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An Annular Lipid Belt Is Essential for Allosteric Coupling and Viral Inhibition of the Antigen Translocation Complex TAP (Transporter Associated with Antigen Processing)*

Sabine Eggensperger†, Olivier Fisette†, David Parcej‡, Lars V. Schäfer§, and Robert Tampe†

Background: The antigen translocation complex TAP plays a crucial role in adaptive immunity.

Results: TAP reconstituted in nanodiscs shows a tight coupling between peptide binding and ATP hydrolysis, which is blocked by herpesviral ICP47.

Conclusion: An annular lipid belt is essential for TAP function and high affinity inhibition by ICP47.

Significance: Nanodiscs are a promising approach to dissect the function, lipid interaction, and modulation of membrane proteins.

The adaptive immune system eliminates virally or malignantly transformed cells when cytotoxic T-lymphocytes recognize antigenic peptides presented by MHC class I molecules (MHC I) on the cell surface. The transporter associated with antigen processing (TAP) is one of the key players in this process. Degradation products of the ubiquitin-proteasome pathway are recognized by TAP and further shuttled into the lumen of the endoplasmic reticulum. TAP is the central junction of a multisubunit machinery, which orchestrates the efficient peptide editing and loading of MHC I molecules. Peptide-MHC I complexes traffic to the cell surface and present their antigenic cargo to CD8+ cytotoxic T-lymphocytes (1–3).

TAP belongs to the family of ATP-binding cassette proteins, which transport a wide range of solutes across cell membranes driven by ATP binding and hydrolysis (4). The heterodimeric complex consists of TAP1 (ABCB2) and TAP2 (ABCB3). Each half-transporter contains a transmembrane domain of six transmembrane helices (TMs), which are involved in peptide binding and translocation (5, 6), followed by a C-terminal nucleotide-binding domain, which coordinates ATP binding and hydrolysis (4). This core-TAP complex is essential and sufficient for peptide binding and translocation (7). In addition, an extra 4-TM bundle at each TAP subunit provides a membrane interaction platform for the MHC I-specific chaperone tapasin and assembly of the peptide-loading complex (7–10). In our working model, peptide and ATP binding occurs independently from each other but together induce an allosteric coupling between transmembrane domain and the nucleotide-binding domain, leading to a dimerization of the two nucleotide-binding domains and a switch of the transmembrane domains from the inward- to the outward-facing conformation, thus moving peptides in the lumen of the endoplasmic reticulum. Finally, ATP hydrolysis resets the transport complex back to the inward-facing, pre-translocation state (11, 12).

TAP translocates most efficiently peptides with 8–12 amino acids in length and displays a specificity by recognizing the three N- and C-terminal residues in the binding pocket of TAP (13–17). Notably, even peptides of 40 amino acids in length and...
peptides with bulky side chains, such as fluorophores, chemical proteases, or polylysine chains, can be transported, however with much lower efficiency (6, 17–19). Various viral proteins, among others such as ICP47 from herpes simplex virus, inhibit TAP. ICP47, which blocks peptide binding to TAP, is the only soluble viral TAP inhibitor known so far (20, 21). Interestingly, the active domain ICP47(3–34), which constitutes two amphipathic α-helices linked by a flexible loop (22–25), inhibits TAP in microsomes with a 50–100-fold higher affinity than TAP solubilized in detergent (24).

A detailed mechanistic understanding of peptide transport and viral inhibition requires the investigation of the TAP complex in an isolated but membrane-embedded state because associated lipids have been found to be essential for TAP function (26). It is further worth mentioning that only a very limited number of detergents maintains the lipid environment crucial for TAP function (26, 27). Here, we report on the tandem affinity purification and first functional reconstitution of the heterodimeric TAP complex in nanodiscs (Nd). These disc-like membrane particles, ranging from 8 to 16 nm in diameter, consist of two membrane scaffold proteins (MSPs) enclosing a lipid bilayer and the membrane protein of interest (28, 29). The design of the MSPs is based on the amphipathic helically structured serum protein apolipoprotein A-1.

Nanodiscs provide several key advantages (29) as follows: (i) small size compared with liposomes (30, 31); (ii) stoichiometry and composition of membrane proteins and lipids can be controlled precisely (28); (iii) substrate, ligand, and protein interactions can be studied in a close-to-native lipid environment with access to both sides of the membrane protein complex (32–36). Here, we demonstrate that TAP reconstituted in nanodiscs displays an allosteric coupling between peptide binding and ATP hydrolysis. The lipid environment, in particular the annular lipid belt, is essential for the high affinity interaction and inhibition of TAP by the viral inhibitor ICP47.

