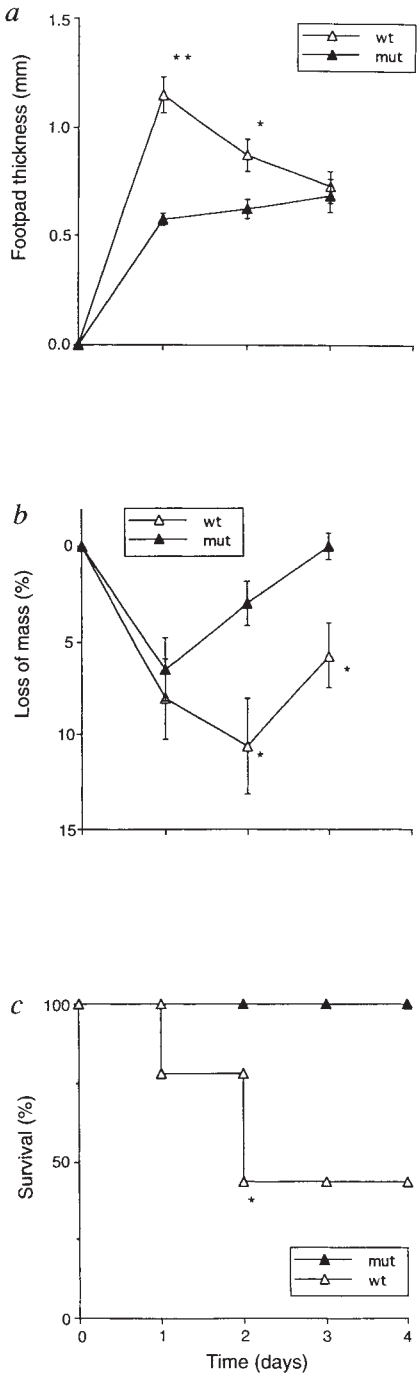

FIG. 3 Mice lacking iNOS *a*, developed significantly less inflammatory response to carrageenin, and *b*, were markedly more resistant to LPS-induced loss of mass and mortality than wild-type mice.
METHODS. *a*, Groups of 5 mice were injected in the right hind footpad with 300 μg lambda carrageenin (Sigma) in 50 μl PBS. The control left hind footpad was injected with 50 μl PBS alone. Footpad swelling was measured with a dial calliper, and the data shown represent mean footpad thickness increase (right footpad—left footpad) ± s.e.m. $*P = 0.02$; $**P < 0.001$. *b*, Groups of 5 mice were injected intraperitoneally with 10 mg per kg LPS in 0.2 ml PBS. The wild-type (wt) mice developed severe symptoms with up to 12% loss of mass. In contrast, mutant (mut) mice showed only minimum symptoms after transient loss of mass within the first 24 h, and they all recovered. $*P < 0.05$. *c*, Groups of 9 mice were injected with 12.5 mg per kg LPS; 5 of 9 wild-type (wt) mice died within 72 h with extensive subcutaneous haemorrhage. In contrast, all the mutant mice recovered from an initial transient loss of mass. Data are pooled from two independent experiments. $*P = 0.016$ (survival test).

demonstration that human monocyte/macrophages express iNOS[15,16] and generate NO in quantities sufficient to kill *Leishmania* parasites[17]. □

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Herpes simplex virus turns off the TAP to evade host immunity

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MANY viruses have evolved mechanisms to avoid detection by the host immune system. Herpes simplex virus (HSV) expresses an immediate early protein, ICP47, which blocks presentation of viral peptides to MHC class I-restricted cells[1]. The properties of the newly synthesized class I molecules in HSV-infected cells resemble those of cell lines deficient in the transporter associated with antigen processing (TAP) in that class I molecules are retained in the endoplasmic reticulum[1,2], and the heavy chain and β_2-microglobulin subunits dissociate in detergent extracts but the complex can be stabilized by peptides[1]. We show here that ICP47 binds to TAP and prevents peptide translocation into the endoplasmic reticulum.

