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Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP

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The herpes simplex virus (HSV) ICP47 protein inhibits the MHC class I antigen presentation pathway by inhibiting the transporter associated with antigen presentation (TAP) which translocates peptides across the endoplasmic reticulum membrane. At present, ICP47 is the only inhibitor of TAP. Here, we show that ICP47 produced in bacteria can block human, but not mouse, TAP, and that heat denaturation of ICP47 has no effect on its ability to block TAP. ICP47 inhibited peptide binding to TAP without affecting ATP binding, consistent with previous observations that the peptide binding and ATP binding sites of TAP are distinct. ICP47 bound to TAP with a higher affinity (K_D ~5× 10^{-8} M) than did peptides, and ICP47 did not dissociate from TAP. ICP47 was not transported by TAP and remained sensitive to proteases added from the cytosolic surface of the membrane. Peptides acted as competitive inhibitors of ICP47 binding to TAP, and this inhibition required a 100- to 1000-fold molar excess of peptide. These results demonstrate that ICP47 binds to a site which includes the peptide binding domain of TAP and remains bound to this site in a stable fashion.

Keywords: herpes simplex virus/ICP47/peptide binding site/TAP

Introduction

CD8+ T lymphocytes recognize peptides, derived primarily from cytosolic proteins, that are presented on cell surface major histocompatibility complex (MHC) class I molecules (Rammensee *et al.*, 1993). Peptides are generated in the cytosol, largely by the action of proteasomes, and gain access to the lumen of the endoplasmic reticulum (ER), where they can associate with newly synthesized class I molecules (reviewed in Heemels and Ploegh, 1995; York and Rock, 1996). Transport of peptides across the ER membrane is mediated by the transporter associated with antigen presentation (TAP) which, like certain components of the proteasome, is encoded in the MHC (reviewed in Yewdell and Bennink, 1992). There is good evidence that TAP is the major source of peptides for class I antigen presentation (reviewed in Heemels and Ploegh, 1995; Howard, 1995; York and Rock, 1996), and mutant mice and human patients lacking TAP show significant defects in class I antigen presentation (van Kaer *et al.*, 1992; de la Salle *et al.*, 1994). In addition, TAP associates with MHC class I heavy chain (HC)–β_2-microglobulin (β_2-m) complexes and this co-localization in the ER membrane may enhance delivery of peptide to newly synthesized class I molecules (Androlewicz *et al.*, 1994; Ortmann *et al.*, 1994; Suh *et al.*, 1994).

TAP is a heterodimer composed of two proteins, TAP1 and TAP2, each with C-terminal hydrophilic domains that bind ATP and more N-terminal hydrophobic domains which may span the membrane six to eight times (reviewed in Townsend and Trowsdale, 1992; Monaco, 1992; Howard, 1995). TAP is a member of the ABC transporter family which includes the mammalian multi-drug resistance (mdr) glycoproteins, the cystic fibrosis transporter, a yeast mating factor transporter and several bacterial amino acid transporters (reviewed in Higgins, 1992). TAP has a relatively broad specificity for small peptides of ~7–12 amino acids, although there are preferences for peptides with hydrophobic or basic C-termini similar to those shown by MHC class I (reviewed in Heemels and Ploegh, 1995). Co-expression of both TAP1 and TAP2 is required for peptide binding and translocation (Androlewicz and Cresswell, 1994; van Endert *et al.*, 1994) and peptide substrates can be cross-linked to residues of both TAP1 and TAP2 (Androlewicz *et al.*, 1994). Hydrolysis of ATP is not required for peptide binding, but is necessary for peptide translocation, and the binding of ATP and peptide to TAP appear to be independent processes (Androlewicz *et al.*, 1993, 1994; Neefjes *et al.*, 1993; Shepherd *et al.*, 1993; Schumacher *et al.*, 1994; van Endert *et al.*, 1994).

TAP expressed in insect cells is capable of translocating peptide, demonstrating that mammalian factors other than TAP are not strictly required for peptide transport (Meyer *et al.*, 1994; van Endert *et al.*, 1994). Studies of TAP expressed in insect cells have suggested that TAP can exist in at least two states, one having a higher affinity for peptide and favoured in the absence of ATP and below 37°C, while the other, with lower affinity for peptide, is induced on binding of ATP (van Endert *et al.*, 1994). Similar observations have been made with other members of the ABC transporter family (Covitz *et al.*, 1994). However, at present, the molecular details of how TAP binds peptides and mediates their transport across the ER membrane are not well understood.

Herpes simplex virus (HSV) infection of human

fibroblasts causes rapid inhibition of MHC class I antigen presentation and class I HC–β_2-m complexes accumulate in the ER in a peptide empty form (Hill *et al.*, 1994; York *et al.*, 1994). We demonstrated that an HSV immediate early polypeptide, ICP47, is both necessary and sufficient to cause this inhibition (York *et al.*, 1994). ICP47 binds to TAP and inhibits peptide translocation in permeabilized mammalian cells (Fruh *et al.*, 1995; Hill *et al.*, 1995). ICP47 did not bind to TAP1 or TAP2 when the proteins were expressed individually (Hill *et al.*, 1995) and, thus, as with peptides, binding of ICP47 to TAP apparently requires contributions from both members of the heterodimer. ICP47 is the only known inhibitor of TAP, and it appears likely that analysis of the effects of ICP47 should provide valuable new information about TAP, as well as further elucidating an interesting strategy for evasion of the immune system.

Here we describe the mechanism by which ICP47 inhibits TAP-mediated peptide transport into the ER. ICP47 inhibited peptide binding to TAP, but there was no effect on ATP binding to TAP. Characterization of the binding of ICP47 to TAP indicated that ICP47 bound to a site which overlaps the peptide binding site, but with a higher affinity than peptides, and ICP47 remained accessible to proteases added from the cytoplasmic side of membranes. Therefore, ICP47 blocks TAP by binding in a relatively stable fashion to a domain of TAP which includes the peptide binding site.

Results

ICP47 produced in bacteria inhibits TAP

In order to study interactions between ICP47 and TAP, ICP47 was expressed in bacteria. The ICP47 gene was fused to glutathione-*S*-transferase (GST) sequences, so that the GST and ICP47 polypeptide sequences were separated by a thrombin-sensitive cleavage site. GST–ICP47 was purifed using glutathione–Sepharose and the ICP47 protein released from the GST by using thrombin which was then inactivated with phenylmethylsulfonyl fluoride (PMSF) (Figure 1A).

To determine whether ICP47 produced in bacteria (rICP47) was able to block TAP activity in cells, we introduced rICP47 into streptolysin O-permeabilized human fibroblasts as previously described (Hill *et al.*, 1995). The rICP47 inhibited TAP-mediated peptide transport and the half-maximal inhibitory concentration (IC_{50}) was ~0.3 μM (Figure 1B). When rICP47 was heated to 95°C for 30 min and cooled, the protein retained most or all of its ability to block TAP (Figure 1B). The inhibition of TAP function by rICP47 required the presence of intact rICP47 protein and was not mediated by residual thrombin because PMSF-inactivated thrombin alone, without rICP47, did not inhibit transport. In addition, ICP47 preparations treated with trypsin, followed by inactivation of the trypsin with trypsin inhibitor, abrogated the ability of rICP47 to interfere with peptide transport, demonstrating that the effect was due to the protein in the preparation.

The ability to introduce ICP47 directly into cells allowed us to address two questions raised by previous studies. First, is TAP-dependent transport in lymphoblastoid cell lines inhibited by ICP47? This question arose because of the previous observation that ICP47 expressed by HSV-1

