Peptides Containing Membrane-transiting Motifs Inhibit Virus Entry*

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Several exceptional peptides have been identified that can cross plasma membranes and deliver various covalently linked moieties into cells. We report the surprising observation that each of four structurally distinct transiting peptides tested displayed antiviral activity and inhibited herpes simplex virus entry into cells. All four peptides inhibited infection at concentrations in the low micromolar range. Some of the peptides selectively and reversibly blocked entry without inactivating virions in a persistent manner. For other peptides, the effects on virus entry were not readily distinguishable from virus inactivation. High concentrations of nearly all peptides lead to irreversible inactivation of virions. By various criteria, the peptides differed in their ability to inactivate virions and in the temperature dependence of inactivation. Testing of peptides with modifications known to disrupt transport revealed that, in some instances, transport activity did not correlate with antiviral activity. These results identify inhibition of viral entry as another common property of membrane-transiting peptides in addition to their ability to cross membranes and transport materials into cells. These or related peptides may be useful as agents to prevent infection and to study the process of viral entry.

Protein-protein interactions are critical in many biological processes including numerous steps in viral infection. Peptides that interrupt protein-protein interactions thus have considerable potential as tools to elucidate the basic mechanisms underlying these processes and as pharmaceutical agents. As an example, the carboxyl terminal 9 amino acids of the small subunit of herpes simplex virus (HSV) 3 ribonucleotide reductase (RR2) can disrupt the RR1:RR2 complex and inhibit enzymatic activity (1, 2). A peptidomimetic derived from the nonapeptide was subsequently shown to have antiviral activity and inhibit replication in vivo (3). A series of synthetic peptides have also been used to identify potential heparin sulfate binding sites in the HSV gC protein (4), and a similar strategy was used to map the interaction site between the HSV UL42 protein and DNA polymerase (5). These findings support the potential use of peptides for numerous purposes; however, since the majority of peptides do not enter cells, their potential as antiviral agents is severely limited.

Recently, several exceptional peptides have been identified that can cross plasma membranes and deliver various covalently linked moieties into cells (6–10). Initially, we were interested in determining whether the membrane-transiting proteins (MTPs) could transport a known protein-protein disrupting peptide into cells, resulting in inhibition of viral replication. We chose to use the carboxy terminal nonapeptide from the HSV RR2 (1, 2) coupled to one or more MTPs to test the strategy. While testing these MTPs for their ability to co-transport potential antiviral peptides into cells we previously found that a modified version of one, denoted EB, inhibited HSV in the range of 1–10 μM and specifically inhibited viral entry into cells (11). In this paper, we report the surprising finding that all MTPs tested to date display antiviral activity, acting to block the entry step in infection. At low peptide concentrations, inhibition of entry was clearly the dominant effect. The different MTPs, however, did not act uniformly but differed in their ability to inactivate virions and in the temperature dependence of inactivation. Antiviral activity was also not directly correlated with transport activity for some of the peptides. Our results identify inhibition of viral entry as another common property of these MTPs in addition to their ability to cross membranes and transport materials into cells.

EXPERIMENTAL PROCEDURES

Virus and Cell Culture—Unless otherwise noted, all studies were carried out in Vero cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% calf serum and 5% fetal bovine serum and grown to confluency in 96-well plates (2 × 104 cells per well) over a period of 4 days (11). The HSV-1 KOS mutant hrR3, which expresses Escherichia coli β-galactosidase from the early ICP6 promoter (12), was used for all studies. High titer stocks of early ICP6 promoter (12), was used for all studies. High titer stocks of virus were prepared as described previously (13).

