The Active Domain of the Herpes Simplex Virus Protein ICP47: A Potent Inhibitor of the Transporter Associated with Antigen Processing (TAP)

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The herpes simplex virus type 1 (HSV-1) protein ICP47 binds specifically to the transporter associated with antigen processing (TAP), thereby blocking peptide-binding and translocation by TAP and subsequent loading of peptides onto MHC class I molecules in the endoplasmic reticulum. In consequence, HSV-infected cells are masked for immune recognition by cytotoxic T-lymphocytes. To investigate the molecular details of this, so far, unique transporter-inhibitor interaction, the active domain and critical amino acid residues were identified by using short overlapping fragments and systematic deletions of the viral inhibitor. A fragment of 32 amino acid residues, ICP47(3-34), was found to be the minimal region harboring an activity to inhibit peptide-binding to TAP comparable to the action of the full-length protein and therefore representing the active domain. Further N or C-terminal truncations cause an abrupt loss in activity. Within the identified active domain, various mutants and chimeras of ICP47 derived from HSV-1 and HSV-2 helped to identify amino acid residues critical for TAP inhibition. On the basis of these results, therapeutic drugs could be designed that are applicable in treatment of allograft rejection or in novel vaccination strategies against HSV, restoring the ability of the immune system to recognize HSV-infected cells.

Introduction

Cytotoxic T-lymphocytes recognize and effectively eliminate virus-infected cells. This requires presentation of viral fragments on the surface of the infected cell in association with major histocompatibility complex (MHC) class I molecules.
membrane and a nucleotide-binding domain. TAP mediates peptide transport into the ER lumen (Neeffes et al., 1993; Shepherd et al., 1993; Androlewicz et al., 1993; Meyer et al., 1994) and its function can be dissected into an ATP-independent peptide-binding step and an ATP-dependent translocation step (van Endert et al., 1994). ATP binding itself does not require the presence of peptides (Müler et al., 1994). The peptide-binding step has been identified to be critical for peptide selection in the subsequent translocation step (Androlewicz & Cresswell, 1994; Uebel et al., 1995; van Endert et al., 1995; Uebel et al., 1997).

Due to the effective pathways of antigen processing and presentation, persistent viruses have been forced to evolve strategies to escape immune surveillance (for review, see Hill & Ploegh, 1995). The immediate-early protein ICP47 (IE12) encoded by herpes simplex virus type 1 (HSV-1) was identified to be responsible for the down-regulation of the MHC class I surface expression in human fibroblasts (York et al., 1994). By specifically binding to the TAP-complex, ICP47 blocks TAP-mediated peptide transport into the ER (Fruh et al., 1995; Hill et al., 1995). On the molecular level, we and others have demonstrated that ICP47 inhibits peptide-binding to TAP, thereby blocking the first and essential step in the translocation pathway (Ahn et al., 1996; Tomazin et al., 1996). The interaction of ICP47 with the TAP complex is highly species-specific, since ICP47 has a 100-fold higher affinity for human than for murine TAP (Ahn et al., 1996). Herpes simplex virus type 2 (HSV-2) encodes a related protein (ICP47-2), which has 65% sequence identity with ICP47-1 within the first 52 to 54 residues (Whitton & Clements, 1984; McGeoch et al., 1985; S. Kohlsättig & U. H. Koszinowski, unpublished results). Apart from these variants of ICP47 encoded by different virus strains, no sequence similarity to any other protein was found in the data base.

In addition to its unique primary structure, ICP47 shows very interesting structural aspects. It appears to be loosely folded (random-coiled conformation) in aqueous solution, thereby limiting approaches to solve the structure at high resolution by solution NMR or X-ray diffraction (Beinert et al., 1997). We found that ICP47 interacts specifically with lipid membranes. Interestingly, this membrane association results in profound conformational changes and a more z-helical structure of the viral inhibitor is formed. These results are consistent with predictions of z-helical regions within the N-terminal half of the ICP47 molecule. Fluorescence analysis revealed that the tryptophan residue at position 3, which is part of a predicted z-helix, participates in the membrane-ICP47 interaction (Beinert et al., 1997).