The instability of MHC class I molecules in detergent lysates from ICP47-expressing cells suggested[1] that the class I molecules had not been loaded with peptide, which is necessary for the stable association of β_2-microglobulin and heavy chain and subsequent transport of the class I complex from the endoplasmic reticulum (ER) to the cell surface[3,4]. ICP47 is a cytosolic protein of relative molecular mass (M_r) 9,000 (9K) which lacks a recognizable signal sequence[1]. Transport from the cytosol into the

ER of peptides destined for presentation by class I MHC is mediated by the TAP heterodimer[5,6]. The localization of ICP47 suggested that it might interfere with the production of peptides, their delivery to the TAP transporter or the function of TAP itself.

We therefore investigated TAP transporter activity in cells expressing ICP47. The synthetic peptide TYNRTRALI (single-letter amino-acid notation), and a complex library of 2,304 peptides were used for these experiments. Both H/TYNRTRALI peptide and library peptides contain a tyrosine for radioiodination and the glycosylation consensus motif NXT, required for N-glycan-mediated retention of peptides translocated by TAP[7,8]. TAP function requires hydrolysis of nucleoside triphosphates[9,10,13]; however, in contrast to the results obtained in lymphoid cells[7], we observed decreased recovery of glycosylated peptide with increasing addition of exogenous ATP to permeabilized fibroblasts (Fig. 1*a*). This decrease is due to peptide hydrolysis (data not shown). Without addition of nucleoside triphosphates, glycosylated peptide was recovered on concanavalin A (ConA)-coated beads in TAP-expressing fibroblasts, and the recovery was markedly reduced by hydrolysis of ATP with apyrase (Fig. 1*a*). To verify that peptide translocation was TAP dependent, we carried out assays in human TAP-deficient fibroblasts. Little radioactivity was recovered in fibroblast line derived from a TAP-deficient human patient[14] (Fig. 1*b*). We confirmed the TAP dependence of transport measured in this assay using mouse embryo fibroblasts derived from TAP-positive and TAP-negative mice, and observed recovery of glycosylated peptide only in cells expressing a functional TAP complex (data not shown).

When human fibroblasts were infected with HSV type I strain F, translocation of peptide was inhibited (Fig. 2*a*). By contrast, infection with HSV-1 strain R3631, which lacks the gene encoding ICP47 (ref. 15), did not affect peptide translocation. To verify the role of ICP47 in inhibition of peptide transport, we used a replication-deficient recombinant adenovirus in which the ICP47 gene, driven by the HCMV immediate early promoter, is inserted into the E1 region of the adenoviral genome, so that adenovirus proteins are not expressed[1] and ICP47 is the only viral gene product synthesized in infected cells. In cells infected with the recombinant AdICP47-1, very little of the peptide library was translocated (Fig. 2*b*). In contrast, in cells infected with adenovirus vectors with the E1 region deleted (AddlE1) or expressing the *lacZ* gene (Ad-lacZ), peptide transport was similar to that observed in uninfected cells. The translocation of peptide in AdICP47-1-infected cells was not restored by addition of either ATP or UTP (the latter can activate TAP but has less effect on cytosolic proteases than does ATP) in concentrations up to 10 mM (Fig. 2*c*). Translocation of the peptide TYNRTRALI results in two main species of endoglycosidase H-sensitive peptide detectable by Tris-tricine gel electrophoresis (Fig. 2*d*). The second species is most probably a degradation product of the input peptide, generated in the cytosol or following translocation[16,17]. In AdICP47-1-infected cells, little peptide was recovered, but the material isolated was identical in mobility to that seen in AddlE1-infected and uninfected cells. We conclude that ICP47 inhibits TAP-dependent peptide translocation into the ER.

Does ICP47 interact directly with the TAP proteins? ICP47 was immunoprecipitated from digitonin lysates prepared from HeLa cells treated with interferon-γ (IFN-γ) and infected with AdICP47-1. Two sets of polypeptides of around 70K and 43K were coprecipitated (Fig. 3*a*) and comigrated with the TAP1/TAP2 complex and MHC class I heavy chain respectively (Fig.