Peptides—Synthesis and analysis of peptides were done at the Biotechnology Center of the University of Wisconsin-Madison. Synthesis was carried out at a 25-μmol scale using an automated synthesizer (Applied Biosystems model 432A “Synergy”) following the principles initially described by Merrifield (14) with modifications by Meienhofer et al. (15) and Fields et al. (16). The cleaved peptides were precipitated with cold t-butylmethylether and dissolved in water. The h2STAT-9 peptide was obtained by selectively acetylated an aliquot of the h2STAT-9 peptide with acetic anhydride in solution. The relative mass of all peptides was confirmed by electrospray ionization mass spectrometry. The purity of the peptides was determined by analytical HPLC. Some peptides were further purified by preparative HPLC. The purity...
of peptides used in these studies is listed in Table I. Peptide concentrations were determined by absorbance readings (11), and we always refer to concentrations of the pure peptide.

Antiviral Assays—For comprehensive treatments, cells and virus were exposed to peptide for 1 h prior to infection and were kept in media containing peptide until the number of infected cells was scored 8 h later. Cells were infected at a m.o.i. of 0.08, and infection was measured by staining cultures with X-gal (11). For antiviral assays with preadsorbed virus, precooled cells were infected at an m.o.i. of 0.025 for 1 h at 4 °C to allow the virus to adsorb to the cells. Unadsorbed virus was rinsed off with ice-cold serum-free DMEM, and cultures were exposed to concentrations of the pure peptide. 

Antiviral activity was measured by absorbance readings (11), and we always refer to concentrations of the pure peptide. Amino acid sequences are given in single letter code; charged lysine and arginine residues are shown in bold letters; dextral amino acids are indicated by d; a biotin-aminohexanoyl; underlining highlights structural differences among peptides.

### RESULTS AND DISCUSSION

To determine whether antiviral activity was a common property of the MTPs, we chose four of the best characterized peptides containing differing membrane-transiting motifs. The sequences of the peptides are shown in Table I. To EB peptide, included for comparison, consists of the FGF4 signal peptide (h-region (18)) attached to an RRKK tag to enhance solubility. The HOM peptide is a wholly synthetic amphipathic helical 18-mer that has been shown to enter cells (19). The HOM peptides are derived from the homeodomain of the Drosophila antennapedia protein (residues 48–58) in which the glutamic acid at position 50 was replaced by proline to target the peptide to the cytoplasm (20). Finally, the TAT peptides include the membrane-transiting sequence of the human immunodeficiency virus tat protein (residues 47–58 (21)), which also functions as a nuclear localization sequence (22). The antiviral activities of the peptides were examined in three standard assays with the mutant HSV-1 KOS hrR3, which expresses β-galactosidase from an early promoter (12). In the first assay, which we refer to as comprehensive, virus and cells were exposed to peptide for 1 h prior to infection, and peptide was present during adsorption and continuously thereafter for a total of 10 h. In the second assay, virus was allowed to attach to cells at 4 °C before peptide was added for 1 h at 4 °C and 1 h at 37 °C. The cells were then switched to peptide-free medium for an additional 8 h. Finally, to test for direct inactivation of virions, virus was incubated with peptide for 1 h in solution and then titrated for remaining infectivity.

As shown in Fig. 1 and Table II, all peptides containing intact membrane-transiting motifs inhibited infection of Vero cell cultures in comprehensive assays at low micromolar concentrations (IC50 = 2–12 μM). The peptides also inhibited preadsorbed virus, although eight times more peptide was required (IC50 = 7–26 μM) when adjusted for the different inputs of virus. The ability of the peptides to inhibit preadsorbed virus indicated that they acted subsequent to viral attachment to cells. Furthermore, inhibition of the preadsorbed virus was irreversible or at least not fully reversible during the first 8 h following removal of the peptide.

