Heres Simplex Virus Inhibitor ICP47 Destabilizes the Transporter Associated with Antigen Processing (TAP) Heterodimer*

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Chemical cross-linking of the transporter associated with antigen processing (TAP) heterodimer was used to determine whether the herpes simplex virus inhibitor of TAP, ICP47, induces a conformational change in TAP. Cross-linking of TAP in cellular membranes produced a major species of ~220 kDa which was comprised solely of TAP.1 and TAP.2 and most likely represents the TAP heterodimer. Interestingly, prior treatment of TAP-containing membranes with TAP peptide substrates stimulated the formation of the cross-linked TAP heterodimer, whereas pretreatment of membranes with ICP47 completely blocked the formation of the cross-linked heterodimer. These data suggest that suitable substrates for TAP stabilize the TAP heterodimer, whereas ICP47 destabilizes the heterodimer. The results indicate that subtle conformational changes occur in the TAP heterodimer upon the binding of peptides and the inhibitor ICP47 and that ICP47 has a deleterious effect on TAP heterodimer structure, in addition to its role as a potent blocker of substrate binding to TAP.

The transporter associated with antigen processing (TAP) plays a critical role in the MHC class I antigen processing pathway by transporting antigenic peptides from the cytosol into the endoplasmic reticulum for assembly with MHC class I heavy chain and β<sub>2m</sub>. Once assembled, the stable complex of class I heavy chain, β<sub>2m</sub>, and peptide, is released from the endoplasmic reticulum and expressed on the cell surface for recognition by CD8<sup>+</sup> T cells. Research over the past few years has greatly expanded our knowledge of the early events involved with the peptide loading of class I molecules. Central to this knowledge was the discovery of the transporter associated with antigen processing genes TAP.1 and TAP.2 (1–4). TAP is a member of the ATP-binding cassette family of transporters (for review, see Ref. 5), which includes P-glycoprotein (6), the cystic fibrosis transmembrane conductance regulator (7), and the sterile 6 transporter in yeast (8). Previous evidence has shown that both TAP.1 and TAP.2 must be expressed to produce a functional transporter (9–11), and therefore the minimal functional unit of TAP is thought to be a heterodimer of TAP.1 and TAP.2. Indeed, stoichiometric and size analysis of the TAP complex suggests that TAP exists as a heterodimer (12, 13). Recent evidence also indicates that TAP physically associates with other molecules that are involved in the loading of MHC class I molecules. These include the class I molecules themselves (14, 15), the newly identified 48-kDa glycoprotein tapasin (12, 13, 16), and the molecular chaperones calnexin and calreticulin (12, 16). The precise roles that these accessory molecules play in TAP-dependent peptide transport and MHC class I loading remain to be seen; however, it is thought that their physical association with TAP helps to maximize peptide transport and loading.

The herpes simplex virus immediate early gene ICP47 encodes a small polypeptide (88 amino acids) which can dramatically inhibit peptide transport by TAP in humans (17, 18). ICP47 binds tightly to the peptide binding site of TAP and prevents the binding of other peptides that are suitable substrates for TAP (19, 20). ICP47 effectively shuts down the TAP-dependent flow of peptides into the endoplasmic reticulum and severely impairs the assembly of MHC class I molecules. As a result, the presentation and recognition of herpes simplex antigens by CD8<sup>+</sup> T cells are restricted, enabling the virus to persist longer within the infected cell. The functional domains of ICP47 have been delineated recently (21, 22). Interestingly, the minimal functional region of the ICP47 molecule is comprised of residues 3–34 (22). This means that more than half the molecule is not required for the inhibition of TAP. The current model for the action of ICP47 proposes that residues 3–13 interact with the endoplasmic reticulum membrane through a membrane-induced α-helix, whereas residues 14–34 interact with the peptide binding site of TAP. Although it is clear that ICP47 competes efficiently for peptide binding to TAP, it is not clear what other effects ICP47 may have on TAP which facilitates its inhibition of TAP function.