To identify the active region and essential amino acid residues of the ICP47-TAP interaction, we tested a variety of different deleted or mutated ICP47 molecules, including ICP47 chimeras of HSV-1 and HSV-2, for their ability to block peptide-binding to the human TAP complex. ICP47(3-34) was identified to be the shortest region that reveals nearly the same activity as the full-length protein. Therefore, this 32mer fragment represents the active domain of the viral inhibitor. Within this interacting domain, several charged amino acid residues were found to be essential for inhibition of TAP.

Results

Overlapping 25mer fragments of ICP47 fail to interact with TAP

Peptide-binding was identified to be a prerequisite step in the translocation mechanism of the TAP transporter. HSV escapes immune surveillance by blocking this peptide-binding step, thereby preventing loading of MHC class I molecules. ICP47 reveals a higher affinity to human TAP (Kd 50 nM) than all peptides analyzed so far, thereby being most effective in blocking peptide-binding to TAP. Thus, we were interested in the mechanism underlying this high affinity and in whether ICP47 contains small fragments that are able to block TAP function. In general, human TAP preferentially binds peptides of eight to 16 amino acid residues (van Endert et al., 1994). In order to bypass binding due to a given peptide length, we synthesized overlapping fragments of 25 amino acid residues, which cover the entire sequence of the viral inhibitor, and determined their activity using TAP inhibition assays. Interestingly, none of these fragments was able to inhibit peptide-binding to TAP (Figure 1a). In addition, we analyzed various combinations of these fragments. Although fragments 1-25, 27-51, and 53-77 nearly cover the entire ICP47 molecule, none of these combinations can complement the function of the full-length protein (data not shown). The residual potential of these 25mer fragments to interfere with peptide-binding to TAP might be due to the fact that they still are somehow poor substrates for TAP.

The failure of these peptides to restore ICP47 function might indicate that multiple contact sites embodied by a larger fragment are required. In order to identify these contact sites, we used the 25mer fragments to disturb the interaction between ICP47 and TAP. For this analysis, inhibition of peptide-binding by full-length ICP47 was adjusted to 50%, while, in addition, a large molar excess of different fragments was present. As shown in Figure 1b, none of these fragments, or a combination of these (not shown), was able to interfere with the interaction of ICP47 and TAP. In summary, the failure of these short fragments to block TAP function directly or to affect the ICP47-TAP interaction might indicate that the structural entity of a larger domain is required for the activity of ICP47.

Identification of the active domain of ICP47

In order to identify the essential region required for TAP inhibition, we synthesized N and C-termi-
Finally truncated fragments of ICP47. The purity and activity of these fragments were analyzed by reverse phase chromatography and TAP inhibition assays, respectively (Figure 2). Starting from the C-terminus, deletions revealed that fragment 1-53 still has the same activity as the full-length protein. By Scatchard analysis as well as by competition assays, the affinity constant of ICP47(1±53) was determined to be 50(±12) nM for human TAP (Figure 2b), which is, within the range of error, identical with that of the full-length protein (Kd 50 nM) (Ahn et al., 1996; Tomazin et al., 1996). Moreover, ICP47(1-53) has a 50-fold lower affinity for murine TAP than for human TAP (Figure 2b). Thus, we conclude that deletion of one-third of the protein at the C-terminus does not affect neither the activity nor the species-specificity of the viral TAP-inhibitor. Further deletions of the C-terminus beyond fragment 1-53 initially resulted in a slight decrease in activity, as shown in Figure 2c. However, full activity was found for fragments 1-40 to 1-34. Fragments shorter than ICP47(1-31) fully lost their ability to block peptide-binding to TAP. These results were confirmed by the affinity constants determined for these ICP47 fragments (Table 1). From this set of data, we conclude that the first 34 amino acid residues of ICP47 are essential and sufficient for TAP inhibition.

In analogy, we performed N-terminal truncations of ICP47. Here, only the first and second amino acid residues were found to be dispensable. Further truncations of tryptophan and alanine at positions 3 and 4 resulted in loss of activity, indicating that these residues are critical for ICP47 function (Figure 2d). These results are confirmed by the affinity constants determined for various fragments (Table 1). Combining the data from the C and N-terminal deletions, we finally synthesized fragment ICP47(3–34) and determined its affinity constant, Kd = 89 nM (Table 1). The minor decrease in affinity of ICP47(3–34) as compared to full-length ICP47 might be due to a cumulative effect of the slightly reduced affinities of fragment ICP47(3–53) and ICP47(1–34) and appears not to be very drastic in contrast to the loss of activity observed for shorter fragments. Therefore, ICP47(3–34) was identified to be the active domain of the viral inhibitor for blocking TAP function.