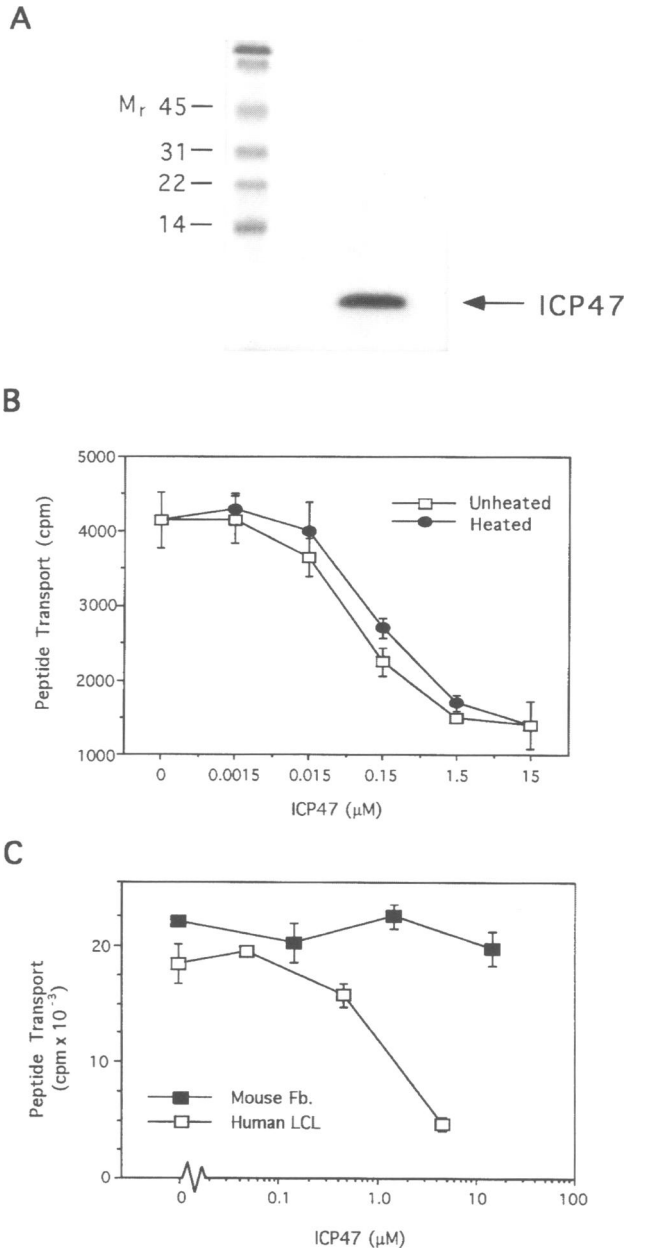

Fig. 1. Expression of HSV-1 ICP47 in bacteria and inhibition of TAP-mediated transport in permeabilized cells. (**A**) The GST–ICP47 fusion protein bound to glutathione–Sepharose was cleaved using thrombin. The thrombin was inactivated by PMSF and the rICP47 was subjected to SDS–gel electrophoresis and stained with Coomassie Blue. Marker proteins are shown on the left of the gel. (**B**) Human fibroblasts were permeabilized using streptolysin O and incubated with various quantitites of untreated rICP47 (open symbols), or rICP47 that had been heated to 95°C for 30 min (closed symbols) as well as the ^{125}I-labelled peptide library for 10 min at 37°C. (**C**) Mouse fibroblasts (closed symbols) or human B lymphoid cells (LCL) were permeabilized using streptolysin O and incubated with various quantities of rICP47 and ^{125}I-labelled peptide library for 10 min at 37°C. The cells were lysed using 0.5% NP-40 and cell lysates incubated with conA–Sepharose, the conA–Sepharose was washed and the glycosylated peptides were eluted with α-methylmannoside and counted.

or HSV-2 or using adenovirus (Ad) vectors inhibited antigen presentation in fibroblasts but not in human B lymphocyte lines (York *et al.*, 1994). When rICP47 was introduced into permeabilized B cells, peptide transport

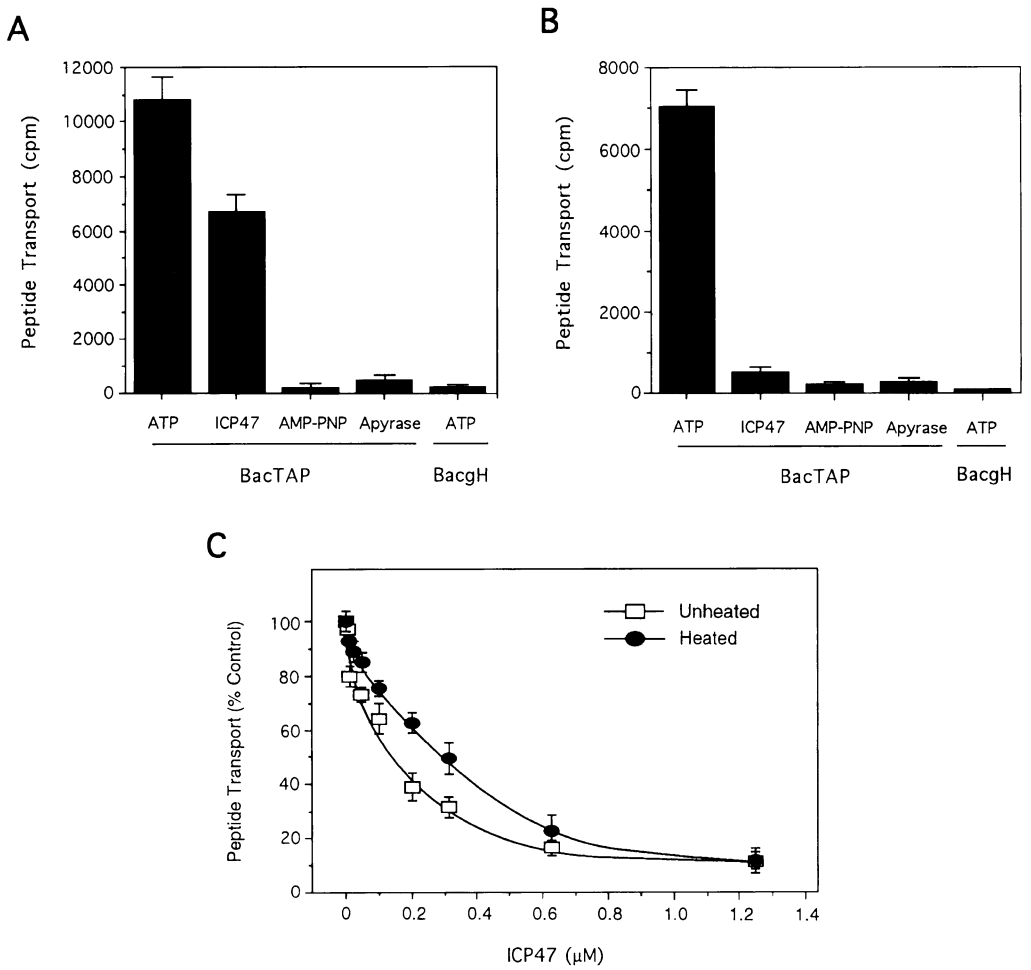

Fig. 2. Inhibition of TAP-mediated peptide translocation in insect microsomes by ICP47. Microsomes derived from insect cells infected with baculovirus vectors expresssing TAP1 and TAP2 (BacTAP) or HSV glycoprotein H (BacgH) were incubated with ATP (5 mM), rICP47 (1 μM in A and B) in the presence of ATP (5 mM), AMP-PNP (5 mM) for 5 min at 4°C or apyrase (20 U/ml) for 5 min at 37°C then the ^{125}I-labelled peptide library was added for 15 min at 37°C (**A**) or 23°C (**B** and **C**). In (C), the rICP47 was heated to 95°C for 10 min (closed symbols) or not heated (open symbols). The microsomes were washed twice, then lysed by using 1% NP-40, lysates clarified and incubated with conA–Sepharose. Radioactivity eluted with 200 mM α-methylmannoside was counted.

was inhibited and the IC_{50} was similar to, or perhaps marginally higher than, that observed with fibroblasts (Figure 1C). Therefore, the rICP47 effectively blocks TAP-mediated peptide transport in both human cell types. Second, is ICP47 able to block the function of mouse TAP? We had observed earlier that intracellular transport of class I molecules and presentation of viral antigens was normal in HSV-infected mouse cells (York *et al.*, 1994). When rICP47 was introduced into mouse fibroblasts (Figure 1C) or mouse EL4 lymphoid cells (not shown), there was little or no inhibition of TAP activity even when 15 μM rICP47 was used. Parenthetically, the ability of rICP47 to inhibit TAP in human cells and not mouse cells strengthens the conclusion that the inhibitory activity is due to specific protein–protein interactions, and is not attributable to non-specific toxicity of the preparation.

