

Herpes Simplex Virus Type 2 ICP47 Inhibits Human TAP but Not Mouse TAP

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Herpes simplex virus serotype 1 (HSV-1) expresses an immediate-early protein, ICP47, that effectively blocks the major histocompatibility complex class I antigen presentation pathway. HSV-1 ICP47 (ICP47-1) binds with high affinity to the human transporter associated with antigen presentation (TAP) and blocks the binding of antigenic peptides. HSV type 2 (HSV-2) ICP47 (ICP47-2) has only 42% amino acid sequence identity with ICP47-1. Here, we compared the levels of inhibition of human and murine TAP, expressed in insect cell microsomes, by ICP47-1 and ICP47-2. Both proteins inhibited human TAP at similar concentrations, and the K_D for ICP47-2 binding to human TAP was 4.8×10^{-8} M, virtually identical to that measured for ICP47-1 (5.2×10^{-8} M). There was some inhibition of murine TAP by both ICP47-2 and ICP47-1, but this inhibition was incomplete and only at ICP47 concentrations 50 to 100 times that required to inhibit human TAP. Lack of inhibition of murine TAP by ICP47-1 and ICP47-2 could be explained by an inability of both proteins to bind to murine TAP.

Previously, we showed that herpes simplex virus serotype 1 (HSV-1) ICP47 (ICP47-1) caused major histocompatibility complex (MHC) class I proteins to be retained in the endoplasmic reticulum (ER) of cells and that antigen presentation to CD8⁺ T cells was inhibited after ICP47-1 was expressed in human fibroblasts (9). ICP47-1 blocked peptide transport across the ER membrane by TAP (2, 6), so that, without peptides, class I proteins were retained in the ER. By contrast, ICP47 did not detectably inhibit MHC class I antigen presentation in mouse cells (9) and inhibited murine TAP poorly (2, 6). ICP47-1 inhibited peptide binding to TAP without affecting the binding of ATP (1, 7) and bound with high affinity, and in a stable fashion, to human TAP (7). Peptides could competitively inhibit ICP47 binding to TAP, consistent with the hypothesis that ICP47-1 binds to a site which includes the peptide binding domain of TAP (7). Others have suggested that the present data do not exclude a distortion in TAP caused by the binding of ICP47 at a site distant from the peptide binding site (3). This seems improbable given our observations that ICP47 inhibits peptide binding and that peptides competitively inhibit ICP47 binding. In order for peptides to inhibit ICP47 binding and vice versa, one would have to invoke allosteric inhibition by both ICP47 and peptides, a highly unlikely prospect.

The predicted amino acid sequence of HSV type 2 ICP47 (ICP47-2) was recently described (3), and it was of some interest that ICP47-1 and ICP47-2 share only 42% amino acid identity (see Fig. 1A). Most of the homology is near the N termini and in the central regions of the molecules. A peptide including residues 2 to 35 of ICP47-1 blocked human TAP in permeabilized cells (3). This observation was somewhat sur-

prising given that this peptide did not include residues 33 to 51, a sequence that is most homologous between ICP47-1 and ICP47-2. Presumably, this conserved domain, and even the C-terminal third of the protein, is important in virus-infected cells for stability or for functions that are not apparent in this in vitro assay involving detergent-permeabilized cells.

Given the differences between the primary structures of ICP47-1 and ICP47-2, we were interested in whether ICP47-2 might inhibit the murine TAP. If this were the case, it would make possible animal studies of the effects of ICP47. Here, we have produced a recombinant form of ICP47-2 and compared the effects of ICP47-2 and ICP47-1 on human and murine TAP proteins expressed in insect cell microsomes. Like ICP47-1, ICP47-2 efficiently blocked human TAP but even at high concentrations did not effectively block murine TAP. Moreover, there was little or no significant binding of either protein to insect microsomes containing mouse TAP.