Critical amino acid residues within the active domain

In order to identify critical amino acid residues within the active domain, we pursued two working hypotheses. The first was based on structural aspects predicting a helix-turn-helix motif, whereas the second was derived from a pattern of charged amino acid residues that are highly conserved between the active domain of ICP47-1 and ICP47-2 (Table 2). The first presumption seems particularly attractive, since truncation of the N-terminal predicted α-helix causes a complete loss of activity for ICP47. To see whether a glycine-proline turn motif is essential for ICP47 function, ICP47 mutants were generated that have the turn motif (G18S/P19S) or a segment between both helical regions (HSpH) replaced by serine or glycine residues (Table 2A). As shown in Figure 3, the mutant G18S/P19S has nearly the same activity in inhibition of peptide-binding to TAP as the wild-type fragment, demonstrating that neither the turn motif nor these two amino acid residues are essential for ICP47 function. In contrast, a complete loss of activity was observed for the mutant HSpH, indicating that the regions of predicted α-helicity alone are not sufficient to retain activity. This result led us to the second working hypothesis, based on charged amino acid residues located between the predicted α-helical regions. For technical reasons, during peptide synthesis, we have chosen the active fragment ICP47(1–32), whereby charged residues at positions 20, 24, 31, and 32 were replaced by

Figure 1. The 25mer, overlapping fragments of ICP47 fail to complement the ICP47-TAP interaction. (a) Inhibition of specific peptide-binding (100 nM radiolabeled RRYQKSTEL) to human TAP was assayed in the presence of a twofold (open bars) or a 40-fold molar excess (filled bars) of full-length ICP47 or overlapping 25mer fragments of ICP47. (b) TAP inhibition assays were performed in the presence of 50 nM full-length ICP47 and 5 μM 25mer fragments. The broken line indicates the inhibition of peptide-binding by full-length ICP47 in the absence of fragments.
Figure 2. Inhibition of peptide-binding to TAP by truncated ICP47 fragments. (a) The purity of the ICP47 fragments was analyzed by reversed phase HPLC. ICP47(1-53) (broken line) and ICP47(1-34) (continuous line) was eluted from a C2/C18 column by using an acetonitrile gradient (0.1% TFA) and monitored at 216 nm. (b) Using a TAP inhibition assay the blocking of specific peptide-binding (100 nM radiolabeled RRYQKSTEL) to human TAP (triangles) or murine TAP (circles) was analyzed in the presence of various concentrations of ICP47. The reporter peptide has the same affinity for human and murine TAP. (c) and (d) To identify the active domain of ICP47, the inhibition of peptide-binding to human TAP was assayed in the presence of a tenfold molar excess of full-length, C-terminally (c), or N-terminally truncated ICP47 (d). Data were obtained by duplicate or triplicate measurements (error bars: ±SD).

Table 1. Affinity constants of ICP47 fragments for human TAP.

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<thead>
<tr>
<th>fragments of ICP47</th>
<th>$K_d$ [nM]</th>
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<tr>
<td>full-length (HSV-1)</td>
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<tr>
<td>C-terminal deletion (HSV-1)</td>
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<td>N-terminal deletion (HSV-1)</td>
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<td>shortest active fragment (HSV-1)</td>
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<td>ICP47 (HSV-2)</td>
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Affinity constants were determined by TAP inhibition assays. Human TAP-containing microsomes were incubated at 4°C for 45 minutes with 100 nM radiolabeled RRYQKSTEL in the presence of various concentrations of the ICP47 fragments or non-labeled RRYQKSTEL. The $K_d$ values of the ICP47 variants were determined by comparing the inhibition of peptide-binding caused by the ICP47 fragments with that of the non-labeled RRYQKSTEL. The $K_d$ value of RRYQKSTEL is known to be 145 nM. Data were derived from at least four measurements (error: ±SD).
glycine (Table 2). These mutants were tested in TAP inhibition assays (Figure 3). The single mutants or the double mutant R20G/D24G showed a reduced ability to block peptide-binding to TAP as compared to the wild-type ICP47(1-32). The triple mutant D24G/K31G/R32G lost its activity almost completely. These results demonstrate that the examined charged residues within the active domain are critical for ICP47 function.