Inhibition of TAP activity in microsomes derived from insect or mammalian cells by rICP47

To characterize further the interaction between ICP47 and TAP, we expressed TAP in insect cells by using recombinant baculoviruses. Such vectors have the advantage of producing high levels of TAP in membranes that do not normally possess the TAP proteins. Microsomes were prepared from insect cells co-infected with baculoviruses expressing TAP1 (T1.5) and TAP2 (T2.12) as described previously (van Endert *et al.*, 1994). The membranes were incubated with a radiolabelled peptide library which can be glycosylated and retained in the ER (Heemels *et al.*, 1993). Peptide transport by the insect microsomes containing TAP was inhibited by apyrase treatment or the inclusion of AMP-PNP, a non-hydrolysable analogue of ATP (Figure 2A and B), and is, therefore, ATP dependent. In addition, transport was TAP dependent as indicated by use of microsomes from cells infected with a control baculovirus vector, BacgH. rICP47 effectively inhibited peptide translocation in TAP-expressing insect microsomes at 23°C and, again, when the protein was heated it retained its ability to block peptide transport (Figure 2B and C). Peptide transport in these insect microsomes was not affected by 10 μM GST, an irrelevant protein produced in bacteria (not shown). The IC_{50} of rICP47 inhibition of TAP-mediated peptide transport at 23°C was ~0.2 μM (Figure 2C), a value similar to that observed when ICP47 was introduced into permeabilized mammalian cells. However, rICP47 was less effective in inhibiting peptide

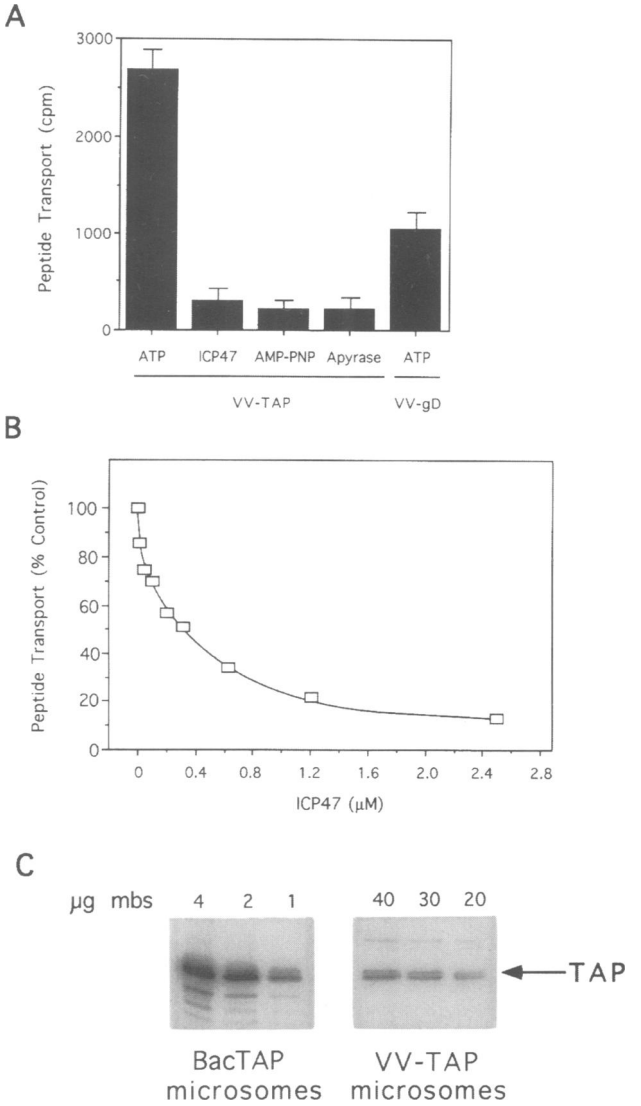

Fig. 3. Inhibition of peptide translocation in human microsomes by rICP47 and comparison of TAP levels in insect and human microsomes. (**A**) Microsomes derived from human KB cells infected with vaccina vectors expressing TAP1 and TAP2 (VV-TAP) or HSV glycoprotein D (VV-gD) were incubated with ATP (5 mM), rICP47 (1 uM) in the presence of ATP (5 mM), AMP-PNP (5 mM) for 5 min at 4°C or with apyrase (20 U/ml) for 5 min at 37°C then the ^{125}I-labelled peptide library was incubated with the membranes for 15 min at 37°C. (**B**) Microsomes from human cells infected with VV-TAP were incubated with various quantities of ICP47 in the presence of 5 mM ATP and radiolabelled peptide library for 15 min at 37°C. The membranes were washed, lysed in 1% NP-40 and radioactivity associated with conA–Sepharose counted. (**C**) Various quantities (μg of total membrane protein/lane) of insect microsomes containing TAP (BacTAP) or human microsomes containing TAP (VV-TAP) were subjected to electrophoresis using SDS–polyacrylamide gels, proteins transferred to nitrocellulose and the blots incubated with anti-TAP1 serum then washed and incubated with [^{125}I]protein A. The TAP1 band was quantified using a PhosphorImager and compared with a TAP1 standard that had been immunoprecipitated from insect cells and the quantity of TAP1 was determined by silver staining.

transport in these insect microsomes at 37°C, even though peptide transport was more efficient at the higher temperature (Figure 2A). This may be related to previous observations that peptide binds poorly to these membranes at 37°C (van Endert *et al.*, 1994) or that TAP can lose immunoreactivity when cell extracts are incubated at 37°C (Russ *et al.*, 1995), although there was no evidence of proteolysis of TAP in our microsomes at 37°C.

To examine rICP47's effects on TAP expressed in mammalian cell microsomes, we expressed TAP in human KB cells using a vaccinia virus vector, VV-TAP1&2 (Russ *et al.*, 1995) and prepared microsomes from the cells. Again, translocation of peptides was ATP dependent; however, with these human microsomes, there was significant endogenous TAP activity associated with microsomes from cells infected with a control vaccinia vector, VV-gD (Figure 3A). rICP47 inhibited the peptide transport by ~85% at 37°C (Figure 3B). Moreover, the IC_{50} of this inhibition was ~0.3 μM, similar to the value observed with insect microsomes at 23°C. Peptide translocation by the VV-TAP1&2 microsomes was also observed at 23°C, although at a reduced level, and ICP47 inhibited this translocation quite effectively (not shown). Therefore, ICP47 can inhibit TAP-mediated peptide translocation in both insect and human microsomes, albeit at different optimal temperatures.

We compared the quantity of TAP expressed in the human microsomes with that in the insect microsomes. The TAP proteins present in the insect microsomes could be visualized directly after samples were subjected to electrophoresis and then stained with silver reagent. In addition, TAP immunoprecipitated from insect cells was used to standardize Western blots. In Figure 3C, microsomes from insect cells infected with BacTAP1&2 or from human cells infected with VV-TAP1&2 were subjected to electrophoresis and blots probed with a polyclonal anti-TAP1 antiserum (Cromme *et al.*, 1994). The anti-TAP1 serum detects TAP1, the lower, more intense band, but also cross-reacts to some extent with TAP2, the upper, less intense band in these blots. PhosphorImager analysis indicated that there was ~60-fold more TAP1 in the insect microsomes than in the human microsomes (21 ng of TAP1/μg membrane protein versus 0.34 ng/μg). However, the total amount of peptide translocated by the human microsomes was only 4- to 6-fold lower than with similar quantities (micrograms of membrane protein) of insect microsomes, suggesting that the peptide translocation activity of TAP in human microsomes was 10- to 15-fold higher than in the insect cell membranes.

ICP47 inhibits peptide binding to TAP but not ATP binding

To study the effect of ICP47 on peptide binding to TAP, we used insect microsomes because the lower level of TAP in the human microsomes made analysis of peptide binding more difficult and control membranes devoid of TAP were available in the insect system. Insect microsomes were treated with apyrase and then incubated with the radioiodinated peptide library at 4°C, to ensure that peptide transport was kept to a minimum (van Endert *et al.*, 1994). Peptide binding was TAP dependent (Figure 4A) and was inhibited by rICP47 (IC_{50} ~0.2 μM) (Figure 4B), in agreement with the inhibition of peptide transport.

ATP binding to TAP was measured in the absence and presence of ICP47 by using 8-azido [^{32}P]ATP which can be cross-linked to TAP (Muller *et al.*, 1994; Russ *et al.*, 1995). Insect microsomes were incubated with 8-azido [^{32}P]ATP, then this reagent was cross-linked to TAP by using UV light and samples were immunoprecipitated

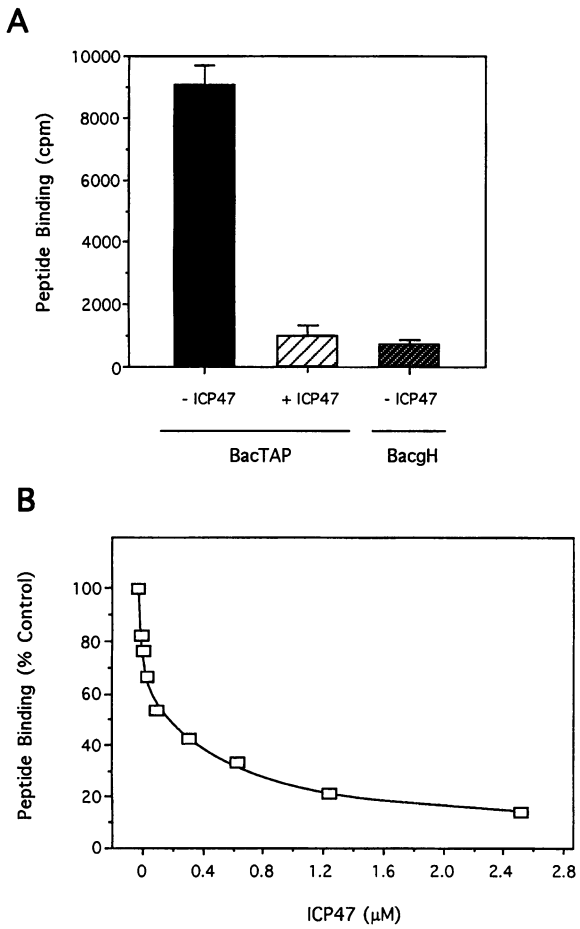

Fig. 4. Inhibition of peptide binding to TAP by rICP47. Microsomes derived from insect cells infected with BacTAP or with BacgH were treated with apyrase, washed in assay buffer, then incubated with or without rICP47 [1 μM in (**A**), or various concentrations in (**B**)] for 5 min at 4°C. The ^{125}I-labelled peptide library was added to the membranes for a further 20 min at 4°C. The membranes were washed twice in assay buffer, pelleted by centrifugation and radioactivity associated with the microsomes counted.