**EXPERIMENTAL PROCEDURES**

**Materials—**Escherichia coli polar lipid was purchased from Avanti Polar Lipids. Digitonin was from Carl Roth or Calbiochem. Chemicals were ordered from Sigma and Carl Roth. 

**Protein Expression and Membrane Preparation—**TAP was expressed in *Pichia pastoris* as described recently (26, 37). Briefly, *P. pastoris* strain SMD1163 was co-transformed with two pPICZC plasmids (Invitrogen) harboring either core-TAP1 (Q03518; residues 227–808) followed by a tobacco etch virus protease cleavage site without a linker region (MGSSHHHHHHENLYFQG-MSP). Purified TAP complexes and MSPs were mixed with either sodium cholate- (100/25 mM lipid) or digitonin (4/25 mM lipid)-solubilized *E. coli* polar lipid at a TAP/MSP/lipid molar ratio of 1:10:300 for MSP1 and 1:10:850 for MSP1E3D1. Empty nanodiscs were formed by MSPs and *E. coli* polar lipid in a molar ratio of 1:35 (MSP1) or 1:90 (MSP1E3D1). If the final detergent concentration was less than 12 mM (cholate) or 0.5 mM (digitonin), the corresponding detergent was added. After incubation at 4 °C for 30 min, 100 mg of SMD-2 Biobeads (Bio-Rad) (dry weight) per mg of protein were added in four portions (1 h, overnight, two times for 2 h). After removal of the Biobeads, TAP complexes reconstituted in nanodiscs were concentrated to 2–5 μM at 1500 × g in Amicon Ultra 0.5-mL centrifugal filters with a 100-kDa cutoff (Millipore).
**Electron Microscopy**—Samples (3 μl) of SEC-purified empty nanodiscs and TAP/Nd were applied to a carbon-coated and glow-discharged EM grid for 1 min and subsequently stained with 3 μl of 1% uranyl acetate for 1 min. EM was performed with a CM120 (Philips) electron microscope equipped with a 2K x 2K CCD camera (Gatan).

**Peptide and ICP47 Binding**—TAP/Dig (4 μg, 310 nM) was incubated with 1 μM C4 peptide (RRYC\(^6\)KSTEL, cysteine-labeled with fluorescein) in the presence and absence of the unlabeled competitor peptide RRYQKSTEL (R9LQK, 400 μM) in binding buffer (PBS, pH 7.4, 0.05% (w/v) digitonin). After incubation at 4 °C for 15 min, the mixture was transferred onto a filter plate (0.65 μm; Hydrophilic Low Protein Binding Durapore\(^{\circledR}\) membrane, Millipore) and washed three times with 250 μl of binding buffer at 4 °C. Bound peptides were solubilized on the filter with PBS, pH 7.4, 1% SDS. After 15 min of incubation and denaturation of the fluorescent fusion proteins at 95 °C for 5 min, the eluted peptides were quantified by fluorescence at λ\(_{ex/em}\) 485/520 nm. Peptides were synthesized by Fmoc (N-(9-fluorenylethoxycarbonyl)) solid-phase chemistry.

Alternatively, peptide and ICP47 binding to TAP solubilized in digitonin and TAP reconstituted in nanodiscs were analyzed by MC-FSEC. TAP/Dig or TAP/Nd\(_d\) were incubated with 1 μM of the fluorescent peptide RRYC\(^{Atto655}\)KSTEL (C4\(^{Atto655}\) for 30 min on ice in either 20 mM Hepes, pH 7.4, 200 mM NaCl, 50 mM KCl, 15% (v/v) glycerol, 0.05% (w/v) digitonin (TAP/Dig), or 10 mM Tris/HC1, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol (TAP/Nd). 1 or 12 μM of full-length ICP47\(^{Helicobacter pylori}\) were incubated for 60 min with TAP/Nd or TAP/Dig, respectively. The samples were analyzed by MC-FSEC (Shodex KW404–4F column) in SEC buffer complemented with 10% glycerol at 0 °C to minimize ligand dissociation. Fluorescence detectors were set to λ\(_{ex/em}\) 435/470 and 563/589 nm, respectively.

