c and T2ctr with a low affinity. Complexes with $K_D$ values weaker than 1 μM would typically not be detectable using the co-precipitation-based assay system, and thus, if binding occurs between T1ctr and T2c, the $K_D$ value must be weaker than 1 μM. Contacts mediated by N-terminal residues contained in T2c may also stabilize and enhance the affinity of the T1c/T2c NBD complex, as the observed association levels in anti-His immunoprecipitations are 0.13–0.21 for the T1c/T2ctr complex compared with 0.40–0.85 for the T2c/
T1c complex (Table 1). Although many factors could influence the observed association levels, these results might also be explained by the greater stability of the T1c/T2c complex compared with the T1c/T2ctr complex. Further experiments will be required to investigate this possibility.

The observation that significant levels of T1c/T2c but not T1ctr/T2ctr complexes are formed indicates that membrane-proximal regions N-terminal to the core NBD of TAP1 and TAP2 are likely sites for TAP1/TAP2 contacts on the cytosolic surface of the ER membrane. Previous studies indicated that the same regions directly participate in peptide binding (13), for which both TAP1 and TAP2 are required. Thus, residues N-terminal to the core NBD might be involved in coupling ATP binding and hydrolysis to peptide binding and translocation. One possible mechanism is that ATP hydrolysis-induced structural changes cause a transient disruption of the peptide binding surface, which results in the release of bound peptides and their subsequent translocation. Such a mechanism is analogous to models that have been proposed for P-glycoprotein-mediated drug transport. Transmembrane segments 6 and 12 (TM6 and TM12) of P-glycoprotein interact with each other, and the interface between these domains forms part of the drug binding pocket (11). There is recent evidence that TM6 and TM12 undergo conformational changes during drug-stimulated ATPase activity (12), which may be responsible for the reported reduction in affinity for P-glycoprotein substrates upon ATP hydrolysis (24).

To investigate the possibility that ATP hydrolysis causes a destabilization of TAP1/TAP2 interaction in membrane-proximal regions of the NBD, we investigated the stabilities of the T1c/T2c, T1c/T2wt and T1wt/T2c complexes in the presence or absence of ATP. Preliminary results indicate that the presence of ATP or non-hydrolyzable ATP analogs does not significantly affect the yield of T2c or T2wt that co-precipitates with T1c, or the yield of T1c or T1wt that co-precipitates with T2c. Several explanations could account for these observations. Possibilities include (i) that, under the defined experimental conditions (for example, Triton extraction), the ATPase activities of truncated and/or wild type TAP have been destroyed or (ii) that ATP hydrolysis-dependent destabilization of a peptide binding surface is a transient phenomenon that our assay system cannot detect. Consistent with previous results (25, 29), we have thus far been unable to demonstrate ATPase activity by any of the truncated TAP constructs that were detergent-extracted and purified. These assays are currently being attempted in the absence of detergent extraction, in order to investigate whether the Triton extraction procedure destroys the ATPase activity. If one or more of observed various complexes can be reconstituted in vitro, the affinities and ATP dependence of the interactions can be more accurately quantitated using physical techniques such as surface plasmon resonance or fluorescence.

For the generation of the different truncated TAP constructs, the lack of precise experimental data on the topology of TAP1 and TAP2 made it difficult to predict the exact boundaries between the final transmembrane domain and the cytosolic regions. Sequence alignments of different mammalian ABC transporter transmembrane domains indicate that the T1c, T1ctr, and T2ctr constructs falls outside of the transmembrane segments, but that T2c includes approximately 10 residues that occur in the final transmembrane segment of wild type TAP2 (15). This might explain why the functional refolding of this construct from E. coli inclusion bodies of the protein was unsuccessful (25), and our observations that low concentrations of detergent (for example 0.1% Triton X-100) are required to maintain the solubility of T2c purified from insect cells. The other constructs (T2ctr, T1c, and T1ctr) are stable in the absence of detergent when purified from insect cells. Membrane insertion of T2c, however, is not a requirement for the observed interactions to occur, as T2ctr mediates all of the interactions observed with T2c.

We have attempted to purify T1c/T2c complexes by sequential 6-His and Myc tag-based affinity chromatography for further physical biochemical characterization of the molecular weight of complexes. Although complexes can be purified, further analysis has been difficult because of low yields of purified proteins from cell lysate preparations, and because of the requirement for the presence of detergent to maintain T2c in solution. To enhance yields of purified complexes for structural characterization, we are currently investigating the possibility of secretion-signal based expression systems for T1c and T2ctr, as well as other truncated TAP2 NBD constructs with N-terminal truncations intermediate in length between T2c and T2ctr.

The unexpected observation of the occurrence of self-association between truncated and wild type subunits might be explained by the high degree of sequence similarity between the NBD of TAP1 and TAP2; alignment of the sequences corresponding to the core NBD (residues 468–748 of human TAP1 and residues 453–686 of human TAP2) reveals 58% identity between TAP1 and TAP2 (15). The sequence identity between TAP1 and TAP2 and the NBD of other closely related mammalian ABC transporters is less; for comparison, MDR1 (P-glycoprotein) N- and C-terminal NBD and TAP1 and TAP2 NBD share 37–46% identity between the different NBD pairs (15).

The observation that T1c/T1wt, T2c/T2wt, and T2ctr/T2wt complexes are formed again raises the question of whether NBD-mediated homodimerization and heterodimerization are competing processes in vivo, i.e. might TAP1/TAP1 association and/or TAP2/TAP2 association compete with TAP1/TAP2 association (1, 2)? The observation that truncation of T1c to T1ctr significantly affected interaction not only with T2c and T2wt, but also with T1wt, suggested that the T1c/T1wt interaction is analogous to the T1c/T2wt and T1c/T2c interactions. If this is indeed true, one implication might be that the formation of TAP1 or TAP2 homodimers (non-functional complexes) does compete with the formation of TAP1/TAP2 (functional) heterodimers. However, the ability to specifically form heterodimeric TAP complexes rather than homodimeric TAP complexes may reside in the membrane-spanning segments, which are translated prior to the NBD, and which are less highly conserved between TAP1 and TAP2 than are the NBD (1). Further experiments will be required to unambiguously define whether TAP subunit homodimerization occurs, and whether homodimerization can compete with heterodimerization. These questions might be best addressed by expressing differentially tagged constructs of wild type TAP1 and TAP2. The alternative possibility that heterodimeric TAP1/TAP2 complexes can further dimerize or oligomerize on membranes also remains to be unambiguously defined, although gel filtration studies have indicated that the TAP complex in detergent solution is dimeric and not a larger oligomer (26).

In summary, our results indicate that the formation of wild type TAP1/TAP2 complexes includes interactions mediated by segments contained in T1c/T2c complexes. This does not preclude additional interactions mediated via the hydrophobic segments of TAP1 and TAP2. Residues N-terminal to the core NBD appear to be important for forming stable T1c/T2c complexes, as significant levels of T1ctr/T2c and T1ctr/T2ctr com-
plexes are not detected by our assay system. Can the core NBD of TAP1 and TAP2 heterodimerize in a manner analogous to that described for the crystallographic homodimers of HisP (23)? Our results are consistent with that possibility, as high protein concentrations required for crystallization can induce the formation of dimers that might not be observed in solution at micromolar and sub-micromolar concentrations. Further work will be required to understand the molecular nature of the T1c/T2c and T1c/T2ctr complexes we observe, the specific structural features that may distinguish TAP1 and TAP2 NBD, and the corresponding functional correlates.

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