Here, we use chemical cross-linking of the TAP heterodimer to detect subtle conformational changes between the two subunits. We found that although peptides that are good substrates for TAP stabilize the formation of the TAP heterodimer as shown by cross-linking, ICP47 significantly destabilizes the TAP heterodimer so that no cross-links were detected. The results indicate that ICP47 induces a loose association of TAP.1 and TAP.2 in the heterodimer which most likely further contributes to the inhibition of TAP function by ICP47.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The B lymphoblastoid cell line Raji (23) was grown in RPMI 1640 (Life Technologies, Inc.) medium supplemented with 10% bovine calf serum, 2 mM glutamine, 1 mM sodium pyruvate, and 100 units/ml each of penicillin and streptomycin. The TAP-deficient .174 (24) and the tapasin-deficient 220 (25) cell lines were grown in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum, glutamine, penicillin, and streptomycin as above. The β<sub>2m</sub>-deficient Daudi (26) and calnexin-deficient CEM-NKR (27) cell lines were grown in Iscove’s modified Dulbecco’s
medium supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin as indicated. Serum-free-adapted *Spodoptera frugiperda* (Sf9) cells (Life Technologies, Inc.) were grown in SF-900 II SFM medium (Life Technologies, Inc.). Sf9 cells were grown to 70% confluence in T175 flasks and were infected with TAP1/TAP2 recombinant baculovirus (generously provided by Robert Tampe, see Ref. 10) at a multiplicity of infection of 0.1. Infected Sf9 cells were harvested after growth for 3 days at 27 °C.

**Preparation of Crude Membranes**—Cell pellets were stored at −80 °C (1 × 10⁸ cells each). Each pellet was thawed and resuspended in 2 ml of cold Tris-buffered saline (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.02% NaN₃) containing 0.5 mM phenylmethylsulfonyl fluoride. The suspension was spun at 2,000 × g for 5 min at 4 °C. The supernatant was saved, and the pellet was resuspended in 1 ml of cold buffer (10 mM Tris-Cl, pH 7.4) and spun again at 2,000 × g for 5 min at 4 °C. The two supernatants were pooled and spun at 100,000 × g for 45 min at 4 °C to pellet the membranes. The membrane pellet was resuspended in 1 ml of cold intracellular transport buffer (50 mM Hepes, pH 7.0, 78 mM KCl, 4 mM MgCl₂, 8.4 mM CaCl₂, 10 mM EGTA, 1 mM dithiothreitol, and 4 mg/ml bovine serum albumin, see Ref. 28), snap frozen in liquid N₂, and stored at −80 °C.

**Preparation of Sf9 Microsomes**—The preparation of microsomes from Sf9 cells has been described previously (10, 11). Briefly, TAP-expressing Sf9 cells (1 × 10⁸) were resuspended in 1 ml of cavitation buffer (50 mM Tris-Cl, pH 7.4, 0.4 M sucrose, 25 mM KOAc, 5 mM MgOAc, 0.5 mM CaOAc) with protease inhibitor mix (0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 0.7 μg/ml pepstatin) and lysed by repeated drawing through a 26-gauge needle. The lysed cell suspension was spun at 1,000 × g for 5 min at 4 °C. The supernatant was diluted 6.4-fold with 2.5 mM sucrose in gradient buffer (50 mM Tris-Cl, pH 7.4, 150 mM KOAc, 5 mM MgOAc), overlaid with 1.9 mM sucrose and subsequently 1.3 mM sucrose (both in gradient buffer). The step gradient was spun at 100,000 × g overnight at 4 °C. The microsomes were collected at the 1.8 M/1.3 M sucrose interface, diluted 2-fold with phosphate-buffered saline containing 1 mM dithiothreitol, and spun at 200,000 × g for 2 h at 4 °C. The microsome pellet was resuspended in 1 ml of cold intracellular transport buffer, snap frozen in liquid N₂, and stored at −80 °C.