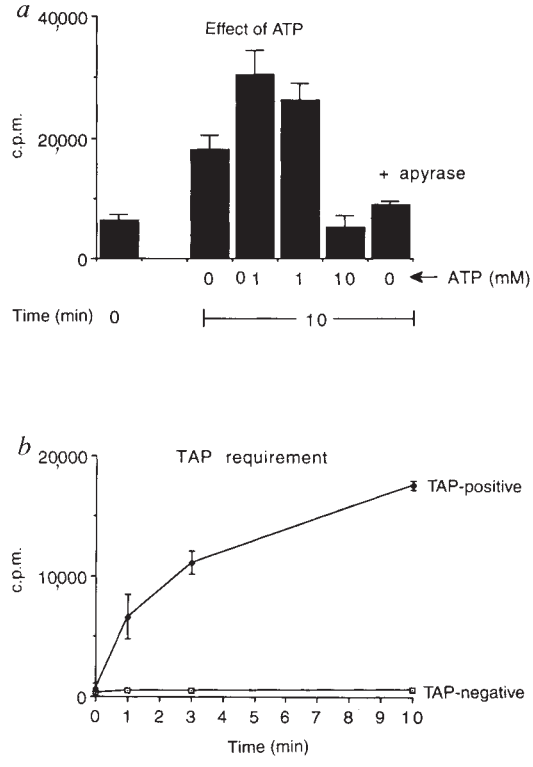


FIG. 1 Peptide translocation in human fibroblasts is dependent on TAP and ATP. *a*, Peptide translocation requires a source of energy. ATP or apyrase at the concentrations indicated were added to human fibroblasts permeabilized with streptolysin O and incubated with the peptide library for 10 min at 37 °C. Cells were lysed and the radioactivity recovered on ConA-Sepharose was measured. The 0 min sample was washed and lysed immediately after addition of peptide at 4 °C. Results shown are the mean of duplicates. Whereas addition of exogenous ATP is inhibitory, hydrolysis of NTPs by apyrase demonstrates the dependence of the assay on a source of ATP. *b*, Peptide transport is TAP dependent. TAP-positive (CCD18-Lu) and TAP-negative (Fib-EMO) fibroblasts were permeabilized, incubated with labelled peptide library and the ConA-recovered peptide measured at various times. Peptide accumulation required an active TAP complex.

METHODS. Transport assay: Human fibroblast lines had been grown from fetal lung (HFLFB)[21], the lung of a 2-month-old female (CCD-18Lu) (ATCC CCL 205), or from a skin biopsy of a 6-yr-old male carrying a mutation in TAP-2 rendering TAP nonfunctional (Fib-EMO)[14], the gift of Henri de la Salle. They were maintained as adherent monolayers in Dulbecco's modified Eagle's medium, 5% newborn calf serum and 5% fetal calf serum (DMEM/10% serum). The cells were detached with trypsin/EDTA, washed three times in DMEM/10% serum, and washed once in transport buffer at 4 °C. (transport buffer: 130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM Hepes, pH 7.3 with KOH). 10^6 cells in transport buffer were transferred to eppendorf tubes, centrifuged (3,000 r.p.m. in a microfuge), and resuspended in 80 μl transport buffer containing 1–2 U ml^{-1} streptolysin O (Wellcome) at 4 °C. The amount of streptolysin O used was determined for each cell line and condition of virus infection and resulted in >60% permeabilization as assessed by trypan blue exclusion. Cells were incubated at 37 °C for 10 min, then transferred to ice for addition of peptide (10 μl), and ATP (Sigma) or apyrase (10 μl, 50 U ml^{-1}) (Sigma) where indicated. They were then incubated for the indicated times (usually 0 and 10 min) at 37 °C. The transport assay was terminated and cells washed by addition of 1 ml 'stop buffer' (transport buffer + 10 mM EDTA), spun (14,000 r.p.m.) and lysed in 0.5% NP40, 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5 (lysis buffer). After pelleting the nuclei, 100 μl ConA-Sepharose beads (Pharmacia) were added to the supernatants and rotated gently at 4 °C for 1 h. The beads were washed four times in lysis buffer and radioactivity measured by γ-spectrometry. A complex library of 2,304 peptides has been previously described[8]. They were radiolabelled with ^{125}I by chloramine-T catalysed iodination, and used in the assay from a stock solution of 10–50 μM in 10 mM ascorbic acid.