Chimeras of ICP47 derived from HSV-1 and HSV-2

In general, chimeras constructed between two homologous proteins harboring distinct activities are helpful tools to identify critical amino acid residues or essential regions involved in the function of a given protein. As shown in Table 2B, the first 52 to 54 residues covering the active region are highly conserved between ICP47 encoded by HSV-1 and HSV-2. Interestingly, we found that ICP47 fragments derived from HSV-2 show a significantly reduced activity in blocking peptide-binding to TAP in comparison to ICP47(1-32). The triple mutant D24G/K31G/R32G lost its activity almost completely. These results demonstrate that the examined charged residues within the active domain are critical for ICP47 function.

Table 2. Primary and predicted secondary structure of ICP47 encoded by HSV-1 or HSV-2.

| A | Secondary structures are predicted by MacMatch 1.1d (University of California, San Francisco). Similar results were obtained by using the BCM Protein Secondary Structure Prediction Program (NNSSP), the PredictProtein Service (EMBL, Protein Design Group, or the Genetic Computer Group (GCG, Madison, Wisconsin). A or T indicate the \( \alpha \)-helix or turn motifs, respectively. The first helical region is well determined (sources from 8 to 9), whereas the second is less defined (sources from 5 to 8). For ICP47(3-45), mutated residues are underlined and highlighted by superscription. B, Sequence comparison of full-length ICP47 derived from HSV-1 and the partial sequence of ICP47-2 as found in the data base (Whitton & Clements, 1984). |

| B | The sequences of these mutants are summarized in Table 2A. |

which cover most of the divergent residues of the homologous region. Two different approaches were followed. First, chimeras containing only small regions in the active ICP47(1-32) replaced by the type 2 sequence were analyzed (Figure 4a). The insertion of the HSV-2-encoded sequence MPS at positions 10 to 12 causes a drastic reduction of its activity, whereas exchanges of the other divergent amino acids lead to only minor effects. Substitutions of the sequence KTT and CA at positions 6 to 8 and 26-27 resulted in a slightly enhanced activity. In a second approach, chimeras were generated in which the type 1 sequence was transformed step-by-step into an ICP47-2 (Figure 4b). Chimeras that possess the HSV-2 sequence up to position 10 were as active as the wild-type fragment, whereas the insertion of proline at position 11 causes the drastic loss of its ability to inhibit TAP function. These data are in line with the results of the first approach. Combining both sets of data, the proline residue at position 11 was identified to be destructive for the activity of ICP47. This conclusion was independently confirmed by the mutant F11P of ICP47(1-32)-1, which has a significantly lower activity (data not shown). Interestingly, this proline residue is located within an \( \alpha \)-helical region predicted for ICP47-1. Thus, the loss in activity caused by the proline substitution in ICP47-2 might correlate with disrupting the \( \alpha \)-helix in this region. As ICP47(1-32) underwent further metamorphosis, only slight variations in the low activity are observed, indicating that the rest of the divergent residues between ICP47-1 and ICP47-2 play only a minor role in TAP inhibition.
Discussion

The herpes simplex virus protein ICP47 blocks peptide transport into the ER by preventing peptide binding to the TAP complex. As a consequence, virus-infected cells escape immune recognition by cytotoxic T lymphocytes. Using overlapping or stepwise truncated fragments of the viral TAP-inhibitor, a 32mer peptide, ICP47(3-34), was identified as the active domain, revealing nearly the same activity as the full-length protein in blocking TAP function. Further truncations at the N or C terminus result in a rapid loss of activity. Furthermore, we have identified critical charged residues within the active domain that are highly conserved between ICP47 encoded by HSV-1 and HSV-2. Substitution of aspartate, lysine and arginine with glycine at positions 24, 31 and 32 causes a complete loss of activity. By contrast, similar modifications at positions 18 and 19 or most of the divergent residues between the ICP47 sequence of HSVI-1 and HSV-2 had no, or only a minor, effect on the activity of ICP47. Because peptides require free N and C termini for their binding to TAP (Androlewicz & Cresswell, 1994; Uebel et al., 1997), one might speculate that the basic and acidic side-chains of ICP47, which are separated by a distance resembling that between peptides ideal for recognition by TAP, mimic the free terminal carboxy or amino group. However, fragment 14-38 alone, containing the conserved, charged residues, but lacking the first predicted helical region, is not sufficient to block TAP function.