**Microscale Thermophoresis**—Microscale thermophoresis (MST) is based on the movement of fluorescent peptides along a temperature gradient induced by an infrared laser (39, 40). The change in fluorescence was monitored using a Monolith NT.115 (pico) MST with red LED Laser (λ\(_{ex/em}\) 598–650/674–693 nm). The fluorescent peptide C4\(^{Atto655}\) (25 nm) was titrated with increasing concentrations of TAP/Dig or TAP/Nd at 22 °C and transferred into standard capillaries. The dilution buffer for TAP/Dig was 20 mM Hepes, pH 7.4, 200 mM NaCl, 50 mM KCl, 15% (v/v) glycerol, 0.05% (w/v) digitonin and for TAP/Nd was 10 mM Tris/HC1, pH 8.0, 150 mM NaCl. The change in fluorescence ΔF was derived from F\(_{hot}/F\(_{cold}\) where F\(_{hot}\) and F\(_{cold}\) are the mean fluorescence at the end of the measurement and directly (0.6 s) after turning on the infrared laser, respectively. To determine the equilibrium dissociation constant (K\(_D\)), the data were fitted to Equation 1, with [A\(_0\)] and [T\(_0\)] representing the known concentration of C4\(^{Atto655}\) and TAP, respectively.

\[
B = \frac{1}{2} \left( ([A_0] + [T_0] + K_D) - \sqrt{(A_0) + [T_0] + K_D}^2 - 4([A_0][T_0]) \right) \quad (\text{Eq. 1})
\]

Hence, B represents the concentration of peptide C4\(^{Atto655}\)-bound TAP complex. In addition, the IC\(_{50}\) value of TAP inhibition by ICP47 was determined. TAP/Dig or TAP/Nd (500 nm each) was incubated with different concentrations of ICP47 (2–34) for 30 min. C4\(^{Atto655}\) was added (25 nm final) and incubated for another 30 min. Thermophoresis was conducted at 22 °C for 30 s. Background binding was determined with a 300-fold excess of unlabeled peptide R9LQK. K\(_D\) values were calculated according to Equation 2, where B is the concentration of bound C4\(^{Atto655}\).

\[
K_D = \left( \frac{B}{IC_{50}} \right) + B - \left( \frac{[A_0] + B - K_D}{[A_0]} \right) \quad (\text{Eq. 2})
\]

**ATP Hydrolysis**—The ATPase activity of TAP was determined by a colorimetric assay based on the complex formation of free inorganic phosphate and ammonium molybdate with malachite green (42). The assay was performed with purified TAP/Dig and TAP/Nd as described (27). TAP (500 nm) was incubated with 3 mM ATP and 5 mM MgCl\(_2\) as well as either the high affinity epitope R9LQK, the nonbinder peptide EPGYTNSTD (E9D, 1 μM each) or the 9-mer RRYC\(^6\)KSTEL, 18-mer RRYQKSTELRRC\(^6\)KSTEL, and 27-mer RRYQKSTELRRYQKSTELRRC\(^6\)KSTEL peptides (10 μM each, cysteine labeled with fluorescein). TAP/Dig samples additionally contain 20 μM MSP1. The peptide-stimulated ATPase activity was inhibited by 10 μM ICP47 (2–34). The assay was conducted in 25 μl for 30 min at 37 °C in corresponding binding buffer supplemented with a final concentration of 1 mM ouabain, 50 μM EGTA, and 5 mM Na\(_2\). The reaction was stopped with 175 μl of 20 mM H\(_2\)SO\(_4\). The release of inorganic phosphate was visualized by adding 50 μl of malachite green solution (3.5 mM malachite green, 0.2% (v/v) Tween 20, 0.8% (w/v) ammonium molybdate in 20% (v/v) H\(_2\)SO\(_4\)) followed by absorbance at 620 nm.

**Peptide Transport**—Peptide transport was analyzed in microsomes isolated from human B-cell lymphoma Raji cells. Rough microsomes were prepared as described (43). 10 μM RRYQNSTC\(^6\)L (9-mer), RRYQKSTELRRYQNSTC\(^6\)L (18-mer), or RRYQKSTELRRYQKSTELRRYQNSTC\(^6\)L (27-mer; cysteine labeled with fluorescein; N-core glycosylation site is underlined) were incubated with 40 μg of microsomes and 5 mM MgCl\(_2\) in PBS, pH 7.0. Peptide transport was started by adding 3 mM ATP and was performed for 2 min at 37 °C. The transport reaction was stopped with 10 mM EDTA in PBS, pH 8.0, supplemented with 500 μM R9LQK to block any fluorescent peptide bound to TAP. Microsomes were pelleted (20,000 × g, 8 min) and washed with PBS, pH 8.0, 10 mM EDTA. Pellets were lysed in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM MnCl\(_2\), 1% Nonidet P-40 for 15 min at room temperature. Debris was pelleted and the supernatant was bound to concanavalin A beads for 1 h at 4 °C. Bound peptides were eluted with lysis buffer, 200 mM methyl-α-D-mannopyranoside for 30 min at room temperature. Fluorescence was detected at λ\(_{ex/em}\) 485/520 nm.