The HSV-2 ICP47 gene was subcloned from plasmid pBB17, which contains a *Kpn*I-*Hind*III 8,477-bp fragment derived from the genome of HSV-2 strain HG52 inserted into pUC19, by using PCR to amplify ICP47-2 coding sequences. One PCR primer hybridized with the 5' end of the ICP47-2 coding sequences and extended 5' to generate a new *Bgl*II site just upstream of the initiation codon. The second PCR primer hybridized with 3' sequences of the ICP47-2 gene, then diverged to produce an *Eco*RI site just downstream of the translation termination codon. After PCR, the DNA fragment was digested with *Eco*RI and inserted into the *Hinc*II (blunt) and *Eco*RI sites of pUC19, producing plasmid pUC47-2, which was subjected to DNA sequencing. The ICP47-2 coding sequences were excised from pUC47-2 with *Bgl*II and *Eco*RI and inserted into the *Bam*HI and *Eco*RI sites of pGEX-2T to generate a fusion protein with glutathione *S*-transferase (GST). The ICP47-GST fusion protein was expressed in bacteria and purified by using glutathione-Sepharose, and then the GST sequences were removed with thrombin as described previously for ICP47-1 (7). A comparison between the predicted amino acid sequences of ICP47-2 and ICP47-1 is shown in Fig. 1, with

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A

ICP47-1: **MSWAL**E**MA**D**T** **FLD**T**M**R**V**G**P****R** **TY**A**D**V**R**D**E****I****N** **KR**G**RED**R**E****A****A** ...

ICP47-2: **MSWAL**K**T**T**D****M** **FLD**S**S**R**C**T**H****R** **TY**G**D**V**C**A****E****I****H** **KR**E**RED**R**E****A****A** ...**

ICP47-1: ...**RTAV**H**D**P**E**R**P** **LL**R**S**P**G**L **L**P**E** **I**A**P**N**A**S**L**G**V****A** **H**R**R**T**GG**T**V**T****D** **S**P**R**N**P**V****T****R******

ICP47-2: ...**RTAV**T**D**P**E**L**P** **LL**C**P**D**V**R**S****D** **P**A**S**R**NP**T**Q**Q****T** **R**G**C**A**R**S**N**E**R**Q** **D**R**V**L**A****P**

B

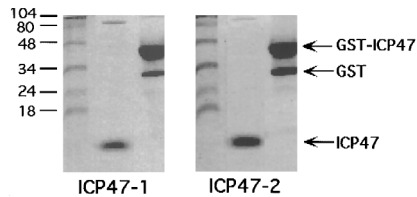


FIG. 1. Comparison of ICP47-1 and ICP47-2 protein sequences and preparation of purified proteins. (A) The predicted amino acid sequences of ICP47-1 derived from HSV-1 strain 17 (6a) and of ICP47-2 derived from HSV-2 strain HG52 (3) are shown. The boldface, underlined letters denote identical amino acids, and the italicized letters denote conserved residues. (B) ICP47-1 and ICP47-2 were produced in *Escherichia coli* by expressing the proteins as GST fusion proteins by fusing the ICP47 coding sequences to GST sequences in plasmid pGEX-2T as described previously (7). Lysates from bacteria were incubated with glutathione-Sepharose and washed several times, and then ICP47-1 or ICP47-2 was eluted by incubation with thrombin, which cleaves between the GST and ICP47 sequences (7). The thrombin was inactivated with phenylmethylsulfonyl fluoride, and the ICP47 preparations were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Bradford protein analysis. The positions of GST-ICP47, GST, and ICP47 protein, as well as those of molecular weight markers 104, 80, 48, 34, 24, and 18 kDa in size, are indicated.

a comparative gel (Fig. 1B) showing the purified preparations of ICP47-1 and ICP47-2 from bacteria.

Microsomes purified from Sf9 insect cells infected with baculoviruses expressing human TAP1 and TAP2 have been described previously (7, 8), as were microsomes from *Drosophila* cells expressing murine TAP1 and TAP2 (1). We previously estimated that approximately 2% of the protein associated with the insect microsomes was human TAP (7), and the microsomes containing mouse TAP possessed similar TAP activity (see below). Peptide translocation by these microsomes was measured by using a library of ^{125}I -labelled peptides (5) that are glycosylated after transport into the ER. Radioactive peptides able to bind to concanavalin A were quantified as an indirect measure of peptide transport (6). Over a range of membranes from 2.5 to 20 μl , with protein concentrations of 10 to 12 mg/ml for human TAP microsomes and 5.0 to 7.0 mg/ml for mouse TAP microsomes, there was a linear increase in peptide transport (Fig. 2). Thus, peptides and ATP were not limiting. Peptide transport was specific because the transport observed with control membranes not containing TAP amounted to less than 1% of that observed when microsomes contained TAP. The levels of peptide transport associated with microsomes containing human or mouse TAP were also compared and standardized. Thus, in subsequent assays, 7.5 to 10 μl of microsomes exhibiting similar amounts of TAP activity were used.