To rule out cytotoxic effects, we measured cell viability in the presence of various concentrations of peptide using trypan blue staining. The results are summarized in Table II. In comprehensive assays and in assays with preadsorbed virus, the inhibition of infection was independent of any cytotoxic effects of the peptides. The EB and bTAT-9 peptides were more cytotoxic in serum-free than in serum-supplemented medium, despite the fact that treatments with peptides in serum-free medium lasted for only 2 rather than 10 h. This is most likely due to direct interactions of these peptides with serum proteins.2

2 All but two of the peptides (bHOMFF and bTAT-9X) directly inactivated virions in solution in a way, or in ways, that were not immediately reversible by peptide dilution (Table II, “Direct inactivation of virions” column) or, in the case of EB, by

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**Table I**

| No. | Peptide   | Purity | Sequence                          |
|-----|-----------|--------|-----------------------------------|
| 1   | EB        | 96     | NH2-RKKAALIPAVLLALAP-COOH          |
| 2   | bEB       | 74     | NH2-RKKAALIPAVLLALAP-COOH          |
| 3   | bKL       | 93     | NH2-RKKAALIPAVLLALAP-COOH          |
| 4   | bKLAd     | 98     | NH2-RKKAALIPAVLLALAP-COOH          |
| 5   | bKLAd1    | 97     | NH2-RKKAALIPAVLLALAP-COOH          |
| 6   | bHOM-9    | 79     | NH2-RKKAALIPAVLLALAP-COOH          |
| 7   | bHOMM     | 99     | NH2-RKKAALIPAVLLALAP-COOH          |
| 8   | bHOMd     | 63     | NH2-RKKAALIPAVLLALAP-COOH          |
| 9   | aHOMd     | 81     | NH2-RKKAALIPAVLLALAP-COOH          |
| 10  | bHOMFF    | 87     | NH2-RKKAALIPAVLLALAP-COOH          |
| 11  | bTAT-9    | 81     | NH2-RKKAALIPAVLLALAP-COOH          |
| 12  | bTAT-9X   | 90     | NH2-RKKAALIPAVLLALAP-COOH          |
| 13  | b2aTAT-9  | 96     | NH2-RKKAALIPAVLLALAP-COOH          |

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dialysis (11). We previously showed for EB that although the inactivation of virions was not reversible, peptide exposure did not disrupt the structure of virions (11). Inactivation of virions was, however, not solely responsible for the antiviral effect (Table II and Fig. 1), since peptides differing 10-fold in their ability to inactivate virions inhibited infection at similar concentrations, regardless of whether the peptides were related to each other (e.g. peptides 6 and 7, Table II). Inactivation of virions was temperature-dependent but did not correlate with the effects of temperature on membrane transiting activities. Thus, shifting the temperature from 37 to 4 °C reduced inactivation of virions by EB only 2-fold (Fig. 1B), whereas peptide uptake mediated by the FGF4 signal sequence was abolished at this temperature (18). Conversely, inactivation of virions by

![Image](https://example.com/image1.png)

**FIG. 1. Antiviral properties of peptides with membrane-transiting motifs.** Comprehensive inhibition of infection (△), inhibition of preadsorbed virus (○), and direct inactivation of virions at in solution at 37 °C (●) and 4 °C (○) are shown for four peptides identified below each panel. The IC_{50} values are shown in Table II. The data in this and all other figures and in Tables II and III are means of triplicate determinations with S.E. of the means.

**Table II**

Antiviral and cytotoxic properties of synthetic peptides

All data represent micromolar peptide concentrations required to inhibit hrR3 or trypan blue exclusion (cytotoxicity assay) by 50% (IC_{50}). Where known, the range of independently determined IC_{50} values are given. ND = not determined. All IC_{50} values were determined from dose-response curves such as those shown in Fig. 1. Underlined peptides have altered membrane transiting sequences.

