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A synthetic peptide from a heptad repeat region of herpesvirus glycoprotein B inhibits virus replication

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Glycoprotein B (gB) is the most conserved glycoprotein of herpesviruses and plays important roles in virus infectivity. Two intervening heptad repeat (HR) sequences were found in the C-terminal half of all herpesvirus gBs analysed. A synthetic peptide derived from the HR region (aa 477–510) of bovine herpesvirus type 1 (BoHV-1) gB was studied for its ability to inhibit virus replication. The peptide interfered with cell-to-cell spread and consistently inhibited replication of BoHV-1, with a 50 % effective concentration value (EC_{50}) of 5 μM. Inhibition of replication was obtained not only with herpesviruses including pseudorabies virus and herpes simplex virus type 1 but also partly with Newcastle disease virus. Possible mechanisms of membrane fusion inhibition by the peptide are discussed.

INTRODUCTION

Enveloped viruses initiate infection by attachment of the virus to a host cell receptor, followed by membrane fusion between the viral and the cellular plasma or endosomal membranes. The fusion proteins of different viruses are synthesized as precursors with a transmembrane (TM) domain near the C terminus and are cleaved into two disulfide-linked subunits by cellular proteases (Freed *et al.*, 1989; Honma & Ohuchi, 1973; Lazarowitz *et al.*, 1973; Lobigs & Garoff, 1990). A hydrophobic stretch of amino acids at the N terminus of the subunit containing the TM domain serves as the fusion domain (Asano & Asano, 1985; Gallaher, 1987; Gething *et al.*, 1986; Levy-Mintz & Kielian, 1991). Two intervening amphipathic heptad repeat (HR) regions have been identified adjacent to the TM and fusion domains in the fusion proteins of orthomyxoviruses, paramyxoviruses and retroviruses (Chambers *et al.*, 1990). Synthetic peptides derived from the HR regions of paramyxoviruses and human immunodeficiency virus (HIV) are potent inhibitors of virus fusion and infection (Lambert *et al.*, 1996; Wild *et al.*, 1992).

Herpesviruses initially attach to heparan sulfate moieties on a host cell and enter the cytoplasm by fusion of the viral envelope with the plasma membrane (Mettenleiter *et al.*, 1990; Okazaki *et al.*, 1991; Spear *et al.*, 2000; WuDunn & Spear, 1989). Glycoprotein B (gB), the most conserved herpesvirus structural component, plays a role in the fusion process (DeLuca *et al.*, 1982; Fitzpatrick *et al.*, 1990; Li *et al.*, 1997; Navarro *et al.*, 1993; Rauh & Mettenleiter, 1991). Bovine herpesvirus type 1 (BoHV-1) gB is synthesized primarily as a 932 aa translation product and, after removal of the signal sequence, the mature form of the glycoprotein has 865 aa (Whitbeck *et al.*, 1988). Fully glycosylated gB (gBa) is a 130 kDa polypeptide and is partially cleaved between Arg-438 and Ala-439 by a cellular protease to yield two subunits, gBb (75 kDa) and gBc (55 kDa), which are covalently linked via disulfide bonds (van Drunen Little-van den Hurk *et al.*, 1984, 1992; Marshall *et al.*, 1986; Okazaki *et al.*, 1986). Despite compelling evidence for the activity of gB as a fusion protein (Fitzpatrick *et al.*, 1990; van Drunen Little-van den Hurk *et al.*, 1992), no hydrophobic fusion domain has been identified near the cleavage site of the glycoprotein.

Here, we report the identification of HR regions within gBs of different herpesviruses and demonstrate that a synthetic peptide derived from one of the regions within BoHV-1 gBc bound to the glycoprotein and inhibited the replication of BoHV-1. The peptide did not affect virus entry but interfered with cell-to-cell spread to reduce virus production. The peptide inhibited the replication not only of pseudorabies virus (PRV) and herpes simplex virus (HSV) but also that of Newcastle disease virus (NDV), although to a lesser degree.