**Phospholipid Quantification in Nanodiscs**—SEC-purified nanodisc samples were heated above 200 °C in 450 μl of 8.9 nM H\(_2\)SO\(_4\) for 25 min. Brown color, resulting from proteins, was bleached by 150 μl of 30% H\(_2\)O\(_2\) heated above 200 °C for 30 min. Total phosphorus was visualized by adding 500 μl of 2.5% (w/v)
ammonium molybdate and 500 μl 10% (w/v) ascorbic acid in 3.9 ml of deionized water followed by absorbance at 820 nm.

Computational Techniques—Molecular dynamics (MD) simulations were run with GROMACS 4.6.5 (44), using the Amber99SB-ILDN protein (45, 46) and Stockholm lipid (47, 48) force fields, and TIP3P water (49). Periodic boundary conditions were used and long range Coulomb interactions were treated with a smooth particle mesh Ewald method (50). Temperature and pressure were kept constant at 300 K and 1 bar, respectively. To set up the simulation systems, coordinates of MSP1 and core-TAP were taken from a cryo-EM structure (51) and homology model (52), respectively. Starting from a 96-lipid POPE/POPG (3:1) NdS with embedded core-TAP, we removed 74 lipids in seven consecutive steps (8–12 lipids per step) to finally arrive at a 22-lipid TAP/NdS. Each of these systems was simulated for 50 ns prior to the next lipid removal step, for a total simulation time of 400 ns. Total system size was about 192,000 atoms. The chosen lipid mixture mimics the composition of the E. coli lipid used for TAP reconstitution. In addition, we repeated all simulations with a POPC/POPG (3:1) mixture. For reference, we also carried out a 50-ns MD simulation of core-TAP in a POPC/POPG (3:1) bilayer.

RESULTS

Tandem Affinity Purification of the TAP Complex—We first used an orthogonal purification procedure to prepare stoichiometrically well defined transport complexes. TAP was first purified by metal affinity chromatography via the His10 tag of TAP1 and subsequently via a Strep-Tactin resin. Monodispersity and stoichiometry of TAP1mVenus (yellow-orange) and TAP2mCerulean (blue) was analyzed by MC-FSEC (A280 black line). The fluorescence intensities of mVenus and mCerulean are calibrated to equal concentrations (37). The apparent molecular mass of TAP/Dig (red arrow) was estimated by the elution (black arrows) of dextran blue (2 MDa), bovine thyroglobulin (669 kDa), and β-amylase (200 kDa). B, purified TAP complexes were analyzed by SDS-PAGE (9%), Coomassie, in-gel fluorescence, and immunoblotting using TAP1- and TAP2-specific antibodies. C, activity of purified TAP was determined by a peptide binding filter assay. 4 μg of TAP (310 nM) was incubated with 1 μM C4F peptide. Binding was competed with a 400-fold molar excess of unlabeled peptide R9LQK. a.u., arbitrary unit; mAU, milli-absorption unit.