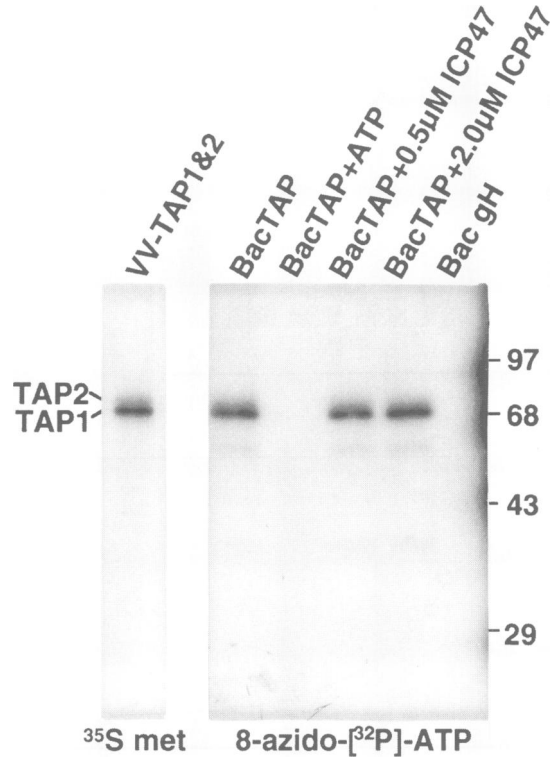

Fig. 5. Effects of rICP47 on ATP binding to TAP. Microsomes derived from insect cells infected with BacTAP or BacgH were washed in buffer and incubated with nothing, 5 mM ATP, 0.5 μM rICP47 or 2 μM rICP47 for 15 min at 4°C then 8-azido-[^{32}P]ATP was added for 15 min at 4°C in the dark. Samples were subjected to 254 nm light from a 1000 W source for 10 s then diluted with buffer containing 1% NP-40 and 0.5% DOC and TAP immunoprecipitated using anti-TAP1 antibodies. For comparison, the TAP complex was radiolabelled with [^{32}S]methionine in human cells infected with VV-TAP1&2 and immunoprecipitated using anti-TAP1 antibodies.

using anti-TAP1 antibodies. We observed radiolabelled TAP1 after immunoprecipitation with the anti-TAP1 antiserum, with only a small amount of TAP2 co-precipitated (Figure 5). 8-Azido [^{32}P]ATP labelling of TAP was inhibited by 5 mM ATP and was not observed with insect microsomes infected using a control baculovirus, BacgH (Figure 7), or in samples not photolysed (not shown). Incubation of insect microsomes with rICP47 had no effect on ATP binding to TAP (Figure 5), even at concentrations of rICP47 higher than required to inhibit translocation (0.5 and 2 μM). Therefore, while ICP47 inhibits binding of peptides to TAP, it does not affect ATP binding.

Properties of ICP47 binding to TAP and competitive inhibition by peptides

Since ICP47 could inhibit the binding of peptides to TAP, it was of interest to determine the properties of the ICP47 binding site on TAP. Microsomes derived from BacTAP-infected insect cells bound ^{125}I-labelled rICP47 and this binding was saturable (Figure 6A). Binding to control microsomes lacking TAP (derived from BacgH-infected cells) was <10% of that observed with TAP-containing microsomes (not shown), and this non-specific binding was subtracted from the binding observed with BacTAP membranes. Binding of rICP47 to BacTAP microsomes reached a plateau at ~1 μM ICP47 in assays involving ~0.3 μM TAP. Thus, it is possible that more than a single ICP47 molecule binds per TAP complex. Scatchard analyses of these data suggested that rICP47 bound to TAP complexes with a K_D of 5.2×10^{-8} M (Figure 6B). By comparison, peptides that are capable of binding to TAP with relatively high affinity can display K_D values in the range of 4×10^{-7} M, although most peptides bind with lower affinities (van Endert *et al.*, 1994). Gel electrophoresis of the ICP47 which bound to insect microsomes containing TAP did not reveal degradation of the protein (not shown) and previously we demonstrated that full-size ICP47 was associated with TAP in cells (Hill *et al.*, 1995) and, thus, there was no evidence that fragments of ICP47 were inhibiting TAP. Other experiments in which ICP47 binding was measured at 23°C revealed similar binding characteristics, although at 37°C the affinity of ICP47 for TAP-containing insect microsomes was reduced (results not shown).

Two types of competition experiments were performed to examine the relative affinities of ICP47 versus the peptide library for TAP. In the first, various quantities of labelled rICP47 were mixed with unlabelled peptide library and then immediately added to BacTAP microsomes and binding of rICP47 measured. The peptide library inhibited