| No. | Peptide | Comprehensive virus inhibition | Inhibition of preadsorbed virus | Direct inactivation of virions | Cytotoxicity |
|-----|---------|-------------------------------|-------------------------------|-----------------------------|-------------|
|     |         |                               | at 37 °C                       | at 4 °C                     | 10 h        | 2 h        |
| 1   | EB      | 4                             | 15–26                         | 44                          | 5–60        | 100–150    |
| 2   | bEB     | 11                            | 15                            | 35                          | 100–200     | 110        |
| 3   | EBX     | 23                            | >200                          | 110                         | >600        | >200       |
| 4   | bKLA    | 4                             | 11                            | 45                          | 15          | >100       |
| 5   | bKLA_{11,12} | 4                        | 12                            | 60                          | 30          | 30–60      |
| 6   | bHOM-9  | 6–9                           | 9–15                          | 110                         | >600        | >250       |
| 7   | bHOM    | 11                            | 15                            | >1000                       | >600        | >200       |
| 8   | bHOMd   | 2–3                           | 7                             | 15                          | 160         | 41–66      |
| 9   | aHOMd   | 5                             | 9                             | 300                         | >600        | >250       |
| 10  | bHOMFF  | 34                            | 40                            | >600                        | >600        | >200       |
| 11  | bTAT-9  | 8–12                          | 23                            | >600                        | >600        | >200       |
| 12  | bTAT-9X | 16                            | 35                            | >600                        | >600        | >200       |
| 13  | b2aTAT-9 | 62                       | >200                           | ND                          | ND          | ND         |

![Image](https://example.com/image2.png)
bHOM-9 was abolished at 4 °C (Fig. 1C), whereas cellular uptake of the HOM-MTP was only slightly reduced (18). These results indicate that viral inactivation, inhibition of infection, and membrane translocation are not always correlated.

Comparisons of the effects of sequence modifications demonstrated that for each of the four types of peptides, the presence of the membrane-transiting motifs was critical for antiviral activity. Confirming previous results using slightly different conditions (11), we found that the scrambled EBX peptide was less effective than the EB peptide (Table II, numbers 1 and 3). We also found that biotinylation of EB did not greatly alter antiviral properties (Table II, peptide 2). For the bKLA peptide, replacing the L-amino acids at positions Leu11 and Lys12 with the corresponding D-amino acids (bKLAd11,12) reduced uptake inhibitory if the incubations were prolonged (Fig. 2). Lower concentrations of the bHOMFF peptide were not blocking activity depended on the presence of intact MTP motifs (20).

The bHOM-9 peptide consists of the HOM-MTP coupled via a proline-glycine linker to the RR2-nonapeptide, a specific inhibitor of ribonucleotide reductase oligomerization (1, 2). Inactivation of virions by bHOM-9 was reduced 10-fold in the bHOM peptide lacking the RR sequence (Table II, “Direct inactivation of virions” column, numbers 6 and 7), indicating that the RR sequence was not irrelevant “cargo” but that it could alter the activity of the HOM-MTP. Inhibition of cellular infection did not depend on the RR-sequence but only on the HOM-MTP (Table II, “Comprehensive virus inhibition” and “Inhibition of preadsorbed virus” columns, numbers 6 and 7). All-D isomers of the HOM-MTP not only retained MTP activity (20) but also displayed antiviral activity, regardless of whether they were biotinylated (bHOMd) or acetylated (aHOMd, Table II, numbers 7–9). Thus antiviral, like membrane-transiting properties, do not depend on chirality at least for the HOM peptides. Replacing the two tryptophan residues in the HOM-MTP by phenylalanine substantially reduced membrane transiting (20) and antiviral activities (Table II). The ability to inactivate virions was lost in the bHOMFF peptide (Table II, number 10).

In the bTAT-9 peptide, the TAT-MTP was coupled to the RR2-nonapeptide via a proline-glycine linker. Again, the antiviral activity did not reside in the RR2-sequence but in the MTP. Thus, the bTAT-9X peptide, which included a scrambled version of the RR2-sequence, retained most of the antiviral activity, whereas selective acetylation of the two lysine residues in the TAT-MTP reduced antiviral activity 5–10-fold (Table II, numbers 11–13). Subsequent experiments have shown that the TAT-MTP by itself blocks HSV-1 infection.3

We found that all peptides except EBX blocked HSV entry within a narrow concentration range (6–25 μM; Fig. 2 and Ref. 11). As illustrated for the bHOM-9 peptide, concentrations that could completely block entry (Fig. 2A) had no effect on the expression and activity of β-galactosidase once the virus had entered the cells (Fig. 2B). As shown for two of the HOM peptides, bHOMFF and bHOMd (Fig. 2C), the efficacy of entry blocking activity depended on the presence of intact MTP motifs. Lower concentrations of the bHOMFF peptide were not inhibitory if the incubations were prolonged (Fig. 2C, △), suggesting that bHOMFF merely alters the kinetics of entry.