FIGURE 1. Tandem affinity purification of the TAP complex. A, 500 mg of crude membranes were solubilized at 10 mg/ml in 2% (w/v) digitonin. TAP was purified by immobilized metal affinity chromatography and subsequently via a Strep-Tactin resin. Monodispersity and stoichiometry of TAP1mVenus (yellow-orange) and TAP2mCerulean (blue) was analyzed by MC-FSEC (A280 black line). The fluorescence intensities of mVenus and mCerulean are calibrated to equal concentrations (37). The apparent molecular mass of TAP/Dig (red arrow) was estimated by the elution (black arrows) of dextran blue (2 MDa), bovine thyroglobulin (669 kDa), and β-amylase (200 kDa). B, purified TAP complexes were analyzed by SDS-PAGE (9%), Coomassie, in-gel fluorescence, and immunoblotting using TAP1- and TAP2-specific antibodies. C, activity of purified TAP was determined by a peptide binding filter assay. 4 μg of TAP (310 nM) was incubated with 1 μM C4F peptide. Binding was competed with a 400-fold molar excess of unlabeled peptide R9LQK. a.u., arbitrary unit; mAU, milli-absorption unit.
tively. Nanodiscs were formed by mixing TAP with cholate or digitonin-solubilized E. coli polar lipid and MSPs, followed by detergent removal with polystyrene beads, schematically illustrated in Fig. 2A. Based on systematic screening, a molar TAP/MSP/lipid ratio of 1:10:300 for TAP/NdS and 1:10:850 for TAP/NdL was found to be optimal for reconstitution. After self-assembly, the lipid particles were analyzed by MC-FSEC. TAP1 and TAP2 in nanodiscs were detected via mVenus and mCerulean, respectively (Fig. 2, B and C). The major elution peak corresponds to heterodimeric TAP1-TAP2 complexes in nanodiscs as confirmed by SDS-PAGE (Fig. 2D). As expected, the apparent molecular mass slightly shifts with the size of nanodiscs (320 and 340 kDa for TAP/NdS and TAP/NdL, respectively). Negative stain electron microscopy confirms the assembly of disc-like nanoparticles. EM images illustrate that SEC-purified TAP/NdL complexes are reasonably homogeneous particles with the expected diameter of ~12 nm (Fig. 2E). In contrast, TAP/NdS particles appeared in a larger disc size than expected (data not shown). Similar results were reported for ATP-binding cassette importers, including MalFGK2 (53) or OpuA (54) incorporated in nanodiscs.

**TAP in Nanodiscs Is Active in Peptide Binding**—After successful reconstitution, we analyzed the peptide binding activity of TAP/NdS by MC-FSEC (Fig. 3). Binding of the fluorescent high affinity peptide C4Atto565 was detected by the overlapping TAP signal detected via mCerulean, whereas free peptides elute at larger volumes (Fig. 3A). Under saturating conditions, peptide binding to TAP in nanodiscs was similar to TAP solubilized in digitonin (Fig. 3C). We next determined the peptide binding affinity to TAP reconstituted in nanodiscs. Because TAP/Nd could not be separated from free peptides by filter or centrifugation assays, we had to establish a new homogeneous interaction assay. MST reports on a direct ligand-protein interaction by minute changes in the diffusion of particles in a microscopic temperature gradient (39, 40). Accordingly, the change in fluorescence (ΔF) of C4Atto655 reflects the concentration of peptide-TAP complexes (decrease in fluorescence signal, Fig. 4A). The equilibrium dissociation constants K_D determined by MST are in the same range, with 54 ± 12 nM for TAP/Dig, 91 ± 15 nM for TAP/NdS, and 120 ± 16 nM for TAP/NdL (Fig. 4, B–D). Competition with an excess of unlabeled peptides demonstrates that the binding is peptide-specific (see Fig. 6B).

**Allosteric Coupling between Peptide Binding and ATP Hydrolysis**—We investigated the ATPase activity of TAP reflecting peptide translocation because it is not possible to follow the transport process directly in nanodiscs. ATP hydrolysis by TAP was determined in the presence and absence of peptides. Notably, the high affinity peptide R9LQK stimulated ATP hydrolysis of TAP reconstituted in NdS and NdL (Fig. 5A). In the absence of peptides or in the presence of the nonbinder peptide E9D (16), TAP/Nd and TAP/Dig show only background levels of the ATPase activity. This behavior was similar to TAP solubilized in digitonin. The ATPase activity of TAP was not affected by the size of the nanodiscs,
indicating that the amount of lipids does not strongly influence the peptide-stimulated ATPase activity.

We next examined the ATP turnover of TAP/NdL stimulated by peptides of different lengths (9, 18, and 27 amino acids) (Fig. 3B). These peptides are concatemers of the peptide epitope R9LQK, preserving all high affinity binding motifs (16). By competition MST (increase in fluorescence signal), their binding affinity was determined to be similar (IC₅₀ of 0.9 μM for

![Figure 3. Peptide binding to TAP in nanodiscs analyzed by MC-FSEC.](image)