**Peptides and Peptide Iodination**—The peptide KB11 (AKVPRPM-KBB) was synthesized by the Keck Foundation Biotechnology Resource Laboratory at Yale University and was kindly provided by Dr. Peter Cresswell. The peptide, which possesses a biotinylated lysine residue at position 11, was conjugated to the UV-inducible cross-linker, N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) and iodinated to specific activity of 15–25 cpm/fmol as described previously (29). ICP47 peptide fragment 1–42, 1–28, and 3–34 were kindly provided by Dr. Robert Tampe (Max Planck Institute, Martinsried).

**Photolabeling and Chemical Cross-linking of TAP in Membranes**—Aliquots (100 μl) of cell membrane or Sf9 microsomes (from 1 × 10⁷ cells each, 0.4 mg total protein) were placed in the wells of a 96-well plate and incubated on ice for 30 min, in the dark, in the presence of ¹²⁵I-KB11-HSAB (4 μM). The samples were then exposed to UV light (254 nm, Spectroline model XX-15F; Spectronics Corp., Westbury, NY) for 6 min. For chemical cross-linking experiments, the homobifunctional cross-linker EGS (ethylene glycol bis(succinimidyld succinate); Pierce Chemical Co.) was added at this point (0.5, 1, 2, or 4 μM final concentration), and the samples were transferred to 1.5-ml microcentrifuge tubes and rotated for 30 min at 4 °C. The cross-linked membranes were washed twice with 1 ml of protein G-Sepharose (50% suspension; Amersham Pharmacia Biotech) and lysed by repeated cavitation using 1 ml of 100 mM Tris-Cl, pH 7.4, 0.4 M sucrose, 25 mM KOAc, 1 mM EDTA, 0.5 mM MgOAc, 5 mM NaOAc, and 1 mg/ml bovine serum albumin. The photolabeled membranes were solubilized in SDS sample buffer, and the labeled proteins were analyzed by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Integrated Separation Systems/Owl Scientific, Chicago, IL), blotted with either anti-TAP.1 (148.3) (10) or anti-TAP.2 (435.3) antibodies, and detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

**RESULTS**

**Chemical Cross-linking of the TAP Heterodimer**—In an effort to cross-link the TAP heterodimer physically, cellular membranes containing TAP were photolabeled using the photoreactive peptide ¹²⁵I-KB11-HSAB (30) and then subjected to chemical cross-linking with EGS as described under “Experimental Procedures.” Panel A, the cross-linked membranes were solubilized in SDS sample buffer and analyzed directly by SDS-PAGE (5–15% gradient gel) and PhosphorImager. Panel B, the cross-linked membranes were first solubilized in Tris buffer, pH 7.4, containing 1% Triton X-100; the solubilized TAP molecules were immunoprecipitated with the anti-TAP2 monoclonal antibody 435.3 and then analyzed by SDS-PAGE (5–15% gradient gel) and PhosphorImager.

![Fig. 1. Chemical cross-linking of photolabeled TAP yields an adduct of 220 kDa.](image-url)

**FIG. 1.** Chemical cross-linking of photolabeled TAP yields an adduct of 220 kDa. Raji membranes (0.4 mg total protein) were photolabeled with ¹²⁵I-KB11-HSAB, washed, and then subjected to chemical cross-linking with EGS as described under “Experimental Procedures.” Panel A, the cross-linked membranes were solubilized in SDS sample buffer and analyzed directly by SDS-PAGE (5–15% gradient gel) and PhosphorImager. Panel B, the cross-linked membranes were first solubilized in Tris buffer, pH 7.4, containing 1% Triton X-100; the solubilized TAP molecules were immunoprecipitated with the anti-TAP2 monoclonal antibody 435.3 and then analyzed by SDS-PAGE (5–15% gradient gel) and PhosphorImager.
belonging TAP with $^{125}$I-KB11-HSAB. The results showed that in the presence of EGS a distinct cross-linked species of $\sim 220$ kDa was detected (p220). The p220 adduct was detected best at the lower concentrations of EGS, i.e. 0.5 or 1.0 mM (lanes 2 and 3). Cross-linking at higher concentrations of EGS resulted in a loss of labeled material which can be detected by SDS-PAGE. It is not clear why this loss occurs. Interestingly, the cross-linked TAP heterodimer runs higher than expected on SDS-PAGE (220 kDa). That the heterodimer runs aberrantly may not be completely unexpected, as chemical cross-linking can induce dramatic physical changes in proteins and alter their SDS binding capacities. Furthermore, the lack of any intermediate cross-linked species argues against the notion that p220 is actually a TAP heterotrimer, heterotetramer, or a complex of other TAP-associated proteins.