3*a*). ICP47 is small (89 amino acids) and migrates at the dyefront of these gels. TAP and class I molecules were coprecipitated from AdICP47-1-infected cells but not from AddlE1-infected cells. The identities of the 70K and 43K proteins as TAP and class I respectively were confirmed by first dissociating the immune complexes and then performing a second immunoprecipitation using anti-TAP (Fig. 3*b*) or anti-class I (Fig. 3*c*) antibodies. The presence of class I heavy chain in the complex indicates that ICP47 interacts with either the TAP complex or the TAP–class I complex[18,19].

TAP is expressed at relatively low levels in human fibroblasts and without IFN-γ treatment it was difficult to detect. We used recombinant vaccinia vectors expressing one (VV-TAP1 or VV-TAP2) or both (VV-TAP1&2) of the TAP subunits to assess whether the TAP heterodimer was required for the observed interaction with ICP47. Anti-ICP47 antibodies coprecipitated TAP1 and TAP2 from extracts of cells coinfected with AdICP47-1 and VV-TAP1&2, but not when cells were coinfected with AddlE1 and VV-TAP1&2. When cells were coinfected with AddlE1 and VV-TAP1 or VV-TAP2, only a

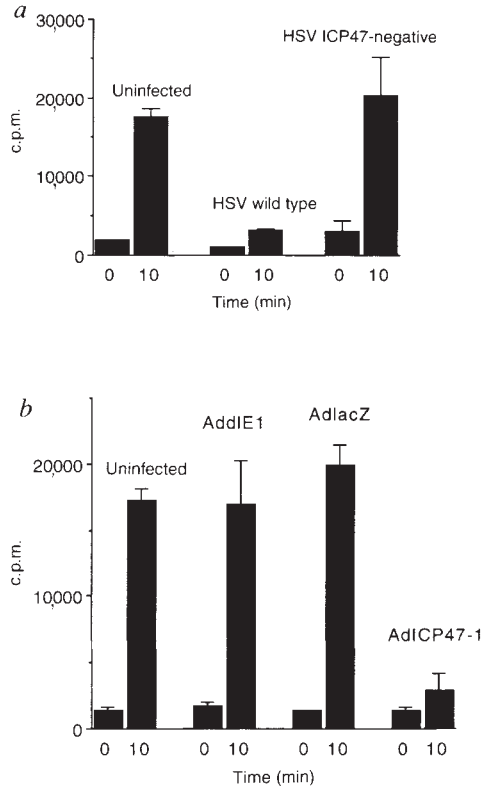
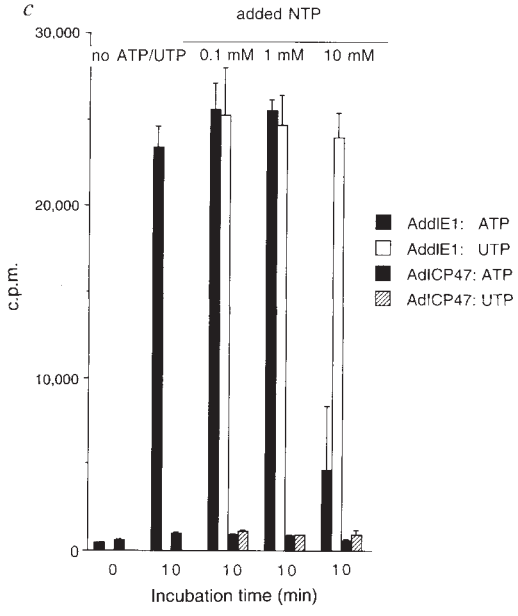
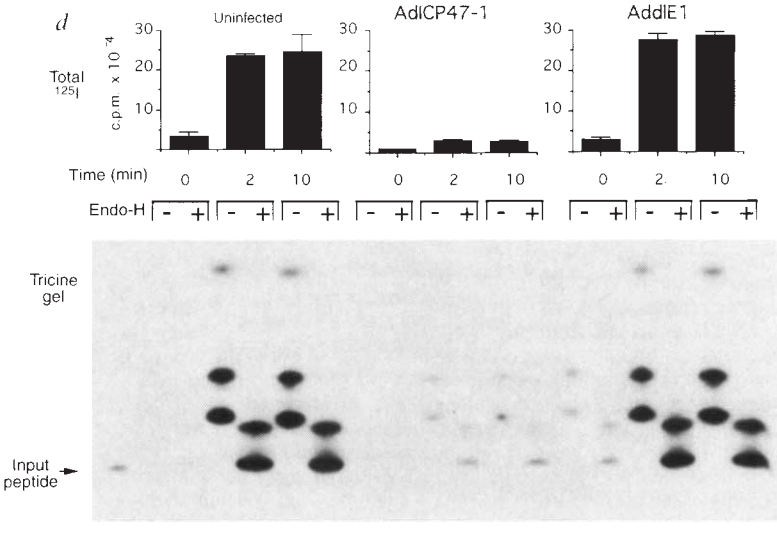