METHODS

Cells and viruses. Madin–Darby bovine kidney (MDBK) cells and Vero E6 cells were cultured in minimal essential medium (MEM; Nissui) containing 10 % calf serum and 5 % fetal calf serum (FCS), respectively. BoHV-1 strain Los Angeles, PRV strain YS-81 and NDV strain TCND were propagated in MDBK cells. HSV-1 strain F was grown in Vero E6 cells. 293T cells were cultured in Dulbecco's modified MEM supplemented with 10 % FCS and used for the expression of gB.

Peptide. Peptides B477–510 and B1–20, which correspond to the HR1 region and the N-terminal end of the mature form of BoHV-1 gB, respectively, were purchased from Sigma Genosys. The amino acid sequences were those from the Cooper strain of BoHV-1

(Whitbeck *et al.*, 1988). The purities of the peptides were 99·1 % (B477–510) and 98·2 % (B1–20). A human 33 aa peptide, S182 (aa 345–377), was purchased from Phoenix Pharmaceuticals and its purity was 99·4 %.

Virus growth in the presence of the peptide. Cell monolayers were infected with the viruses at an m.o.i. of 0·01 in the presence of different concentrations of the peptide and incubated at 37 °C for 48 h. In the one-step growth experiment, the cells were infected at an m.o.i. of 10 and incubated for 24 h. The supernatants were clarified by centrifugation and viral titres were determined by plaque assays on appropriate cell monolayers.

Fluorescent antibody staining. MDBK cell monolayers were infected with BoHV-1 in the presence (100 μM) or absence of the peptide and incubated at 37 °C for 18 h. After fixation with methanol, the cells were incubated with monoclonal antibody (mAb) specific for BoHV-1 gD (Okazaki *et al.*, 1986), washed with PBS and exposed to FITC-conjugated anti-mouse IgG.

Expression of BoHV-1 gB. The complete coding sequence of gB was excised from the plasmid pVA97, containing the *Hin*dIII–A fragment of the Los Angeles strain genome, by digestion with *Sal*I and *Kpn*I and cloned into the *Sal*I/*Kpn*I sites of pUC19. The resulting plasmid was completely digested with *Eco*RI and partially with *Pst*I and a 3·0 kb fragment was cloned into the *Eco*RI/*Pst*I sites of pUCBM21 (Boehringer), which contained an in-frame deletion in the leader sequence but retained the authentic signal sequence of gB. The altered coding sequence of gB was excised by digestion with *Hin*dIII and *Eco*RI and cloned into the *Hin*dIII/*Eco*RI sites of pcDNAI/Amp (Invitrogen) to express the full-length mature form of gB.

Transfection of 293T cells was carried out as described previously (Okazaki *et al.*, 1991). At 48–60 h post-transfection, cells were harvested and lysed with 1 % Triton X-100/1 % sodium deoxycholate, followed by centrifugation to remove debris.

Surface plasmon resonance (SPR) spectrometer analysis. The interaction of the synthetic peptide with gB was evaluated by SPR spectroscopy using SPR-670M (Nippon Laser & Electronics Laboratory). mAb 185/2 (Okazaki *et al.*, 1986), specific for BoHV-1 gB, was immobilized via 4,4′-dithiodibutyric acid on to the gold film in the SPR cells standing in a row in the SPR instrument. After blocking with 1 mg casein ml^{-1}, the cell lysate containing gB was injected and washed with PBS at the rate of 15 μl min^{-1}. After baseline stabilization, 50 μl of the peptide B477–510 or B1–20 was injected in parallel and washed with PBS. The changes in the SPR signal were monitored at the rate of 15 μl min^{-1}.