The p220 Cross-linked Species Is Derived from TAP Subunits Only—To rule out further the possibility that p220 was the combination of a TAP heterodimer and an associated protein, we performed cross-linking experiments on membrane preparations from other cell lines that lacked particular TAP-associated molecules, i.e. MHC class I heavy chain, tapasin, and calnexin. The analysis was performed on the cell lines .220, tapasin-negative (25); Daudi, $\beta_2$m-deficient (26); (class I heavy chains do not associate with TAP in $\beta_2$m-deficient cells; see Ref. 14); CEM-NKR, calnexin-deficient (27); and TAP.1/TAP.2 expressing SI9 cells (10). The results, shown in Fig. 2, revealed that p220 is derived from TAP subunits only. For all of the cell lines tested, a p220 band was detected at varying degrees. From this we can infer that p220 is not comprised of a TAP heterodimer and tapasin, class I heavy chain, or calnexin. This is supported further by the finding that p220 was also detected in the SI9 insect cells that express TAP.1 and TAP.2. It is known that MHC class I molecules are not expressed in insect cells, and it is highly unlikely that tapasin is expressed in insect cells; therefore, the existence of p220 most likely reflects a complex that is derived from TAP subunits only. The p220 band was weak in the .220 cells (see lane 4) because of the lower level of TAP expression in .220 cells due to their lack of tapasin (31).

The Formation of the Cross-linked TAP Heterodimer Is Not Dependent on Prior Photolabeling with $^{125}$I-KB11-HSAB—To rule out the possibility that the formation of the TAP heterodimer was indirectly due to photolabeling TAP with $^{125}$I-KB11-HSAB prior to cross-linking with EGS, we cross-linked TAP in Raji membranes and analyzed the cross-linked products directly by Western blot using $\alpha$-TAP.1 and $\alpha$-TAP.2 antibodies. As shown in Fig. 3, p220 was detected in the absence (lanes 2 and 6) or presence (lanes 4 and 8) of KB11-HSAB. This confirms that the TAP heterodimer can be cross-linked in the membrane without prior photolabeling with peptide and that both TAP.1 and TAP.2 are components of the heterodimer.

The TAP Heterodimer Is Stabilized by TAP Substrates and Destabilized by ICP47—To determine the effect of peptides and ICP47 on the cross-linking of the TAP heterodimer, we performed the cross-linking analysis in the presence and absence of suitable TAP substrate peptides RRYQKSTEL (B27#3) (32), QVLRPMTYK (Net7B) (33), KB11, and the TAP inhibitor ICP47. We determined the level of TAP expression in .220 cells due to their lack of tapasin. To determine the effect of ICP47 on cross-linking of the TAP heterodimer, we tested the minimal active and maximal inactive fragments of ICP47, which were available, in the TAP cross-linking assay. The results of this analysis are shown in Fig. 4C. The minimal active fragment (residues 3–34 as described by Neumann et al., Ref. 22) resulted in the complete blockage of TAP heterodimer cross-linking (lane 5), as did residues 1–42 (lane 3). In contrast, ICP47 inhibited the formation of p220 to essentially background levels of quantitation, and a p220 band in the presence of ICP47 could not be detected on the Western blot.