The question remained as to whether blocking virus entry was merely due to inactivation of adsorbed virus prior to entry or whether some entry process per se, such as membrane fusion, was inhibited by one or more peptides. The fact that virus inactivation by the bHOM-9-MTP was highly temperature-dependent offered a unique opportunity to test whether, at least in this case, inactivation of adsorbed virions preceded entry. At 200 μM, bHOM-9 could inactivate hrR3 in solution with a temperature coefficient (50% inactivation) of 21 °C (Fig. 3A, ■), well below the temperature required for normal viral entry, which occurred at 34 °C (Fig. 3B, ●). The bHOM-9 peptide also inactivated preadsorbed virus well below temperatures permitting entry. For example, at 25 °C when less than 5% of the virions were internalized (Fig. 3B, ●), 12 and 100 μM bHOM-9 inhibited preadsorbed virus by 25 and 50%, respectively (Fig. 3B, ▲ versus △).

The inhibitory effects of bHOM-9 were not due to the release of infectious virus into the culture medium. For instance, after exposure to 100 μM bHOM-9 at 30 °C, the recovered culture medium contained <3% of the virus infecting the control cells (Fig. 1B, ○), even though at this temperature, the peptide had inhibited, and could have released, 70% of the preadsorbed virus (Fig. 3B, △). No additional infectious virus could be recovered from media adjusted to 0.5 M NaCl, even though control experiments showed that adsorbed hrR3 released by heparin could be successfully recovered as infectious virus after treatment with 0.5 M NaCl (data not shown). The results are consistent with the notion that the bHOM-9 peptide can irreversibly inactivate preadsorbed virus prior to entry without releasing the virions from the cell surface. This, however, appears to be only a secondary effect of bHOM-9 seen at higher concentrations.

The primary target of low concentrations of bHOM-9 seemed to be the actual entry process itself. This was shown in experiments, in which inhibition of virus entry and virus inactivation were measured simultaneously after preadsorbed virus

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was exposed to peptide for 1 h at 33 °C, and cultures were switched to peptide-free medium for another 8 h at 37 °C. The fraction of virus entering cells (30–70% of untreated controls) was determined in cultures treated with low pH citrate buffer following the incubation at 33 °C. The fraction of inactivated virus was estimated in mock-treated cultures. As shown in Fig. 4A, bHOM-9 preferentially blocked virus entry (•) and inactivated virus only at 10-fold higher concentrations (□). The bHOM peptide was even more selective in that it blocked entry almost completely before any virus was inactivated (Fig. 4B).

The IC$_{50}$ values measured for the HOM peptides and representatives of the three other groups of peptides are given in Table III, where peptides are listed in order of their preferential inhibition of entry. The bTAT-9 peptide was at least as selective as the bHOM peptide, whereas EB and bKLA had little or no selective effects on entry.

Inhibition of entry by some entry blockers was readily reversible once the peptides were removed. This was shown by following the resumption of virus entry in peptide-free medium at 37 °C, subsequent to partial or complete inhibition of entry during the first hour at 37 °C. One-hour treatments with 10 μM bHOM-9 inhibited entry by >90% (Fig. 4C, •) at zero time and inactivated about one-third of the virus (Fig. 4, □). Like the control virus, all infectious virus entered cells within the first hour after removal of the peptide. Entry was also fully restored

![Image](http://www.jbc.org/)

**Table III**

| Peptide   | Inhibition of entry | Virus inactivation |
|-----------|---------------------|--------------------|
| bTAT-9    | 0.8                 | 75                 |
| bHOM      | 2.6                 | 200                |
| bHOM-9    | 1.6                 | 21                 |
| EB        | 1.1                 | 4.3                |
| bKLA      | 7.8                 | 10                 |