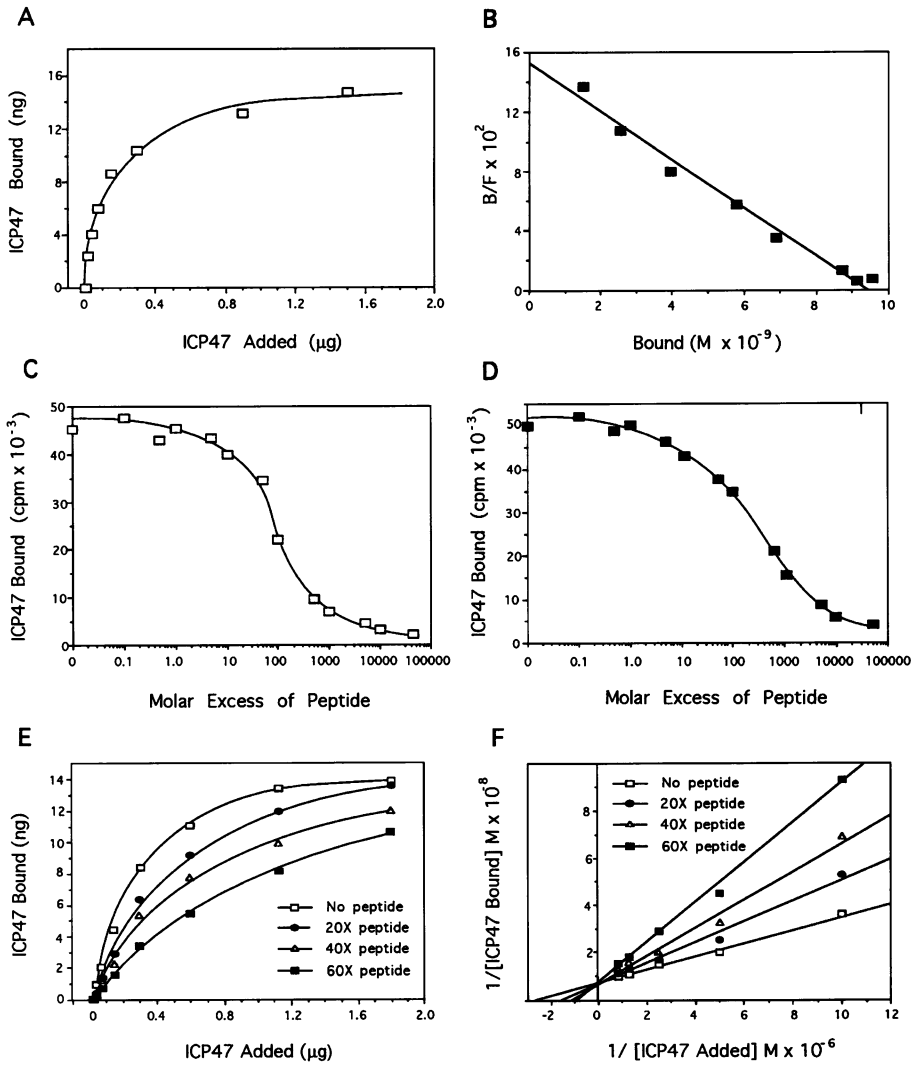

Fig. 6. Properties of ICP47 binding to TAP and competition for ICP47 binding by peptides. (**A**) ^{125}I-Labelled rICP47 was incubated with microsomes derived from insect cells infected with BacTAP or BacgH for 45 min at 4°C in assay buffer. The membranes were washed twice in assay buffer, pelleted by centrifugation and radioactivity associated with the microsomes was counted. For each concentration of ICP47, binding to the BacgH membranes was subtracted from the binding observed with BacTAP membranes. (**B**) Scatchard analysis of the binding of rICP47 was performed as described previously (Johnson *et al.*, 1990). (**C**) Insect microsomes containing TAP were mixed with ^{125}I-labelled ICP47 (0.2 μM) and, at the same time, the peptide library (unlabelled), at various molar ratios relative to the ICP47, for 45 min at 4°C. (**D**) ^{125}I-Labelled rICP47 was incubated with the microsomes for 15 min at 4°C, then various quantities of the peptide library were added for an additional 30 min at 4°C. The membranes were washed and radioactivity associated with the membranes counted. (**E**) Binding of radiolabelled rICP47 was measured as in (A) except that a constant amount of unlabelled peptide library: 6 μM (20×), 12 μM (40×) or 18 μM (60×) was present for each plot. (**F**) A plot of the reciprocal of substrate (ICP47) versus the reciprocal of substrate bound was performed on the data shown in (E).

binding of rICP47 to the microsomes in a dose-dependent manner, so that a 120-fold molar excess of peptide over ICP47 was required to inhibit ICP47 binding by 50% (Figure 6C). In a second experiment, rICP47 was incubated with microsomes for 15 min at 4°C then peptides were added for an additional 30 min and rICP47 binding was measured. In this case, a 450-fold molar excess of peptide over ICP47 was required to reduce binding of ICP47 by 50% (Figure 6D). Thus, ICP47 binds to TAP with a higher affinity than does peptide.

To test more directly whether ICP47 could bind to the peptide binding site of TAP, we determined whether peptides could act as competitive inhibitors of rICP47 binding. Binding experiments involving ^{125}I-labelled rICP47 similar to those described in Figure 6A were performed, except that ICP47 was titrated in the presence of a fixed concentration of peptide (Figure 6E). Plots of the reciprocal of substrate (ICP47) versus the reciprocal of the substrate bound (Segel, 1976) produced lines that intersected at a point close to the *y*-axis (Figure 6F). Therefore, at infinite concentrations of ICP47, its binding is unaffected by different quantities of competitor (peptide). This demonstrates classical competitive inhibition and is strong support for the hypothesis that ICP47 binds to a site on TAP which includes the peptide binding domain of TAP.

The off-rate of ICP47 and peptides from TAP

To examine the stability of the binding of ICP47 to TAP, we compared the dissociation of ICP47 from TAP with that of peptide from TAP. Insect microsomes were incubated with either radiolabelled rICP47 or radiolabelled

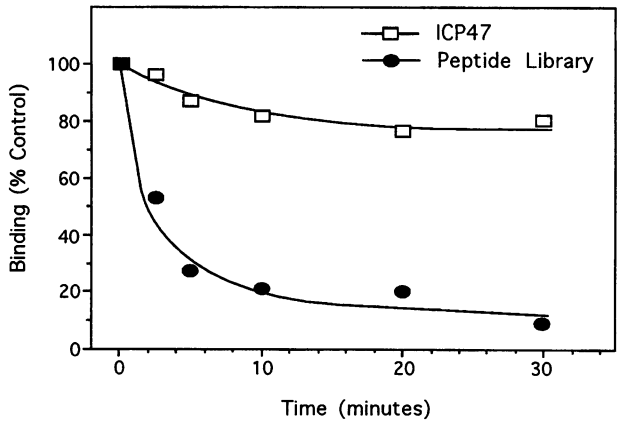

Fig. 7. Comparison of the off-rate of rICP47 versus peptides from TAP. Microsomes derived from insect cells infected with either BacTAP or BacgH were treated with apyrase, washed and then incubated with either radiolabelled rICP47 (open symbols) or radiolabelled peptide library (closed symbols), each at 0.2 μM, for 20 min at 4°C. Membranes were washed once and then resuspended in assay buffer containing 10 mM AMP-PNP. At various times, aliquots of the membranes were pelleted for 5 min and the radioactivity associated with the pellet counted. Binding of peptide or rICP47 to BacgH microsomes was subtracted from that observed with BacTAP microsomes.

peptide library, then the membranes were washed and suspended in buffer containing AMP-PNP. Previously, van Endert *et al.* (1994) showed that ATP or a non-hydrolysable ATP analogue increased the rate of peptide removal from TAP. At various times, the membranes were pelleted and the radioactivity associated with the membranes measured. More than 80% of the rICP47 was present after 30 min of incubation, whereas >90% of the peptides were displaced during this period (Figure 7). In other experiments, in which the microsomes were suspended in buffer containing ATP, similar results were obtained. Therefore, once bound, ICP47 remains associated with TAP in a stable fashion but peptides dissociate more readily.

Membrane topology of the ICP47 binding site

It is conceivable that ICP47 is itself translocated across the membrane, either entirely or in part, to yield the inactive ICP47–TAP complex. We performed proteolysis on TAP-containing microsomes incubated with rICP47 and assayed degradation of ICP47 and TAP. As a control, membranes containing a soluble form of HSV glycoprotein H (gH), which is transported across the ER and lacks a cytosolic domain, were similarly treated with protease. Western blots were performed to detect proteolysis of TAP and rICP47 in the BacTAP microsomes and of gH in the BacgH microsomes. ICP47 was completely destroyed following incubation with proteinase K for 5 min; a polyclonal serum directed to the entire protein did not recognize ICP47 fragments (Figure 8). Similarly, TAP was degraded after a 5 min incubation with proteinase K, although fragments of TAP1 were observed. The gH protein was largely resistant to proteolysis, although there was a faster migrating form which was sensitive to protease and was apparently not glycosylated and inserted into the membrane (Figure 8). gH was degraded when membranes were treated with proteinase K in the presence of detergents (not shown). The sensitivity of ICP47 to

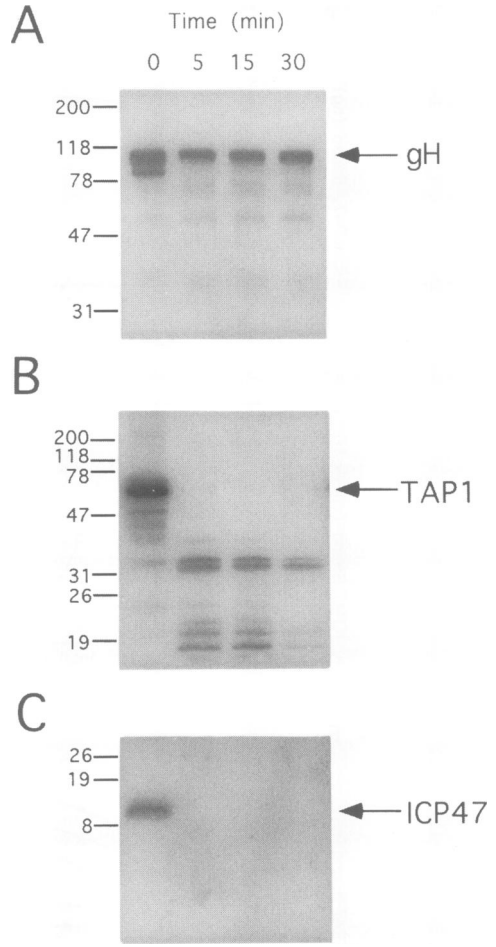

Fig. 8. Proteolysis of TAP and ICP47 bound to TAP in insect microsomes. (**A**) Microsomes from insect cells infected with BacgH were incubated for 30 min at 23°C in the presence of 5 mM ATP. (**B**) and (**C**) Microsomes from BacTAP-infected cells were incubated for 30 min at 23°C in the presence of 5 mM ATP and rICP47 (0.5 μM). Both sets of membranes were washed and resuspended in PBS containing 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 1 mM DTT. Proteinase K (100 μg/ml) was added and incubated with the microsomes for 0, 5, 15 or 30 min then the proteinase K was inactivated by using 1 mM PMSF and the samples were mixed with 2% SDS and 2% β-mercaptoethanol and immediately heated to 100°C for 10 min. The samples were subjected to electrophoresis in SDS gels, proteins transferred to nitrocellulose and blots incubated with either anti-TAP1, anti-gH or anti-ICP47 serum, followed by incubation with [^{125}I]protein A. The blots were dried and exposed to X-ray film.

proteolysis suggests that ICP47 remains confined to the cytosolic side of the microsomal membrane.