To document further the effect of ICP47 on cross-linking of the TAP heterodimer, we tested the minimal active and maximal inactive fragments of ICP47, which were available, in the TAP cross-linking assay. The results of this analysis are shown in Fig. 4E. The minimal active fragment (residues 3–34 as described by Neumann et al., Ref. 22) resulted in the complete blockage of TAP heterodimer cross-linking (lane 5), as did residues 1–42 (lane 3). In contrast, the maximal inactive fragment of ICP47 (residues 1–28) had no effect on the formation of p220 (lane 4), and produced an amount of heterodimer which was very similar to that produced in the absence of any peptide
peptide substrates and inhibited by ICP47. Raji membranes (0.4 mg total protein) were pretreated with the indicated peptides (10 μM each) for 10 min at 37 °C before chemical cross-linking with EGS. The membranes were analyzed by SDS-PAGE and Western blot analysis using the anti-TAP2 antibody 435.3 as described in the legend to Fig. 3. Panel A, chemical cross-linking was performed in the presence of a series of TAP peptide substrates (B27#3, Nef7B, and KB11) and the TAP inhibitor ICP47 (residues 1–42). Panel B, the p220 bands shown in panel A were quantitated using the ImageQuant software program (Molecular Dynamics) and displayed in histogram format. Panel C, chemical cross-linking was carried out in the presence of ICP47 fragments; 1–42 (lane 3), 1–28 (lane 4), and 3–34 (lane 5). These data support the notion that ICP47 specifically inhibits the formation of a TAP heterodimer as measured by chemical cross-linking. The precise active and inactive fragments that have been defined for inhibition of TAP function are shown here also to correlate with the stable formation of the TAP heterodimer.

**DISCUSSION**

We have shown that the TAP heterodimer can be chemically cross-linked using the homobifunctional cross-linker EGS. The major cross-linked species is 220 kDa, which, we argue, represents the TAP heterodimer. First, if it represented a TAP heterotrimer or heterotetramer one would expect to see intermediate cross-linked species. However, we do not detect any such intermediate species. Second, it is not unusual for cross-linked proteins to run aberrantly on SDS-PAGE, as the cross-links could induce structural changes and different SDS binding properties. Third, all attempts to detect another additional protein within the p220 complex have failed (see Fig. 2). In cell lines that lack class I heavy chain association with TAP (Daudi), lack calnexin (CEM-NKR), or lack tapasin (.220), the p220 adduct was still detected. Indeed, p220 was formed in TAP-expressing insect cells that do not express several of the molecules that associate with TAP. Fourth, p220 was generated from TAP heterodimers that were solubilized in Triton X-100, which is known to dissociate TAP from its accessory molecules (data not shown). Finally, when the cross-linker EGS was cleaved under basic conditions, p220 fell apart into sub-units of ~70 kDa only (data not shown) (note, any unlabeled molecules associated with TAP would not be detected under these conditions). For all of these reasons we feel that p220 is the cross-linked TAP heterodimer.

Furthermore, we show here that the herpes simplex virus inhibitor ICP47 destabilizes the TAP heterodimer as revealed by sensitivity to cross-linking with EGS. This finding provides significant insight into the mechanism of action of ICP47. Not only does ICP47 block peptide binding to TAP and therefore inhibit TAP function, but it also appears to affect the conformation of the TAP heterodimer such that the TAP.1 and TAP.2 subunits are in a “loose” association. We also provide data to show that the converse is true, i.e. suitable peptide substrates for TAP stabilize the heterodimer. This is not necessarily surprising, as the addition of exogenous peptides have been shown to stabilize the TAP heterodimer during reconstitution experiments. From the data, we can put forth a theory with regard to the conformation of the TAP heterodimer. In a completely unoccupied state the TAP heterodimer takes on a neutral or default conformation as indicated by a minimal degree of cross-linking (see Fig. 4). In the presence of peptide the heterodimer takes on a more stabilized or “tight” conformation as shown by an increase in cross-linked heterodimer. In contrast, in the presence of ICP47 the heterodimer is destabilized to the point where no efficient cross-links can be formed. It should be made clear, however, that ICP47 does not cause the complete dissociation of the heterodimer into individual subunits. The TAP.1 and TAP.2 subunits still remain associated as indicated by coimmunoprecipitation experiments (data not shown). Thus we find that the changes induced into the TAP heterodimer by peptides and inhibitors are subtle compared with the forces holding the two subunits together. However, these subtle conformational changes could have a dramatic effect on TAP function. In the case of ICP47, destabilization of the TAP heterodimer works in concert with the blockade of substrate binding to generate a potent inhibitor of TAP.

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