FIG. 2 ICP47 inhibits TAP-dependent peptide translocation. *a*, HSV infection inhibits peptide translocation. Human fibroblasts (CCD18-Lu) were infected using 10 plaque-forming units (PFU) per cell with wild-type HSV type I strain F or R3631, a mutant lacking the genes US11 and US12 (which encodes ICP47)[1]. Nine hours later the cells were trypsinized and a transport assay with the peptide library performed as for Fig. 1. *b*, Expression of ICP47 is sufficient to inhibit TAP-dependent peptide transport. Human fibroblasts (CCD18-Lu) were left uninfected or infected using 60 PFU per cell with recombinant adenovirus expressing ICP47 (AdICP47-1), β-galactosidase (AdlacZ) or with a deletion of the E1 region (AddlE1). Sixteen hours later a transport assay with the peptide library was performed as described for Fig. 1. *c*, Neither ATP nor UTP abolishes the effect of ICP47 on transport. Transport assays were performed as in Fig. 1 using the peptide library and cells infected with the viruses indicated at 60 PFU per cell, either with no NTPs added, with ATP added, or with UTP added at the concentrations indicated. *d*, Analysis by Tris-tricine electrophoresis of peptide recovered from ConA-Sepharose. CCD-18Lu cells were infected with AddlE1, AdICP47 or left uninfected. After 18 h a transport assay with the peptide TYNRTRALI was performed as described for the peptide library. After counting, peptide was eluted from the beads, half of the eluate was treated with endoglycosidase-H (endo-H) and the material analysed by Tris-tricine gel electrophoresis.

METHODS. Tris-tricine gel electrophoresis of ConA-Sepharose recovered peptide. ConA-Sepharose beads were washed twice with water at 4 °C and peptide eluted by exposure to 200 μl 1% trifluoroacetic acid (TFA) for 20 min. The supernatant and those of two further washes in 200 μl 1% TFA were pooled and dried overnight. Duplicates were pooled and resuspended in 50 μl 50 mM Sodium citrate pH 5.5, 25 μl of which was treated with 1 μl endoglycosidase H_f (New England Biolabs) for 2 h. The samples were diluted in sample buffer and analysed by Tris-tricine gel electrophoresis as described[22]. HSV stocks were propagated and titred on Vero cells. R3631[15] was a gift from B. Roizman; HSV-1(F) was a gift from P. Spear. The generation of AdICP47-1 and AddlE1 has been previously described[1]. AdlacZ was the gift of B. Panning, MIT.