![Figure 4. Binding affinities of TAP analyzed by MST.](image)
Peptide-stimulated ATP hydrolysis was measured at peptide concentrations of 10 μM for the high affinity peptide R9LQK or nonbinding peptide E9D. Background ATPase activity is given in the absence of peptide. ATP hydrolysis was measured in triplicate. Error bars, S.D. B, TAP/NdS, TAP/NdL, or empty nanodiscs were incubated with 3 mM MgATP and 1 μM of the high affinity peptide R9LQK or nonbinding peptide E9D. Background ATPase activity is given in the absence of peptide. ATP hydrolysis was measured in triplicate. Error bars, S.D. C, peptide transport of the 9-mer, 18- and 27-mer. TAP-containing microsomes were incubated with 10 μM of each peptide, 5 mM MgCl2, and 3 mM ATP. Peptide transport was followed for 2 min at 37 °C. Error bars, S.D. D, peptide binding (9-, 18-, and 27-mer) to TAP/NdS was measured by MST. Increasing concentrations of peptide were incubated with 200 nM TAP/NdS and 25 nM of C4Atto655. The IC50 values are 0.9 μM for 18-mer, and 0.7 μM with a c.i. of 0.8 –1 μM for 27-mer. All binding data were measured in triplicate. Error bars, S.D.

FIGURE 5. Allosteric coupling between peptide binding and ATP hydrolysis. A, TAP/Dig, TAP/NdS, TAP/NdL, or empty nanodiscs were incubated with 3 mM MgATP and 1 μM of the high affinity peptide R9LQK or nonbinding peptide E9D. Background ATPase activity is given in the absence of peptide. ATP hydrolysis was measured in triplicate. Error bars, S.D. B, TAP/NdS, TAP/NdL, or empty nanodiscs were incubated with 3 mM MgATP and 1 μM of the high affinity peptide R9LQK or nonbinding peptide E9D. Background ATPase activity is given in the absence of peptide. ATP hydrolysis was measured in triplicate. Error bars, S.D. C, peptide transport of the 9-mer, 18- and 27-mer. TAP-containing microsomes were incubated with 10 μM of each peptide, 5 mM MgCl2, and 3 mM ATP. Peptide transport was followed for 2 min at 37 °C. Error bars, S.D. D, peptide binding (9-, 18-, and 27-mer) to TAP/NdS was measured by MST. Increasing concentrations of peptide were incubated with 200 nM TAP/NdS and 25 nM of C4Atto655. The IC50 values are 0.9 μM for 18-mer, and 0.7 μM with a c.i. of 0.8 –1 μM for 27-mer. All binding data were measured in triplicate. Error bars, S.D.

A Lipid Interface Is Essential for High Affinity TAP Inhibition—We also examined the effect of the viral TAP inhibitor ICP47 (active domain, amino acids 3–34) (22) on the allos-teric coupling between peptide binding and ATP hydrolysis. ICP47 encoded by HSV-1 blocks the peptide-stimulated ATP hydrolysis by competing with R9LQK for the binding pocket (Fig. 6A). ICP47 alone does not induce ATP hydrolysis of either TAP/Dig or TAP/Nd. Upon addition of ICP47 (10 μM), the peptide-induced ATP hydrolysis of TAP/NdS and TAP/NdL was blocked to background levels, although TAP/Dig was only partially (33%) inhibited. These results point to the important role of the lipid interface, without an influence of the membrane scaffold protein itself, in the mechanism of how ICP47 blocks peptide binding to TAP. For this reason, the inhibition of peptide binding was analyzed by competition microscale thermophoresis (increase in fluorescence signal) with rising concentra-tions of ICP47 (Fig. 6, B and C). 100% competition was recorded at a 300-fold excess of unlabeled peptide (Fig. 6, B and C, dashed line, open rectangle). Remarkably, ICP47 has a drastically higher potency to inhibit TAP in nanodiscs (IC50 1.8 ± 0.3 μM for TAP/NdS and 1.1 ± 0.2 μM for TAP/NdL) (Fig. 6, C and D) as compared with detergent-solubilized TAP (IC50 51 ± 14 μM for TAP/Dig). These results show that the lipids surrounding the TAP complex are essential for high affinity ICP47-TAP interaction. We further wondered whether the different IC50 values are explained by an accumulation at the membrane or a correct folding of ICP47 triggered by the lipid environment in nanodiscs. Therefore, we determined the stoichiometry of ICP47Atto565 binding to TAP/Dig and TAP/Nd by MC-FSEC (37) under saturating conditions (10-fold above the Kd value). As shown in Fig. 6D, ICP47 binds to TAP solubilized in digitonin or TAP reconstituted in nanodiscs in a 1:1 stoichiometry. These findings lead us to the assumption that the membrane environment in nanodiscs facilitates correct folding of ICP47 and high affinity interaction with TAP.