Discussion

The role of TAP in mediating peptide transport into the ER for MHC class I antigen presentation is well established, and some of the biochemical and functional properties of TAP have been described (reviewed in Heemels and Ploegh, 1995; Hill and Ploegh, 1995; Howard, 1995). By analogy with other members of the ABC transporter family, TAP1 and TAP2 each span the membrane six or eight times and possess C-terminal, cytosolic domains which contain Walker box motifs that bind ATP (Russ *et al.*, 1995). Both TAP1 and TAP2 are required for peptide transport (Neefjes *et al.*, 1993; Meyer *et al.*, 1994;

van Endert *et al.*, 1994), and recent studies involving cross-linkable peptide analogues have indicated that the peptides bind to residues contributed by both TAP1 and TAP2 (Androlewicz *et al.*, 1994). However, at present, the detailed properties and composition of the peptide binding site of TAP and the mechanism by which peptide is translocated across the ER membrane are not well understood. Thus, studies of HSV ICP47 protein have the potential to provide valuable information on how TAP functions. In addition, by blocking TAP, ICP47 may be useful as a specific inhibitor of class I antigen presentation *in vitro* and *in vivo*.

Here, we have characterized the mechanism by which HSV ICP47 inhibits TAP-mediated peptide translocation across the ER membrane. ICP47 inhibited binding of peptide to TAP, yet there was no observed effect on ATP binding, consistent with earlier observations that peptide and ATP binding are independent (Shepherd *et al.*, 1993; Androlewicz and Cresswell, 1994; Schumacher *et al.*, 1994; van Endert *et al.*, 1994). In other experiments, we found no evidence that ICP47 could, itself, bind peptide or ATP (P.Jugovic and D.C.Johnson, unpublished). To characterize ICP47 binding to TAP, we measured the affinity of ICP47 for TAP and found that the K_D of this binding was $\sim 5 \times 10^{-8}$. This value is ~10-fold lower than for a peptide, R-9-L, ($K_D = 4 \times 10^{-7}$) (van Endert *et al.*, 1994). This specific peptide had a particularly high affinity for TAP, and the majority of other peptides tested in this previous report exhibited lower (10- to 1000-fold) affinities for TAP in competition assays. In our competition experiments involving a peptide library (composed of >2000 9mers), inhibition of ICP47 binding required peptide concentrations 100- to 1000-fold in excess of the ICP47 concentration. In addition, peptides were displaced from TAP much more rapidly than was ICP47, even at 4°C and under conditions where peptide was not transported. Therefore, it appears that ICP47 has a higher affinity than peptides for TAP and, once bound, ICP47 remains in that form for a relatively longer period.

The observation that ICP47 inhibited peptide binding to TAP combined with the finding that peptides inhibited ICP47 binding suggested that ICP47 binds to a site which includes the peptide binding site of TAP. However, to test this hypothesis more directly, we measured whether the binding of ICP47 was competitively inhibited by the peptide library. The results showed conclusively that ICP47 binds to a site on TAP which includes or overlaps the peptide binding site. However, one must consider that ICP47 is an 88 amino acid protein which presents a substantially larger surface for possible interactions with TAP than would be expected with 8–10 residue peptides. More extensive interactions with TAP may allow ICP47 to bind more tightly than peptides, and its size may preclude transport across the membrane. Indeed, ICP47 remained entirely sensitive to protease treatment after binding to TAP under conditions which promote peptide transport. This result is consistent with the notion that the peptide binding site of TAP is accessible to proteases added from the cytoplasmic surface. We conclude that ICP47 binds with relatively high affinity and in a stable fashion to a domain of TAP which includes the peptide binding site.

Remarkably, ICP47 could be heated to 100°C without loss of its ability to inhibit TAP. More recently, the entire 88 residue ICP47 was chemically synthesized and could also effectively block TAP in permeabilized cells (A.Hill and H.Ploegh, unpublished). These observations suggest that, if folding of ICP47 is necessary for its function, the protein must be able to refold after heating or chemical synthesis and attain a conformation that allows binding to TAP. It is also possible that ICP47 does not attain a terminally folded conformation until binding to TAP. Consistent with this hypothesis, circular dichroism measurements suggest that ICP47 is largely disordered in solution (R.Tomazin, A.Edwards and D.C.Johnson, unpublished). These results suggest that ICP47 or fragments of ICP47 might be the starting points for immunosuppressive drugs that would selectively and specifically inhibit TAP.

Previously, we reported that MHC class I presentation in human B lymphoid cells was not affected by ICP47 expression (York *et al.*, 1994). In these and other studies (Posavad and Rosenthal, 1992; Koelle *et al.*, 1993), ICP47 was delivered into the human B cells by infection with HSV or using recombinant Ad vectors. However, since HSV and Ad vectors infect human lymphoid cells poorly and expression of TAP is higher in lymphoid cells than in fibroblasts, it was conceivable that expression of ICP47 in the B cells did not attain levels sufficient to inhibit peptide transport. The studies described here demonstrate clearly that ICP47 can inhibit TAP in B lymphoid cells, although the IC_{50} was perhaps marginally higher than that observed in fibroblasts. In contrast to the results obtained with human B cells, high concentrations of ICP47 did not inhibit TAP in mouse fibroblasts or lymphoid cells, consistent with our previous observations that class I transport and antigen presentation was normal in mouse cells infected with HSV type 1 (York *et al.*, 1994).

The levels of TAP in the insect cell microsomes were apparently 60-fold higher than that observed in VV-TAP1&2-infected human cells. We found that this high level of expression was necessary to analyse accurately the interactions between ICP47 and TAP. Previously, we were unable to detect binding of ICP47, produced by *in vitro* translation, to microsomes derived from human cells (York *et al.*, 1994), although, when TAP expression was increased in these membranes by using VV-TAP1&2, binding of the *in vitro* translated ICP47 could be observed (R.Tomazin, unpublished). Peptide translocation in the human membranes appeared to display a 10- to 15-fold higher specific activity than that observed with the insect cell membranes, similar to previous observations (van Endert *et al.*, 1994). However, since peptide translocation was not measured directly, it is also conceivable that other components, e.g. glycosylation machinery, were limiting in these insect microsomes. These observations are intriguing and may relate to the absence of mammalian membrane proteins, e.g. MHC class I–β_2-m dimers or cytoplasmic factors, or to differences in lipid content and membrane fluidity between mammalian and insect microsomes.

Accompanying the reduced specific activity of TAP in the insect microsomes, we found that ICP47 inhibited TAP most effectively at 23°C, the optimal temperature for insect cell growth, whereas ICP47 was less effective in inhibiting TAP at 37°C. It has been suggested that TAP may exist in two conformations in the insect microsomes,

so that peptide binds better at lower temperatures and in the absence of ATP, whereas transport is maximal at higher temperatures (van Endert *et al.*, 1994). Together, these observations are consistent with the hypothesis that ICP47 functions more effectively under conditions that favour peptide binding, rather than transport. Nevertheless, ICP47 effectively inhibited peptide transport in both the insect (at 23°C) and mammalian microsomes (at 23 or 37°C) with an IC_{50} of ~0.2 μM, suggesting that, under these conditions, ICP47 could bind similarly to TAP in both mammalian and insect membranes. It is possible that binding of ICP47 to TAP is influenced by MHC class I–β_2-m in the complex or that there are defects in folding of TAP in insect cell membranes at 37°C, in the absence of mammalian proteins.

Given the results presented here, how should we view the effects of ICP47 during the course of an HSV infection in the human host? ICP47 is a member of the first class of proteins that is expressed in infected cells. By binding to TAP in a relatively stable manner and inhibiting peptide transport into the ER, it appears that ICP47 can inhibit MHC class I presentation of all other classes of HSV polypeptides, which make up the vast majority of proteins expressed in infected cells. This inhibition is observed in infected human fibroblasts and keratinocytes, which express relatively low levels of TAP, but not in B cells that express higher levels of TAP (Posavad and Rosenthal, 1992; Koelle *et al.*, 1993; York *et al.*, 1994), and which are not important hosts for the virus. The effects of ICP47, and other viral polypeptides, e.g. the vhs protein (Tigges *et al.*, 1996), appear to explain major defects in the CD8+ T lymphocyte response to HSV observed *in vivo* (reviewed in Schmid and Rouse, 1992; York and Johnson, 1995). Very few CD8+ T cells are observed during early stages of HSV lesions, and those that can be observed recognize primarily virion structural proteins which are delivered into cells on infection (Tigges *et al.*, 1992), and are most likely to bypass a TAP blockade. However, the effects of ICP47 on skin cells appear to be largely overcome by treating the cells with interferon-γ (IFN-γ) which up-regulates TAP and other components of the class I presentation pathway (Tigges *et al.*, 1996; R.Hendricks and D.C.Johnson, unpublished). *In vivo*, CD8+ T cells appear in HSV lesions coincident with or following the expression of IFN-γ (Cunningham and Noble, 1989). Thus, the effects of ICP47 may be important in conferring resistance to CD8+ T lymphocytes during early stages of virus replication, allowing virus replication and dissemination, but compensatory immune mechanisms may ultimately allow for recognition and control of virus spread.