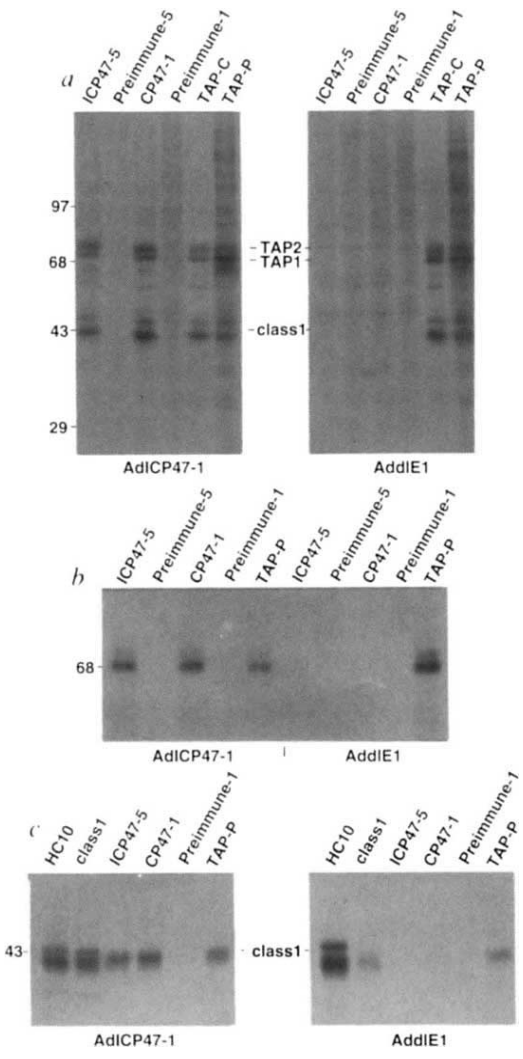


FIG. 3 ICP47 associates with the TAP complex. *a*, HeLa cells treated with IFN-γ and infected with 100 PFU per cell AdlCP47-1 or AddlE1 for 36 h were metabolically labelled for 6 h and lysed in 1% digitonin. Immunoprecipitations with anti-ICP47 antisera, preimmune sera or anti-TAP sera were analysed by SDS-PAGE. *b*, *c*, Proteins immunoprecipitated as in *a* were eluted and subjected to a second round of immunoprecipitation with anti-TAP (*b*) or anti-class I (*c*) antibodies. Only in cells that expressed ICP47 was a complex of ICP47, TAP1 and class I detected.

METHODS. HeLa cells were treated with recombinant human IFN-γ (100 U per ml) for 12 h and infected with AdlCP47-1 or AddlE1 using 100 PFU per cell. Thirty-six hours after infection the cells were labelled with [^{35}S]methionine/[^{35}S]cysteine (Dupont) for 6 h, and lysed in 1% digitonin/TBS (digitonin (Sigma) was dissolved at 1% w/v in 100 mM NaCl, 50 mM Tris (pH 7.5) by boiling, then cooled, filtered, and 2 mg ml^{-1} BSA and 0.5 mM PMSF added). Lysates were precleared by centrifugation at 83,000*g* for 45 min and incubated with the following rabbit antisera: anti-ICP47-1 (raised against a carboxy-terminal peptide of ICP47), anti-ICP47-5 (raised against a full-length ICP47 produced in bacteria), preimmune sera, and anti-TAP1 antisera: anti-TAP-C[18], anti-TAP-P[23]. Immune complexes were recovered on Protein A-Sepharose, washed four times with 0.1% digitonin/TBS; and analysed by SDS PAGE on 10% gels. Sequential immunoprecipitation: immune complexes on protein A-Sepharose were denatured by boiling for 5 min in an equal volume of 25 mM Tris pH 6.2, 2% SDS, 600 mM β-mercaptoethanol. The samples were diluted 10-fold in 1% digitonin/TBS, and immunoprecipitated with either anti-TAP-P, monoclonal antibody HC10, recognizing free HLA-B and C locus heavy chains[24], or polyclonal rabbit serum recognizing denatured HLA class I heavy chains (class I)[25].

small quantity of the TAP complex was precipitated by the anti-ICP47 antibodies alone, probably due to expression of endogenous TAP. Coinfection with VV-TAP1 and VV-TAP2 restored the ability of TAP to precipitate with ICP47. Thus we conclude that the interaction of ICP47 with TAP requires both TAP subunits.