Beside these sequence characteristics, ICP47 has some interesting structural properties. The viral TAP inhibitor appears to be loosely folded (random-coiled conformation) in aqueous solution. However, in the presence of lipid membranes, drastic conformational changes were observed, and upon binding to negatively charged membranes, an \(\alpha\)-helical conformation was induced. Interestingly, full-length ICP47 and ICP47(1-53) behaved nearly identically, indicating that the active domain might be involved in structural reorganization upon binding to the membrane and the TAP-complex. Evidence was provided that the tryptophan residue interacts directly with the membrane (Beinert et al., 1997). These experimental results are in agreement with secondary structure predictions, proposing two \(\alpha\)-helical regions (residues 3 to 13 and 35 to 45) within the first N-terminal half of the protein (Table 2A). Interestingly, the tryptophan and alanine residues at positions 3 and 4, which promote helix formation, are essential for ICP47 function. Vice versa, insertion of proline at position 11, which is known to break \(\alpha\)-helices, causes a drastic loss of activity. Taken together, these data point to the significance of the predicted secondary structural elements. C-terminal truncations and various mutations indicate that the second predicted \(\alpha\)-helical region and the turn-motif is dispensable. In conclusion, we propose a model for the active domain of ICP47 that consists of three sections. The first segment is formed by a predicted \(\alpha\)-helical region (residues 3 to 13). Here, reduction of the probability of helix formation by truncation or proline insertion correlates with a loss in activity. Furthermore, it was demonstrated that this segment, particularly the tryptophan residue at position 3, interacts with the membrane (Beinert et al., 1997). This region might serve as a membrane anchor to increase the local concentration of the TAP inhibitor, as has been observed for various peptide hormone receptors (Schwyzer, 1995). The second segment (residues 14 to 34) is supposed to interact with the substrate binding site via a pattern of charged amino acid side-chains. Interestingly, these charged amino acid residues are conserved between ICP47 encoded by HSV-1 and HSV-2. For specific interaction, these residues must be optimally arranged, possibly due to the help of the first segment, since the first and the second segment are active only...
in combination. This combination, a 32mer fragment, forms the active domain in respect to blocking TAP function. Interestingly, considering that particularly the conserved charged residues are important for interacting with human TAP, the sequence variations found between ICP47-1 and ICP47-2 within that region do not drastically affect the activity of the viral inhibitor. The third segment, residues 35 to 88, is not required for TAP inhibition. Considering that due to evolutionary pressure viruses have been forced to condense multiple functions to a limited genome size, it seems attractive to speculate whether this apparent redundant region has a second, and so far unknown function. Furthermore, in this respect, it is surprising that the most conserved region (residues 35 to 52) with only two divergent residues is not needed for TAP inhibition (Table 2B).

During the refereeing procedure of this paper, Galocha et al. (1997) reported also on the characterization of the active region of ICP47. Their conclusions are largely in agreement with our results, although different assay systems were used. However, in contrast to our study, ICP47 derived from HSV-2 (strain HG52) was found to be as active as ICP47-1. Most interestingly, ICP47 of HSV-2 differed by two amino acid residues (P11F and S12L) from the published, partial ICP47 sequence of HSV-2 strain HG52 (Whitton & Clements, 1984) as well as from the ICP47 sequence of HSV-2 strain 186 (S. Kohlstädt & U. H. Koszinowski, unpublished results), the last two being identical and given in Table 2. Because of the exchange of two amino acid residues and four base-pairs, the difference can hardly be explained by a PCR error during sequencing. Thus, it has to be clarified why both ICP47-2 sequences from HSV-2 strain HG52 submitted by Whitton & Clements (1984) and Galocha et al. (1997) differ by these amino acid residues. The difference confirms our conclusion concerning the proline residue at position 11 to be responsible for the reduced activity of the ICP47-2 protein as examined in this work. In addition, this highlights exciting epidemic aspects of HSV-2. Mutations in various HSV strains could result in variants of ICP47 that harbor different potentials to block TAP function. Thus, altered pathogenicity of HSV strains may be related to variants of ICP47.