An Annular Lipid Belt Remains Around TAP in Small Nanodiscs—With respect to the remarkable difference in the inhibition pattern of ICP47 to TAP, the amount of phospholipids per nanodisc after reconstitution of TAP was determined. We defined that 22 ± 2 and 113 ± 3 lipids remain associated with
TAP/NdS and TAP/NdL, respectively. In empty MSP1 nanodiscs, depending on the lipid species, 110 (POPC) to 160 (DPPC) phospholipids have been found (28). Based on the cross-sectional area of the transmembrane domain of the homology model (52), core-TAP can replace 60 phospholipids. Although the E. coli polar lipids used in this study clearly represent a more complex lipid mixture as compared with the pure phospholipid nanodiscs, the observation that the transporter replaces more than 100 lipids in NdS thus cannot be understood solely by simple geometric considerations. Instead, other mechanisms are at play, such as possible changes in lipid affinity due to the presence of TAP. In conclusion, 22 lipids in TAP/NdS seem to be sufficient for high affinity TAP inhibition by ICP47.

The surprisingly small number of lipids found in the NdS complex (22 ± 2) raises questions about its structure at the atomic level and, in particular, whether as little as 22 lipids are sufficient to keep up a lipid belt around TAP. To address these questions, we used all-atom MD simulations in explicit solvent. The final structure of TAP/NdS obtained from our simulations is shown in Fig. 7A. The POPE and POPG lipids indeed form a belt that entirely encircles the TM region of TAP1/2, isolating the transporter from MSP1. Only a small number of residues on the TAP complex and MSP1 are in direct contact (Fig. 7B). The scaffold proteins accommodate the small nanodisc size by partial unfolding, yet retain a largely helical conformation (average helicity is 50%, compared with 72% in the starting cryo-EM structure). In our reference simulation of TAP in a conventional lamellar bilayer, 50 lipids are in direct contact with TAP. The striking difference with the 22 lipids strongly suggests that structural changes of the lipids in TAP/NdS are required to maintain a complete belt around TAP. We observed tilting of the lipids with respect to the TM helices and displacement of some phosphate headgroups toward the center of the membrane (Fig. 7C), allowing the lipids to cover more surface of TAP in the plane of the Nd. The classical bilayer structure is lost (for NdS 50–60 lipids), and the resulting lipid belt is substantially thinner. Our simulations of the POPC/POPG NdS yielded similar results (Fig. 7C), including the formation of a lipid belt around TAP.

**DISCUSSION**

Functional and structural analyses of membrane proteins are often difficult, especially in the case of multisubunit complexes, where the correct assembly, stoichiometry, and stability must be carefully controlled. In addition, the local lipid environment is emerging as one of the most important of the many factors that govern their stability and function. As TAP1 and TAP2 can form inactive homodimers, we used a tandem affinity purification strategy via a His10 tag at TAP1 and a StrepII-tag at TAP2. C-terminal tagging with mVenus (TAP1) and mCerulean (TAP2) facilitated sensitive quantification of TAP1/2 complexes. Based on the calibrated fluorescence, a monodisperse heterodimeric TAP complex was isolated. TAP function was preserved, resulting in stoichiometric peptide binding. The purified TAP complex was reconstituted in small (9.8 nm) and...
large nanodiscs (12 nm) formed by MSP1 and MSP1E3D1, respectively. Typically, membrane proteins are reconstituted in liposomes to study their function in a physiologically relevant membrane environment. Proteoliposomes are at least 10-fold larger than nanodiscs and display a mixed protein orientation (26). In addition, in nanodiscs the number of lipids and protein subunits is well defined. The limited number of lipids in nanodiscs mimics molecular crowding as recently observed by solid-state NMR, which shows a reduced dynamic of proteorhodopsin in nanodiscs compared with lamellar preparations (31). Therefore, nanodiscs display a more native-like environment compared with liposomes. We demonstrated by MC-FSEC that the apparent molecular mass of 320–340 kDa corresponds to one heterodimeric TAP complex per nanodisc (TAP 200 kDa, MSPs 50–60 kDa and lipids 25–100 kDa). TAP in nanodiscs is active in peptide binding and allosteric coupling between substrate binding and ATP hydrolysis.