RESULTS

Intervening amphipathic HR regions within gBs of different herpesviruses

Two HR regions were detected in the amino acid sequences of gB of all the herpesviruses analysed (Fig. 1a). BoHV-1 gB HR1 region commenced 39 aa from the cleavage site and the HR2 region was 22 aa away from the TM domain. Although the proteolytic cleavage site was absent in HSV-1, HSV-2 and Epstein–Barr virus (EBV) gB, the HR regions were found in the C-terminal half of the glycoproteins. Hydrophobic (I, L, M and V) and aromatic (F, Y and W) amino acids were predominant at the heptad positions of 'a' and 'd' where the sequences were highly conserved. Helical-wheel representations of the HR1 and HR2 of BoHV-1 gB illustrate the amphipathic property of the regions (Fig. 1b). In particular, the HR1 region represented a leucine zipper motif.

Inhibition of virus replication by a synthetic peptide containing the BoHV-1 HR1 sequence

Peptides corresponding to the HR1 region (B477–510) and the N-terminal end (B1–20) of BoHV-1 gB were synthesized. It was assumed that a peptide corresponding to the HR2 region would not be soluble in water, so it was excluded in this study. The two peptides related to BoHV-1 gB and a 33 aa peptide bearing no relation to the virus, S182 (aa 345–377), were assessed for their effect on virus replication by infecting MDBK cells with BoHV-1 at an m.o.i. of 0·01. Infected cells were incubated for 48 h and the virus titres of the culture fluids were determined. As shown in Fig. 2, peptide B477–510 consistently inhibited the replication of BoHV-1 in a dose-dependent manner, whereas no inhibition was observed with the peptides B1–20 or S182. The 50 % effective concentration value (EC_{50}) of peptide B477–510 was approximately 5 μM.

To confirm the specificity of inhibition by peptide B477–510, its activity was tested using the herpesviruses PRV and HSV-1 and the unrelated paramyxovirus NDV. Fig. 3 shows that greater than 90 % inhibition was observed with PRV and HSV-1 with a peptide concentration of 100 μM. The EC_{50} for PRV and HSV-1 was 20 and 40 μM, respectively. However, only 60 % inhibition was observed with NDV at a concentration of 100 μM, although the EC_{50} for this virus was 50 μM.

Direct effect of the peptide on BoHV-1 virions

To address whether peptide B477–510 inactivated the virus, BoHV-1 was mixed with different concentrations of the peptide and incubated at 37 °C for 1 h. Serial 10-fold dilutions of the reaction mixtures were prepared to determine the virus titres by plaque assays on MDBK cells. As shown in Fig. 4, the peptide exhibited no antiviral activity at a concentration of 6·25 μM. Even on treatment with 100 μM peptide, more than 25 % of the viral infectivity remained. These findings indicated that the peptide minimally affected BoHV-1 virions.

Effects of the peptide at different stages of infection

The trypan blue exclusion test with MDBK cells indicated that peptide B477–510 had no toxic effect on the cells at a concentration of 500 μM (data not shown). Due to the amphipathic property of the peptide, the possibility that the peptide affected the plasma membrane and interfered with virus attachment was examined by infecting with virus at an m.o.i. of 0·01. When MDBK cells were incubated with 100 μM of the peptide prior to virus adsorption at 37 °C for 1 h, or during the adsorption at 4 °C for 2 h, the virus yield was decreased only slightly (Fig. 5). The peptide also showed minimal effect on virus replication when