Here, we have identified the active domain and critical residues involved in this unique inhibitor-transporter interaction. Based on these results, it may be possible in the future to design peptidomimetics based on ICP47(3–34) that are specific and highly potent inhibitors of the TAP transporter. By increasing the stability against proteolysis and by solving the targeting aspects of these ICP47 derivatives, therapeutic drugs could be developed that act as effective immune suppressors in the treatment of allograft rejection or MHC class I linked diseases. In addition, novel virus-based gene shuttles can be engineered, encoding the DNA of the active domain and therefore able to protect transformed cells from lysis by virus-specific T-lymphocytes. Finally, novel vaccines could be developed that specifically interfere with the unique TAP-ICP47 interaction and thereby restore the ability of the immune system to eliminate the persistent virus from the human body.

Materials and Methods

Preparation of TAP-containing insect cell microsomes

The heterologous expression of human and murine TAP in insect cells has been reported (Meyer et al., 1994; Ahn et al., 1996). TAP-containing microsomes were isolated by a combination of differential and sucrose density-gradient centrifugation (Meyer et al., 1994; Uebel et al., 1995). The microsomal membranes were resuspended in phosphate-buffered saline, 1 mM 1,4-dithio-DL-threitol, snap-frozen in liquid nitrogen and stored at −80°C.

Isolation of full-length ICP47

Full-length ICP47 was expressed in Escherichia coli as a 6 × His fusion protein (His-ICP47) and purified by metal affinity chromatography and reversed phase HPLC as described (Ahn et al., 1996). Purity of the protein was analysed by SDS-PAGE and reversed phase HPLC.

Synthesis of variants of ICP47

Synthetic fragments of ICP47 were prepared by solid phase synthesis on a multiple peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt; Software Syro, MultiSynTech, Bochum) according to Fmoc/tBu-strategy (Jung & Beck-Sickinger, 1992). Peptides were synthesized on p-benzoyloxybenzyl alcohol resin preloaded with Fmoc-amino acids. Fmoc-protected amino acids were activated with disopropylcarbodiimide/1-hydroxybenzotriazol in DMF and coupled in tenfold excess for 90 minutes. The N-terminal protecting group was cleaved by piperidine/DMF (1:1, v/v). Side-chains of the amino acids were protected as follows: D(tBu), E(tBu), T(tBu), N(Trt), Q(Trt), R(Pmc), H(Boc), C(Trt), W(Boc). Peptides were cleaved from the resin using Reagent K (1 ml) containing 82.5% TFA, 5% (w/v) phenol, 5% (w/v) thioanisol, 2.5% (w/v) ethane dithiol, 5 mM MgCl₂, 0.05 % polyethylene glycol 6000) to a final protein concentration of 25 mg/ml. The protein concentration was determined using the MicroBCA protein assay (Pierce).

TAP inhibition assay

TAP-containing microsomes were diluted with ice-cold assay buffer (phosphate-buffered saline with 1 mg/ml dialyzed bovine serum albumin, 1 mM 1,4-dithio-DL-threitol, 5 mM MgCl₂, 0.05 % polyethylene glycol 6000) to a protein concentration of 25 μg/ml. The protein concentration was determined using the MicroBCA pro-
tein assay (Pierce). The suspension was homogenized by drawing through a 23-gauge needle. From this suspension, 150 μl was incubated with radiolabeled RRYQKSTEL (100 nM) in the presence of a molar excess of the corresponding ICP47 molecule. In order to reach equilibrium for all ICP47 variants, samples were incubated for 45 minutes on ice. During that period peptides or ICP47 molecules are not modified or degraded. Subsequently, 350 μl of cold assay buffer was added and the microsomes were pelleted by centrifugation (12,000 × g for eight minutes at 4°C). After washing with 500 μl of cold assay buffer, microsome-associated radioactivity was quantified by γ-counting. The amount of bound peptide was background corrected for non-specific binding in the presence of a 400-fold molar excess of unlabeled peptide, which corresponds also to 100% inhibition of peptide-binding to TAP. To determine the dissociation constant of ICP47 derivatives (Kd) the inhibitor concentration was varied over three orders of magnitude and the data were fitted by a competition function as described (Uebel et al., 1995). The activity of the ICP47 variants was measured by at least two independent assays. Competition by unlabeled RRYQKSTEL (Kd = 145 nM) served as an internal reference. Peptides were radiolabeled as described (Meyer et al., 1994).

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