ICP47-2 inhibited peptide transport by human TAP, and the inhibition was similar to that of ICP47-1; the 50% inhibitory concentration (IC_{50}) for ICP47-2 was 0.24 μM and for ICP47-1 was 0.27 μM (Fig. 3A). In other experiments the IC_{50} values

for ICP47-1 and ICP47-2 varied from 0.15 to 0.35 μM , and there were no experiments in which there was a significant difference in the abilities of the two proteins to inhibit human TAP. Moreover, the binding properties of ICP47-2 to human TAP were similar to those of ICP47-1. Binding experiments were performed as described previously for ICP47-1 (7) by using membranes containing human TAP and ^{125}I -labelled ICP47-2. Specific binding of ICP47-2 was calculated by subtracting the binding to control microsomes derived from insect cells infected with a baculovirus expressing HSV gH (7). The binding of ICP47-2 was saturable, so that at a protein concentration of 1 μM approximately 16 ng of protein bound to human TAP (Fig. 4A). In previous experiments with a similar preparation of insect microsomes containing human TAP, the binding of ICP47-1 also saturated at 15 to 16 ng (7). The ICP47-2 binding data were analyzed in a standard Scatchard plot, and the K_D was calculated to be 4.8×10^{-8} M (Fig. 4B), compared with 5.2×10^{-8} M for ICP47-1 (7). These values are greater than those of high-affinity peptides that bind to human TAP with affinities reaching 4×10^{-7} M, though the vast majority of peptides bind to TAP with much lower affinities (8).

To determine whether ICP47-2 could inhibit the murine TAP, microsomes from insect cells expressing mouse TAP were incubated with various concentrations of ICP47-1 and ICP47-2 and TAP assays were performed. Inhibition of the mouse TAP was observed with both ICP47-1 and ICP47-2, but relatively high concentrations of both proteins were required (Fig. 3B). The IC_{50} values for ICP47-1 and ICP47-2 in this experiment were 10.8 and 16.2 μM , respectively. However, we were unable to reduce TAP activity beyond approximately 40% with ICP47-1 or ICP47-2 concentrations reaching 30 μM . This was 100 times the concentration required to inhibit human

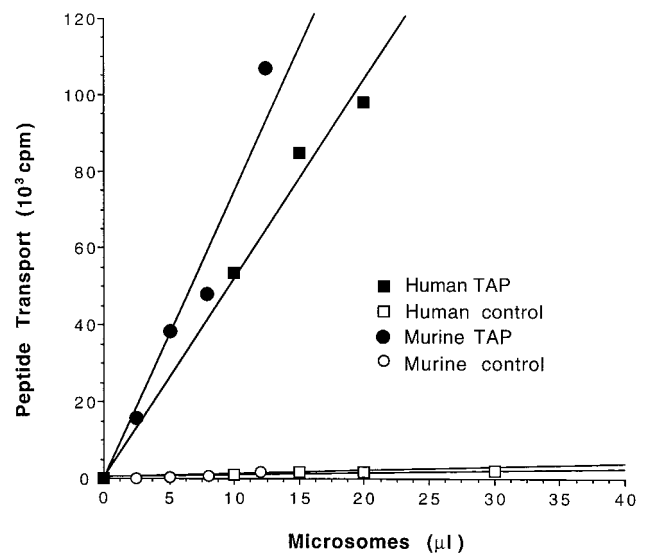


FIG. 2. Peptide transport by insect microsomes containing human or murine TAP. Microsomes were derived from insect Sf9 cells coinfecting with BacTAP1 and BacTAP2 (Human TAP) (7) or from Sf9 cells infected with a control baculovirus, BacgH (Human control). Alternatively, microsomes were derived from *Drosophila* cells induced to express mouse TAP (Murine TAP) (1) or from *Drosophila* cells which were not induced to express mouse TAP (Murine control). Various concentrations of each microsome preparation were incubated with ^{125}I -labelled peptides and 5 mM ATP in a volume of 150 μl for 10 min at 23°C. The microsomes were washed, pelleted, and disrupted in detergent as described previously (7). Peptides able to bind to concanavalin A-Sepharose were eluted with alpha-methylmannoside and quantified (7).