Materials and methods

Production of recombinant ICP47 and radioiodination

The HSV-1 ICP47 coding sequences were fused downstream of the GST gene in the plasmid pGEX-2T (Pharmacia) producing plasmid pGEX-2T-47-1. Cultures of *Escherichia coli* (HB101) containing pGEX-2T-47-1 were grown overnight, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h then the cells were suspended in phosphate-buffered saline (PBS) containing an additional 350 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin) and disrupted using a French press. Cell lysates were centrifuged at 12 000 *g* for 30 min then the supernatants were incubated with glutathione–Sepharose (Pharmacia) for 3 h with constant mixing at 4°C. The glutathione–Sepharose was loaded into a small column and washed three times with PBS containing an additional 350 mM NaCl and 0.1% Triton X-100, washed three times with PBS and then incubated with thrombin (Sigma, 0.35 μg/ml) for 45 min at 22°C in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$. The ICP47 (100–400 μg/ml) was then removed from the column by washing with PBS and the thrombin inactivated with 1 mM PMSF. The ICP47 was aliquoted, snap-frozen in liquid N_2 and stored at –70°C. ICP47 (10 μg) was radioiodinated in 50 mM Tris–HCl, pH 7.5 (250 μl) containing 1 mCi Na[^{125}I] and a single Iodobead (Pierce) for 2–15 min, producing material with a specific activity of 1–6×10^7 c.p.m./μg.

Peptide translocation in permeabilized cells

Human lung fibroblasts (CCD-18LU) were obtained from ATCC. Mouse fibroblasts were prepared from mouse embryos and treated with recombinant IFN-γ (50 U/ml) for 24 h prior to the assay, as we previously found that peptide translocation was difficult to measure in these cells without IFN-γ treatment. Fibroblasts and human B lymphoid cells were permeabilized with streptolysin O (Bio Merieux, France) as previously described (Hill *et al.*, 1995); for each assay shown, at least 60% of the cells were unable to exclude Trypan Blue. The cells were incubated with a complex library of 2304 peptides (Heemels *et al.*, 1993) which had been radioiodinated using chloramine T (Hill *et al.*, 1995). ICP47 was either heated or not for 30 min at 95°C then cooled and introduced into the permeabilized cells in conjunction with the peptides in transport buffer (Hill *et al.*, 1995). The amount of peptide transported into the ER after 10 min at 37°C was assessed by lysing the cells with 0.5% NP-40 and applying lysates to concanavalin A (conA)–Sepharose. The conA–Sepharose was washed four times with lysis buffer and radioactivity associated with the conA–Sepharose was counted.

Preparation of microsomes containing TAP from insect and human cells

The construction and characterization of baculoviruses expressing human TAP1 (T1.5) and TAP2 (T2.12) has been described (van Endert *et al.*, 1994).

Sf9 cells were grown in Sf-900II medium (GIBCO) in suspension (4–5×10^6 cells/ml) and 300–800 ml of these cultures were co-infected with either T1.5 and T2.12 baculoviruses or a baculovirus expressing a soluble form of HSV glycoprotein H (BacgH) for 68–72 h. BacgH expresses a truncated form of HSV type 1 gH in which the transmembrane and cytoplasmic domains of gH were removed (L.Hutchinson, C.Roop and D.C.Johnson, unpublished). The cell pellet was suspended in 10 mM Tris–Ac (pH 7.5), 1.5 mM MgAc, 1 mM DTT and protease inhibitor cocktail for 10 min on ice then the cells were disrupted using a Dounce homogenizer and 40 mM Tris–Ac (pH 7.5), 250 mM sucrose, 25 mM KAc, 3.5 MgAc, 0.5 mM CaAc were added before nuclei were removed by centrifugation at 800 *g* for 10 min. Mitochondria were removed by centrifugation at 10 000 *g* for 10 min then the crude microsomes were pelleted by centrifugation at 100 000 *g* for 60 min. The membranes were resuspended in 50 mM Tris–Ac (pH 7.5), 250 mM sucrose, 25 mM KAc, 5 mM MgAc, 0.5 mM CaAc, 1 mM DTT and the protease inhibitor cocktail (microsome buffer) and further purified by centrifugation through a 1.1 M sucrose cushion at 150 000 *g* for 4–5 h. The membrane pellets were resuspended in microsome buffer at 4–5 mg of membrane protein/ml, snap-frozen in liquid N_2 and stored at –70°C. Microsomes were also derived from human KB cells infected with VV-TAP1&2, a vaccinia virus containing the human TAP1 and TAP2 genes (Russ *et al.*, 1995) or infected with control vaccinia vector, VV-gD, which expresses HSV-1 gD (Cremer *et al.*, 1985). KB cells were grown in suspension in Joklik's-modified MEM containing 2% fetal bovine serum (FBS) and 10% horse serum (HS) at 3–6×10^5 cells/ml, concentrated 10-fold and infected with VV-TAP1&2 or VV-gD (using 5–10 p.f.u./cell) for 2 h. The infected cells were resuspended in Joklik's medium containing 1% FBS and 5% HS at 5×10^6 cells/ml and incubated for 10–12 h. Microsomes derived from the human cells were prepared as described for the insect cells except that the hypotonic buffer was 10 mM triethanolamine (TEA; pH 7.5), 1.5 mM MgAc, 1 mM DTT and the microsome buffer was 50 mM TEA (pH 7.5), 250 mM sucrose, 50 mM KAc, 2 mM MgAc, 0.5 mM EDTA, 1 mM DTT containing the protease inhibitor cocktail.

Analysis of TAP levels in microsome preparations

TAP expressed in insect microsomes could be visualized by silver staining (Bio-Rad). In addition, several micrograms of TAP were partially purified from insect cells by immunoprecipitation (York *et al.*, 1994) and used as a standard on Western blots. Western blot analysis of TAP1

expressed in insect and in human microsomes involved anti-TAP1 antibody (Cromme *et al.*, 1994) and [^{125}I]protein A as described (Brunetti *et al.*, 1994), then protein bands were quantified using a PhosphorImager.

Peptide transport into microsomes

A complex library of 2304 peptides described previously (Heemels *et al.*, 1993) was radioiodinated using chloramine T as described (Hill *et al.*, 1995). Peptide translocation was performed as described previously (Meyer *et al.*, 1994) using 20–25 µl of insect microsomes or 60–80 µl of human microsomes except that the conA–Sepharose incubation was performed for 4 h and elution with α-methylmannoside was for 8–12 h. Microsomes were treated with apyrase (Sigma, 20 U/ml) for 5 min at 37°C, or with AMP-PNP (Sigma, 5 mM).

Peptide and ICP47 binding to microsomes

Insect microsomes (20–25 µl) were treated with apyrase (20 U/ml) for 5 min at 37°C then washed and incubated with the ^{125}I-labelled peptide library for 20 min at 4°C in 150 µl of PBS containing 0.1% dialysed bovine serum albumin (BSA), 10 mM $MgCl_2$, 1 mM DTT and protease inhibitor cocktail mix without PMSF (assay buffer). The membranes were washed twice with assay buffer, pelleted by centrifugation at 20 000 *g*, then disrupted in 1% NP-40, 0.5% sodium deoxycholate (DOC), 50 mM Tris–HCl (pH 7.5), 100 mM NaCl (NP-40/DOC buffer) and radioactivity associated with the microsomes counted. For ICP47 binding, insect microsomes (20–25 µl) were incubated for 45–60 min at 4°C with varying amounts of radioiodinated ICP47 diluted in assay buffer. The membranes were washed and disrupted as above and radioactivity associated with the microsomes counted. The binding of ICP47 to microsomes derived from BacgH-infected cells was subtracted from the binding to microsomes derived from BacTAP-infected cells. Scatchard analysis was performed as described (Johnson *et al.*, 1990). Peptide competition for ICP47 binding was performed by incubating various quantitites of peptide, radiolabelled ICP47 (0.2 µM) and insect microsomes (25 µl) for 45–60 min at 4°C in assay buffer or, alternatively, by incubating microsomes with radiolabelled ICP47 for 15 min at 4°C then adding peptides for an additional 30 min at 4°C. To measure the off-rate of peptides and ICP47 from TAP, microsomes derived from insect cells infected with BacTAP or BacgH were treated with apyrase then incubated with radiolabelled ICP47 (0.2 µM) or radiolabelled peptide library (0.2 µM) at 4°C for 20 min. The microsomes were washed once and resuspended in assay buffer containing 10 mM AMP-PNP. Aliquots were taken at various times then the membranes were pelleted for 5 min using a microfuge and the radioactivity associated with the microsomes was counted.