In this study, we have established several approaches to investigate peptide-TAP, protein-TAP, and lipid-TAP interaction using nanodiscs, microscale thermophoresis, MC-FSEC, and MD simulations. The affinity and maximal binding of antigenic peptides to TAP/Nd is comparable with TAP in digitonin. Hence, the reconstitution procedure maintains the function of peptide binding. Furthermore, the high affinity peptide R9LQK stimulated ATP hydrolysis of TAP in nanodiscs, whereas the nonbinder peptide E9D (16) did not induce ATP hydrolysis above background. These data demonstrate the allosteric coupling between peptide binding and ATP hydrolysis of TAP reconstituted in nanodiscs.

It is known that TAP can translocate peptides of different length, however, with much lower efficiency (15, 19). Here, we show that a 9-, 18-, and 27-mer peptide, bound to the TAP complex with similar affinity, displayed a gradually decreased transport and ATPase activity. At saturating concentrations (5–10 times above \( K_d \) values), the 9-mer peptide led to a turnover of 4 ATP/min, whereas the 18- and 27-mer peptides reduced the turnover to 1 ATP/min and 0.5 ATP/min, respectively. These results were confirmed by a reduced peptide transport (61 and 23%) of the 18-mer and 27-mer, respectively, in comparison with the 9-mer peptide. Assuming that the 9- to 27-mer peptides have similar association rates based on the measured coincident \( K_d \) values, these results show that the movement of longer peptides through the TAP complex becomes rate-limiting.
In addition, we examined whether lipids in nanodiscs are sufficient to promote high affinity protein interaction with the viral TAP inhibitor ICP47. Based on previous findings that ICP47 inhibits TAP in microsomes with a 50–100-fold higher affinity than TAP solubilized in detergent, we speculate that the lipid interface promotes ICP47 binding to TAP via a correct folding of ICP47 by interacting with the lipid-TAP interface (24). Here, we observed a full inhibition of peptide-induced ATP hydrolysis for TAP in nanodiscs, although the peptide-stimulated ATPase of TAP/Dig was only partially blocked by ICP47 (10 μM). These observations were confirmed by competition binding assays, revealing inhibition constants $K_i$ of 0.28 and 0.21 μM of TAP/Nd$_S$ and TAP/Nd$_L$, respectively, which are significantly higher than for TAP in digitonin (5.0 μM). ICP47 binds in a 1:1 stoichiometry to TAP reconstituted in nanodiscs. Thus, the lipid interface in nanodiscs does not lead to an accumulation of the viral factor but may promote the correct folding of ICP47 for the high affinity inhibition of TAP. We therefore determined the number of phospholipids in small and large nanodiscs containing reconstituted TAP. Our MD simulations showed that, by tilting and displacement of their phosphate groups toward the center of the membrane, the 22 lipids in TAP/Nd$_S$ still allow for the formation of an annular lipid belt around the core-TAP complex. This finding is consistent with our experimental observation that the ICP47-TAP interaction is not significantly affected by higher amounts of lipids in TAP/Nd$_L$. Thus, the annular lipid belt surrounding the TAP complex in small nanodiscs is essential and sufficient to promote correct folding of ICP47 for high affinity TAP inhibition.

The allosteric coupling between peptide binding/translocation and ATP hydrolysis is not significantly altered between TAP reconstituted in nanodiscs or TAP in digitonin. We used digitonin for solubilization, which is known to optimally preserve TAP activity (26, 27), forming large lipid-detergent micelles of 75–100 kDa (55, 56). Although these associated lipids are sufficient to preserve TAP function, a lipid interface of 22 annular lipids is essential for high affinity ICP47-TAP interaction.

In conclusion, one stoichiometrically defined heterodimeric TAP complex was reconstituted in nanodiscs. TAP/Nd is active in high-affinity binding of peptides. Furthermore, a peptide-stimulated ATP hydrolysis was observed, reflecting the allosteric coupling between peptide binding and ATP hydrolysis. Steric constraints, such as longer peptides and bulky side chains, which hinder peptide translocation but not binding (18), decelerated the translocation and ATP hydrolysis cycle of TAP. Thus, the peptide-stimulated ATPase activity indirectly reports on the translocation process of TAP in nanodiscs. The peptide-stimulated ATP hydrolysis was blocked by the herpesvirus immune evasion ICP47 by a 1:1 interaction. This allosteric inhibition is significantly decreased in detergent micelles. Thus, nanodiscs provide this essential lipid interface for high affinity ICP47-TAP interaction. The small size, the lipid environment, and the accessibility to both sides of the membrane protein complex make nanodiscs a powerful approach to study the dynamics and interaction mechanism of TAP by biophysical techniques, including single molecule studies.

**REFERENCES**


TAP Function and Viral Inhibition in Nanodiscs