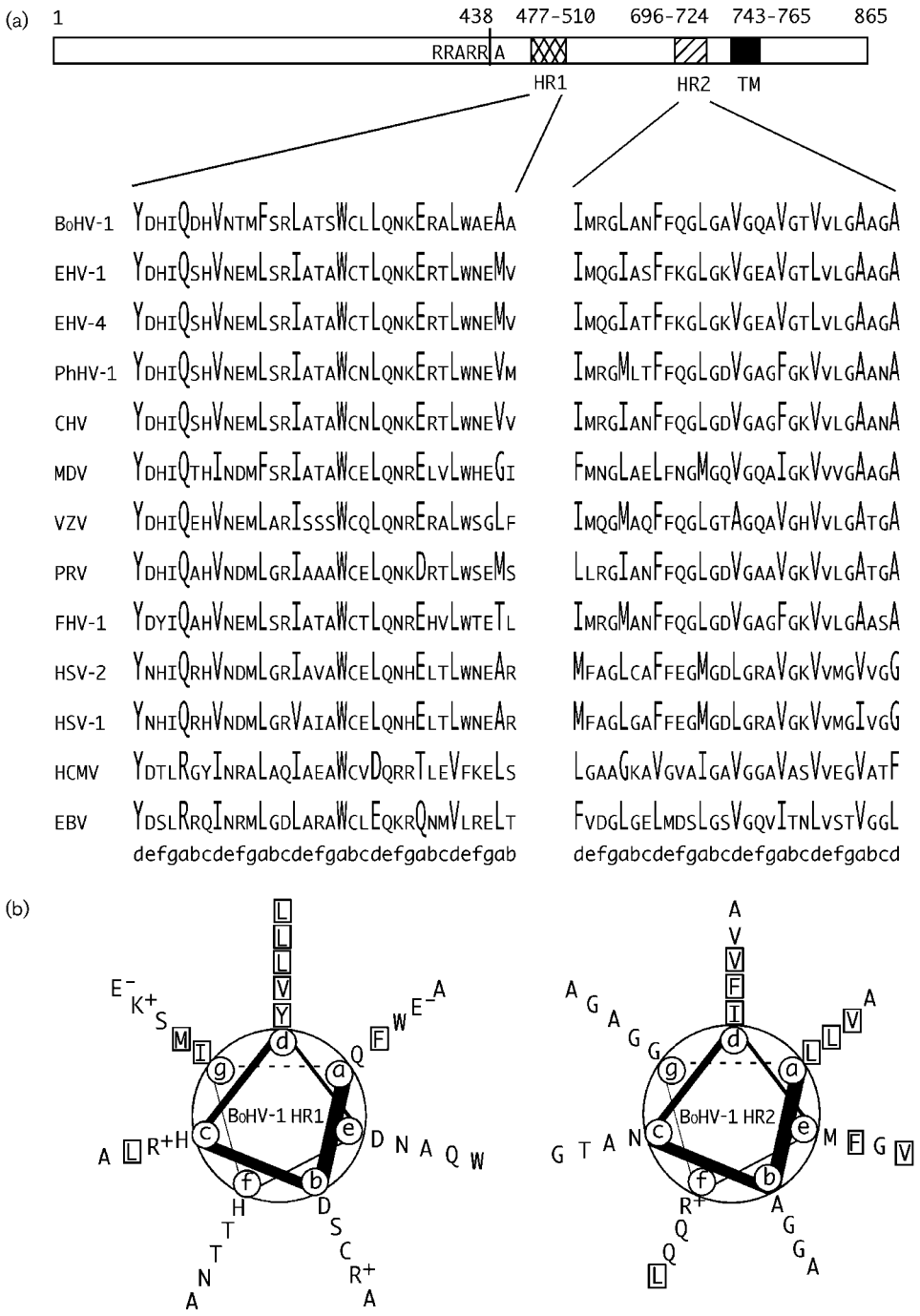

Fig. 1. HR regions of BoHV-1 gB. (a) Diagram of the mature form of BoHV-1 gB and amino acids representing the HR regions of herpesvirus gBs. BoHV-1 gB is cleaved at aa 438 by the cellular protease. Amino acids 477–510, 696–724 and 743–765 correspond to the HR1, HR2 and TM regions of the glycoprotein. Residues in larger type are located in heptad positions, which are shown on the bottom line ('a' and 'd'). GenBank accession numbers: EHV-1 (equine herpesvirus type 1), residues 584–617 and 806–834, BAA00304.1; EHV-4 (equine herpesvirus type 4), residues 579–612 and 801–829, AAA46106.1; PhHV-1 (phocine herpesvirus type 1), residues 488–521 and 710–738, CAA92272.1; CHV (canine herpesvirus), residues 482–515 and 704–732, AAK51052.1; MDV (Marek's disease virus), residues 467–500 and 688–716, BAA02866; VZV (varicella-zoster virus), residues 458–491 and 680–708, AAK19938.1; PRV, residues 537–570 and 754–782, AAA47465.1; FHV-1 (feline herpesvirus type 1), residues 545–578 and 764–792, AAB28559.1; HSV-2, residues 507–540 and 728–756, AAK97852; HSV-1, residues 509–542 and 730–758, AAA45776.1; HCMV (human cytomegalovirus), residues 487–520 and 718–746, P06473; EBV, residues 470–503 and 694–722, AAK95476. (b) Helical-wheel representations of the HR regions of BoHV-1 gB. Hydrophobic and aromatic amino acids are boxed. The charged side chains are indicated.