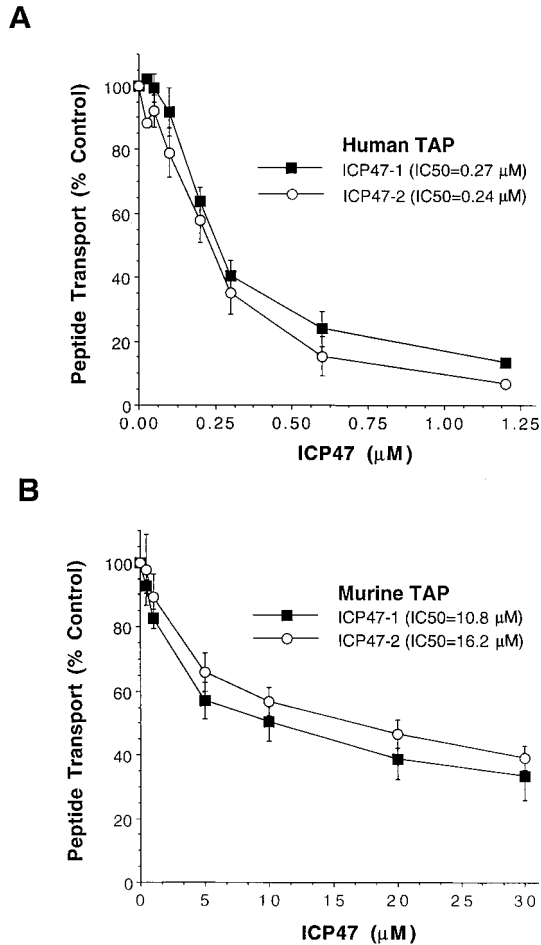


FIG. 3. Inhibition of human and murine TAP-mediated peptide transport by ICP47-1 and ICP47-2. TAP assays were performed as described in the legend for Fig. 2 by using insect microsomes containing human TAP (10 μ l of membranes containing 12 mg of membrane protein per ml) (A) or murine TAP (7.5 μ l of membranes containing 4.8 mg of membrane protein per ml but with equivalent levels of TAP activity compared with microsomes containing human TAP) (B) and various concentrations of ICP47-1 and ICP47-2. The results shown are combined from two separate experiments, each involving human and murine TAP.

TAP by 50%. We attempted to measure the specific binding of radiolabelled ICP47-1 and ICP47-2 to microsomes containing mouse TAP in experiments similar to those shown in Fig. 4. However, there was little specific binding of ICP47-1 and ICP47-2, and it was difficult to measure binding at lower protein concentrations. We therefore measured binding at a single, higher protein concentration (2.75 μ M), one sufficient to inhibit 10 to 20% of the mouse TAP activity and all of the human TAP activity. In this experiment, specific binding to microsomes containing murine TAP was determined by subtracting the binding to microsomes from insect cells that were not induced to express murine TAP (1). The binding of ICP47-1 and ICP47-2 to human TAP was easily measured (Fig. 5), although under these conditions it is important to note that ICP47-1 and ICP47-2 were present at concentrations beyond those required to saturate the TAP (Fig. 4A). By contrast, it was found that there was little or no significant binding of ICP47-1 or ICP47-2 to microsomes containing murine TAP when background binding to control membranes was subtracted. In the experiment shown, specific ICP47-2 binding was