**Fig. 3.** Inactivation of free and preadsorbed virus below temperatures permitting virus entry. A, free hrR3 virus (2.4 × 10$^6$ pfu) in solution was exposed to 200 μg peptide in serum-free medium for 1 h at 4 °C and for an additional hour at the indicated temperatures. The treated virus was then diluted 1000-fold into serum-supplemented medium and titered in Vero cells. The bHOM-9 peptide (■) inactivated virus with a temperature coefficient (50% inactivation) of 21 °C, whereas the effects of the bHOMFF peptide (□) were indistinguishable from those of untreated controls (not shown). B, preadsorbed hrR3 (4.0 × 10$^6$ pfu/well) was exposed to 100 μM (△) or 12 μM (▲) bHOM-9 for 1 h at 4 °C and for an additional hour at the indicated temperatures before peptide was rinsed off and cultures were returned to 37 °C. Serum-supplemented medium was used throughout the assay. Controls (○) were incubated without peptide. No cytotoxic effects were seen in mock-infected trypan blue-stained cells that had been exposed to peptide. Temperature requirements for uninhibited entry of preadsorbed hrR3 in the presence of serum (temperature coefficient: 34 °C) were incubated without peptide. No cytotoxic effects were seen in mock-infected trypan blue-stained cells that had been exposed to peptide. Peptide inhibition of entry virus inactivation

**Fig. 4.** Distinct effects of bHOM-9 and bHOM on entering and pre-entry virus at 33 °C. Preadsorbed virus was treated for 1 h at 33 °C with bHOM-9 (A, C) or bHOM D (B, D), and peptides were rinsed off. To measure the inhibition of entry and the inactivation of virus during the 1-h treatments at 33 °C (A, B), cultures were immediately treated for minute with low pH citrate buffer (●) or PBS (○), respectively. Cultures were kept in peptide-free medium, and infected cells were scored 8 h later. No cytotoxic effects were indicated in trypan blue stained mock-infected cultures. To examine whether inhibition of entry was reversible (C, D), the assay was repeated except that the citrate treatments were done after the times indicated. Also, in addition to untreated controls (●), only two peptide concentrations were tested (10 μM (△) and 100 μM (□)). In the second assay, the extent of virus inactivation was measured by treating some cultures with PBS instead of citrate immediately following peptide removal (open symbols in C and D).
within 1 h following partial or complete inhibition of entry by 10 and 100 μM bHOM (Fig. 4D, ▲ and ■, respectively). Only after inhibition with 100 μM bHOM-9 was entry restored more slowly (Fig. 4C, ■).

At least for some of the peptides, inhibition of entry and virus inactivation of virions appeared to be separate peptide functions that may be independently manipulated. Addition of the hydrophobic RR2-sequence to the bHOM peptide had little effect on virus entry but enhanced the ability of the peptide to inactivate virions nearly 10-fold (Table III). The presence of the RR-sequence by itself, however, was not sufficient for this activity as indicated by the fact that replacement of the HOM-MTP in bHOM-9 by the TAT-MTP resulted in a peptide (bTAT-9) with nearly 4-fold less inactivation ability but no loss of entry blocking activity (Table III). The ability of the EB peptide to inactivate virions also depended on the hydrophobic FGF leader sequence (EB and EBX, Table II).

The ability of MTPs to inhibit entry appears to involve two separate mechanisms: (i) interference with entering virus or some component of the fusion machinery and (ii) irreversible inactivation of adsorbed virions prior to entry. Some of the peptides can selectively block entry without irreversibly inactivating virions. For other peptides, any effects on virus entry are not readily distinguishable from virus inactivation. High concentrations of nearly all peptides lead to irreversible inactivation of virions. These MTPs may ultimately be useful as prophylactic agents to block virus infection and as tools to further our understanding of the processes involved in viral entry into cells.

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