Cross-linking of 8-azido-ATP to TAP

Insect cell microsomes (25–35 µl) were washed and suspended in 300 µl of 25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, and the protease inhibitor cocktail (lacking PMSF). 8-Azido-[^{32}P]ATP (ICN; 3 µCi) was added and incubated with the membranes for 15 min at 4°C in the dark. The microsomes were transferred to cuvettes and photolysed with 254 nm light from a 1000 W mercury source for 10 s. The reactions were then diluted with 700 µl of NP-40/DOC buffer containing BSA (1 mg/ml) and 0.5 mM PMSF and the detergent extracts clarified by centrifugation at 20 000 *g* for 20 min. TAP was immunoprecipitated by using anti-TAP1 antiserum and protein A–Sepharose.

Proteolysis of microsomes

Insect microsomes were washed then incubated with ICP47 (0.5 µM) for 30 min at 23°C in assay buffer containing 5 mM ATP. The membranes were pelleted (20 000 *g* for 5 min) and suspended in PBS containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT. The membranes were cooled on ice for 5 min then 100 µg/ml of proteinase K (Sigma, 20 mAnson U/mg) was added for 0, 5, 15 or 30 min at 4°C. Reactions were stopped by addition of 1 mM PMSF for 2.0 min then 2% SDS, 2% β-mercaptoethanol, 10% glycerol was added and the samples boiled for 10 min before loading on SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and TAP1 was detected using anti-TAP1 antiserum (Cromme *et al.*, 1994), gH was detected using R83 anti-gH serum (Roberts *et al.*, 1991) and ICP47 was detected using anti-ICP47-5 serum (Hill *et al.*, 1995).

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References

Androlewicz,M.J. and Cresswell,P. (1994) *Immunity*, **1**, 7–14.
Androlewicz,M.J., Anderson,K.S. and Cresswell,P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 9130–9134.
Androlewicz,M.J., Ortmann,B., van Endert,P.M., Spies,T. and Cresswell,P. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 12716–12720.
Brunetti,C.R., Burke,R.L., Kornfeld,S., Gregory,W., Masiarz,F.R., Dingwell,K.S. and Johnson,D.C. (1994) *J. Biol. Chem.*, **269**, 17067–17074.
Covitz,K.-M.Y., Panagiotidis,C.H., Hor,L.-I., Reyes,M., Treptow,N.A. and Shuman,H.A. (1994) *EMBO J.*, **13**, 1752–1759.
Cremer,K.L., Mackett,M., Wohlenberg,C., Notkins,A.L. and Moss,B. (1985) *Science*, **228**, 737–740.
Cromme,F.V., Airey,J., Heemels,M.-T., Ploegh,H.L., Keating,P.J., Stern,P.L., Meijer,C.J.L.M. and Walboomers,J.M.M. (1994) *J. Exp. Med.*, **179**, 335–340.
Cunningham,A.L. and Noble,J.R. (1989) *J. Clin. Invest.*, **83**, 490–496.
de la Salle,H., Hanau,D., Fricker,D., Urlacher,A., Kelly,A., Salamero,J., Powis,S.H., Donato,L., Bausinger,H., Laforet,M., Jeras,M., Spehner,D., Bieber,T., Falkenrodt,A., Cazenave,J.-P., Trowsdale,J. and Tongio,M.-M. (1994) *Science*, **265**, 237–241.
Fruh,K., Ahn,K., Djaballah,H., Sempe,P., van Endert,P.M., Tampé,R., Peterson,P.A. and Yang,Y. (1995) *Nature*, **375**, 415–418.
Heemels,M.-T. and Ploegh,H.L. (1995) *Annu. Rev. Biochem.*, **64**, 463–491.
Heemels,M.-T., Schumacher,T.M.N., Wonigeit,K. and Ploegh,H.L. (1993) *Science*, **262**, 2059–2063.
Higgins,C.F. (1992) *Annu. Rev. Cell. Biol.*, **8**, 67–113.
Hill,A. and Ploegh,H.L. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 341–343.
Hill,A., Jugovic,P., York,I.A., Russ,G., Bennink,J., Yewdell,J., Ploegh,H.L. and Johnson,D.C. (1995) *Nature*, **375**, 411–415.
Hill,A.B., Barnett,B.C., McMichael,A.J. and McGeoch,D.J. (1994) *J. Immunol.*, **152**, 2736–2746.
Howard,J.C. (1995) *Curr. Opin. Immunol.*, **7**, 69–76.
Johnson,D.C., Burke,R.L. and Gregory,T. (1990) *J. Virol.*, **64**, 2569–2576.
Jones,T.R., Hanson,L.K., Sun,L., Slater,J.S., Stenberg,R.M. and Campbell,A.E. (1995) *J. Virol.*, **69**, 4830–4841.
Koelle,D.M., Tigges,M.A., Burke,R.A., Symington,F.W., Riddell,S.R., Abbo,H. and Corey,L. (1993) *J. Clin. Invest.*, **91**, 961–968.
Meyer,T.H., van Endert,P.M., Uebel,S., Ehring,B. and Tampé,R. (1994) *FEBS Lett.*, **351**, 443–447
Monaco,J.J. (1992) *Immunol. Res.*, **11**, 125–132.
Muller,K.M., Ebensperger,C. and Tampé,R. (1994) *J. Biol. Chem.*, **269**, 14032–14037.
Neefjes,J.J., Momburg,F. and Hammerling,G.J. (1993) *Science*, **261**, 769–771.
Ortmann,B., Androlewicz,M. and Cresswell,P. (1994) *Nature*, **368**, 864–867.
Posavad,C.M. and Rosenthal,K.L. (1992) *J. Virol.*, **66**, 6264–6272.
Rammensee,H.G., Falk,K. and Rötzschke,O. (1993) *Annu. Rev. Immunol.*, **11**, 213–244.
Roberts,S.R., Ponce De Leon,M., Cohen,G. and Eisenberg,R.J. (1991) *Virology*, **184**, 609–624.
Russ,G., Esquivel,F., Yewdell,J.W., Creswell,P., Spies,T. and Bennink,J. (1995) *J. Biol. Chem.*, **270**, 21312–21318.
Schmid,D.S. and Rouse,B.T. (1992) In Rouse,B.T. (ed.), *Herpes Simplex Virus: Pathogenesis, Immunobiology, and Control*. Springer-Verlag, Berlin, pp. 57–74.
Schumacher,T.N.M., Kantesaria,D.V., Heemels,M.-T., Ashton-Richardt,P.G., Shepherd,J.C., Fruh,K., Yang,Y., Peterson,P.A., Tonegawa,S. and Ploegh,H.L. (1994) *J. Exp. Med.*, **179**, 533–540.
Segel,I. (1976) In *Biochemical Calculations*. 2nd edn. John Wiley, New York, pp. 234–235.
Shepherd,J.C., Schumacher,T.N.M., Ashton-Rickardt,P.G., Imaeda,S., Ploegh,H.L., Janeway,C.A. and Tonegawa,S. (1993) *Cell*, **74**, 577–584.

Suh,W.K., Cohen-Doyle,M.F., Fruh,K., Wang,K., Peterson,P.A. and Williams,D.B. (1994) *Science*, **264**, 1322–1326.
Tigges,M.A., Koelle,D., Hartog,K., Sekulovich,R.E., Corey,L. and Burke,R.L. (1992) *J. Virol.*, **66**, 1622–1634.
Tigges,M.A., Leng,S., Johnson,D.C. and Burke,R.L. (1996) *J. Immunol.*, in press.
Townsend,A. and Trowsdale,J. (1993) *Semin. Cell Biol.*, **4**, 53–61.
van Endert,P.M., Tampé,R., Meyer,T.H., Tisch,R., Bach,J.-F. and McDevitt,H.O. (1994) *Immunity*, **1**, 491–500.
van Kaer,L., Ashton-Rickardt,P.G., Ploegh,H.L. and Tonegawa,S. (1992) *Cell*, **71**, 1205–1214.
Yewdell,J.W. and Bennink,J.R. (1992) *Adv. Immunol.*, **52**, 1–123.
York,I.A. and Johnson,D.C. (1995) In McFadden,G. (ed.), *Viroceptors, Virokines, and Related Immune Modulators Encoded by DNA Viruses*. R.G. Landes Company, Texas, pp. 89–105.
York,I.A. and Rock,K.L. (1996) *Annu. Rev. Immunol.*, in press.
York,I.A., Roop,C., Andrews,D.A., Riddell,S.R., Graham,F.L. and Johnson,D.C. (1994) *Cell*, **77**, 525–535.

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