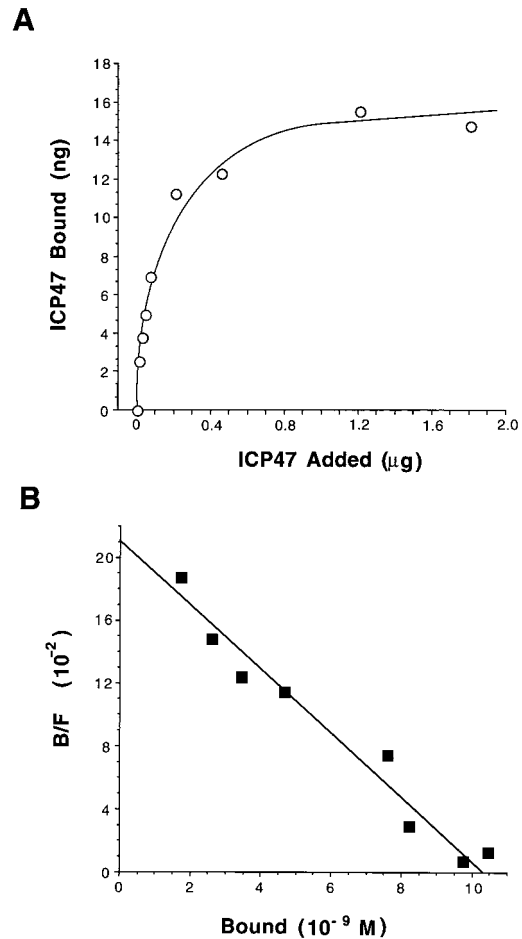


FIG. 4. Binding of ICP47-2 to human TAP. (A) Microsomes (15 μ l of membranes with a 7.5-mg/ml concentration of membrane protein) derived from Sf9 cells expressing TAP1 and TAP2 or expressing HSV-1 gH (control membranes not containing TAP) were incubated with various amounts of 125 I-labelled ICP47-2 for 60 min at 4°C as described previously (7). Binding to control membranes was subtracted from binding to microsomes containing TAP at each point. (B) Scatchard analysis of the data in panel A. The K_D for ICP47-2 binding to TAP was calculated to be 4.8×10^{-8} M.

greater than zero, but in other experiments this binding was less than zero, and thus we concluded that there was no detectable binding overall. In every experiment, it was clear that the level of binding of ICP47-1 and ICP47-2 to murine TAP was at least 25-fold lower than to human TAP. However, the human TAP present in these microsomes was limiting in these experiments, and thus it is very likely that the 25-fold difference between the levels of binding to human and mouse TAP is an underestimate. More likely this difference is 50- to 100-fold. On the basis of the inhibitory concentrations required to block murine TAP and the binding studies described above, estimates of the binding affinities of ICP47-1 and ICP47-2 for murine TAP may fall in the range of 5×10^{-6} M. Therefore, ICP47-1 and ICP47-2 bind poorly to the murine TAP, and this largely accounts for their inability to block mouse TAP peptide transport.

In summary, ICP47-2 and ICP47-1 could block human TAP and bound to TAP with similar high affinities. It was interesting that these two proteins, whose primary structures are only about 40% identical, inhibit human TAP with indistinguishable profiles and bind to human TAP with virtually identical affin-

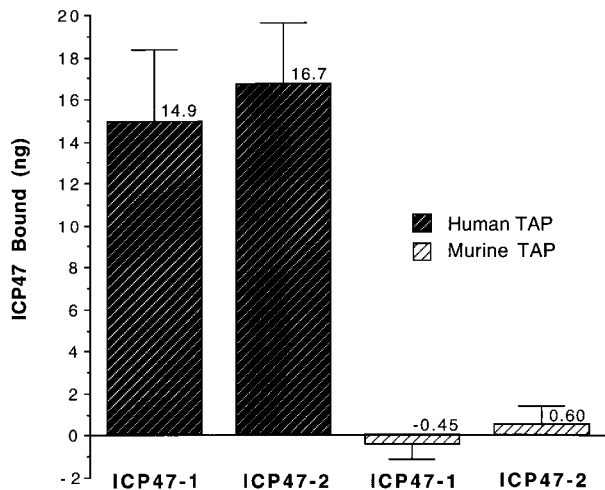


FIG. 5. Binding of ICP47-1 and ICP47-2 to microsomes containing murine TAP. Microsomes containing human TAP or control membranes without human TAP (100 μ g of membrane protein per 150- μ l assay) or microsomes containing mouse TAP or control membranes without mouse TAP (50 μ g of membrane protein with the same TAP activity as with the human microsomes) were incubated with 125 I-labelled ICP47-1 or ICP47-2 at 2.75 μ M for 60 min at 4°C. The microsomes were washed twice, pelleted, and disrupted with detergents as described previously (7). Radioactivity associated with the microsomes was quantified by gamma counting. "ICP47 bound" refers to specific binding, calculated by subtracting the binding to control membranes (without TAP) from that observed with microsomes containing human or murine TAP.

ities. Moreover, both proteins blocked murine TAP poorly and only at high protein concentrations and could not bind to murine TAP. These results, at face value, would suggest that mice will not be an appropriate model in which to test the effects of ICP47 on HSV replication or as a selective inhibitor of CD8⁺ T-cell responses in other systems. However, we recently found that an HSV-1 ICP47 mutant showed dramatically reduced neurovirulence in mice, without altering the course of disease in the cornea (4). Therefore, ICP47 may attain sufficient concentrations in certain cells in the nervous

systems of mice to inhibit TAP. This may be related to the fact that TAP and class I proteins are expressed at low levels in the nervous system. Alternatively, ICP47 may have other functions in the nervous system.

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