We suggest that ICP47 binds to the cytosolic portion of the TAP heterodimer and thereby prevents peptide translocation. It remains a formal possibility that ICP47 interacts with MHC class I heavy chain. We consider this unlikely for two reasons. First, whereas coprecipitation of class I with TAP required the presence of only the TAP1 subunit[19,20], coprecipitation of TAP with ICP47 required expression of both subunits. Second, upregulation of the TAPs by infection with VV-TAP1&2 dramatically increased the amount of TAP1 and TAP2 immuno-

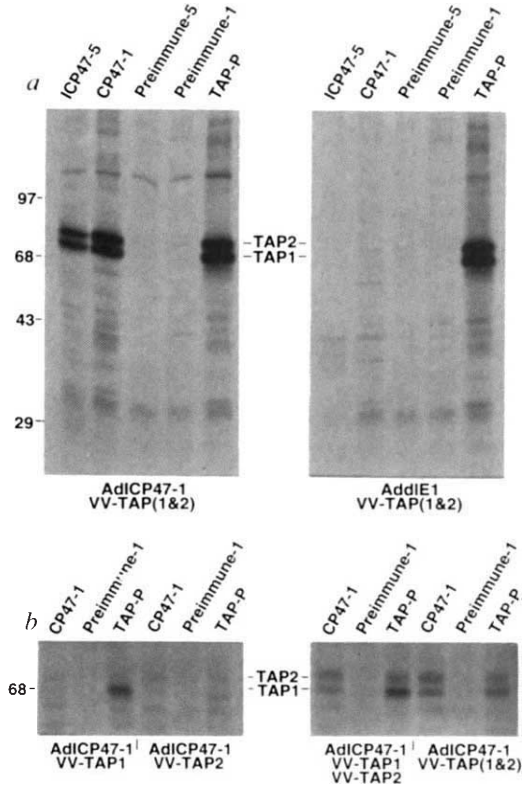


FIG. 4 Association of ICP47 and TAP requires coexpression of both TAP subunits. *a*, HeLa cells were infected with AdlCP47-1 or AddlE1 using 100 PFU per cell for 36 h and then coinfected with recombinant vaccinia viruses expressing both TAP subunits (VV-TAP1&2) for 10 h using 10 PFU per cell; then labelled with [^{35}S] methionine/cysteine for 4 h and immunoprecipitated as in Fig. 3 with the indicated antibodies. Class I is not detected in complexes precipitated from these cells either with anti-TAP antisera or anti-ICP47 antiserum, presumably due to the relative overabundance of the virally expressed TAP and because vaccinia shuts off host protein synthesis. *b*, HeLa cells infected with AdlCP47-1 as above were coinfected with vaccinia viruses expressing TAP1 (VV-TAP1), TAP2 (VV-TAP2), both TAP subunits (VV-TAP1&2) or simultaneously with VV-TAP1 and VV-TAP2, radiolabelled, and immunoprecipitated as above. Only when both TAP1 and TAP2 were expressed in cells did anti-ICP47 antibodies precipitate the TAP complex.

METHODS. Construction of recombinant vaccinia viruses: The single TAP subunit vectors were constructed by homologous recombination of human TAP1a or TAP2b[24] (a gift from T. Spies) cDNA in a modified form of pSC11 into the thymidine kinase region of vaccinia under the control of the p7.5 early/late promoter as previously described[27,28]. VV-TAP1&2 was constructed by inserting a plasmid containing TAP2 cDNA and the HSV thymidine kinase gene into the *Hin*dIII site of the VV-TAP1 and selecting for thymidine kinase expression as previously described[29].

precipitated by anti-ICP47 antibodies, suggesting that a third molecule such as class I does not limit the association. ICP47, when overexpressed, is distributed throughout the cytosol and nucleus as detected by immunofluorescence and ICP47 produced by *in vitro* translation did not associate with ER membranes[1]. These results remain to be reconciled with the present data, but explanations include the failure to detect the relatively small fraction of membrane-associated ICP47 due to the paucity of TAP subunits, and the possibility that ICP47 interacts with TAP indirectly through a cytosolic intermediary that has escaped detection.

We describe interference with peptide translocation into the ER as a novel mechanism by which viruses may evade immune control. The nature of a particular virus–host relationship is illuminated by the elements of the immune response actually targeted by the virus. For instance, four viruses are currently known to interfere post-translationally with MHC class I-restricted antigen presentation: adenovirus, murine and human cytomegaloviruses, and HSV. These viruses have some similarity in their lifestyle, in that they establish either latent or persistent infections. Herpesviruses can proceed to reactive from latency, replicate in the face of a fully primed immune system and infect new hosts. After a short period, the immune system controls the infection and little harm to the host ensues. Transmission of the virus to a new host usually occurs not from the primary infection, but from virus produced by reactivation later in life. We suggest that the ability to create this window of replicative opportunity within an immunized host is facilitated by selective avoidance of the host's CD8-positive T-cell response. □

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A viral inhibitor of peptide transporters for antigen presentation

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CYTOTOXIC T lymphocytes lyse target cells after T-cell-receptor-mediated recognition of class I major histocompatibility complex molecules presenting peptides[1]. Antigenic peptides are generated in the cytoplasm by proteasomes[2] and translocated into the lumen of the endoplasmic reticulum (ER) by peptide transporters (TAP)[3–6]. Herpes simplex virus (HSV) expresses a cytoplasmic protein, ICP47, which seems to interfere with such immune surveillance by mediating retention of 'empty' class I molecules in the ER[7,8]. By expressing ICP47 in HeLa cells under an inducible promoter[9], we show that ICP47 efficiently inhibits peptide transport across the ER membrane such that nascent class I molecules fail to acquire antigenic peptides. This inhibition was overcome by transfecting murine TAP. Further, we demonstrate that ICP47 colocalizes and physically associates with TAP within the cell. Inhibition of peptide translocation by a viral protein indicates a previously undocumented potential mechanism for viral immune evasion.

The observation that class I molecules in ICP47-expressing cells did not contain peptides[8] suggests that ICP47 could limit the supply of peptides required for complete assembly of class I molecules in the ER, and their subsequent transport to the cell surface[10]. This is supported by our observation that the expression levels of class I haplotypes, at the cell surface of ICP47-expressing HeLa cells, correlated with those of respective class I haplotypes expressed in TAP-deficient cell lines (data not shown). Because ICP47 was shown to be localized in the cytoplasm[8], it was possible that interference with antigen processing occurred before peptide translocation, e.g. at the level of proteolytic peptide generation in the cytosol. However, we observed that ICP47 prevented peptide loading of class I molecules even when proteolytic antigen degradation was bypassed by expressing minigenes encoding a class I-binding epitope (data not shown).

These observations suggest that ICP47 might interfere with peptide translocation across the ER membrane. We established a stable HeLa cell line, ICP-O20, in which ICP47 expression can be upregulated by the removal of tetracycline[9]. By using a recently developed assay for peptide transport[5], we compared the peptide translocation activities of human TAP in ICP-O20 cells cultured in the presence of decreasing tetracycline concentrations, thus increasing the expression of the regulated gene product proportionally[9,11]. An inverse correlation was found between levels of ICP47 expression and peptide transport activity (Fig. 1*a*). Even when peptide translocation activity was increased by interferon-γ (IFN-γ) which upregulates TAP expression[3], ICP47 inhibited peptide translocation (Fig. 1*a*). Thus human TAP activity was efficiently blocked by ICP47. By contrast, when murine TAP1 and TAP2 were transiently expressed in ICP-O20 cells, high surface levels of class I

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