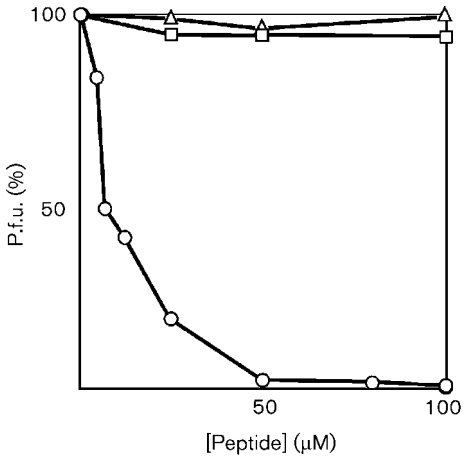

Fig. 2. Inhibition of BoHV-1 replication by the synthetic peptide. Monolayers of MDBK cells were infected with BoHV-1 at an m.o.i. of 0·01 in the presence of different concentrations of the peptide B477–510 (○), B1–20 (△) or S182 (345–377) (□). After a 48 h incubation, virus titres of the supernatants were determined by plaque assays. Data points are from three separate experiments.

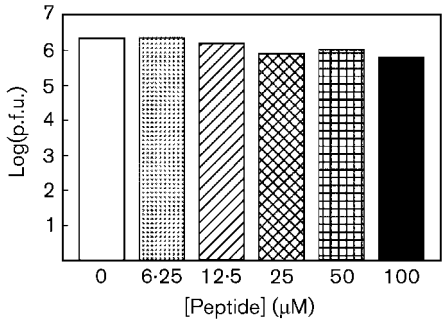

Fig. 4. Minimal effect of peptide B477–510 on BoHV-1 virions. BoHV-1 was mixed with an equal volume of the peptide at different concentrations. After a 1 h incubation at 37 °C, virus titres of the reaction mixtures were determined by plaque assays. The assays were carried out in duplicate.

incubated with cells to which the virus had been adsorbed at 4 °C and incubated for 1 h at 37 °C. On the other hand, virus replication was markedly inhibited when the peptide was added to cells already infected with the virus. These findings suggested that the peptide had little effect on the plasma membrane or initial stages of infection of the virus but interfered with the late stages of infection.

The inhibitory activity of the peptide was further examined by infecting at different m.o.i. values. MDBK cells were inoculated with BoHV-1 at an m.o.i. of 0·01 or 10 and incubated for 24 h in the presence of the peptide. As shown in Fig. 6, the virus yield was minimally affected by the peptide when the cells were infected with virus at a high m.o.i., indicating that the one-step growth of the virus was accomplished in the presence of the peptide. Thus, it appeared that the peptide had minimal effect on the synthesis of viral components, assembly or release of the virions.

Inhibition of cell-to-cell spread of the virus

To assess the effects of the peptide on cell-to-cell spread of BoHV-1, MDBK cells were infected with virus at an m.o.i. of 0·01 and overlaid with the peptide at a concentration of 100 μM. After an 18 h incubation at 37 °C, the cells were examined by fluorescent antibody staining with anti-BoHV-1 gD mAb. As shown in Fig. 7, in the presence of the peptide the foci of specific staining were smaller than those in the absence of the peptide. This finding, together with the above results, suggested that the peptide interfered

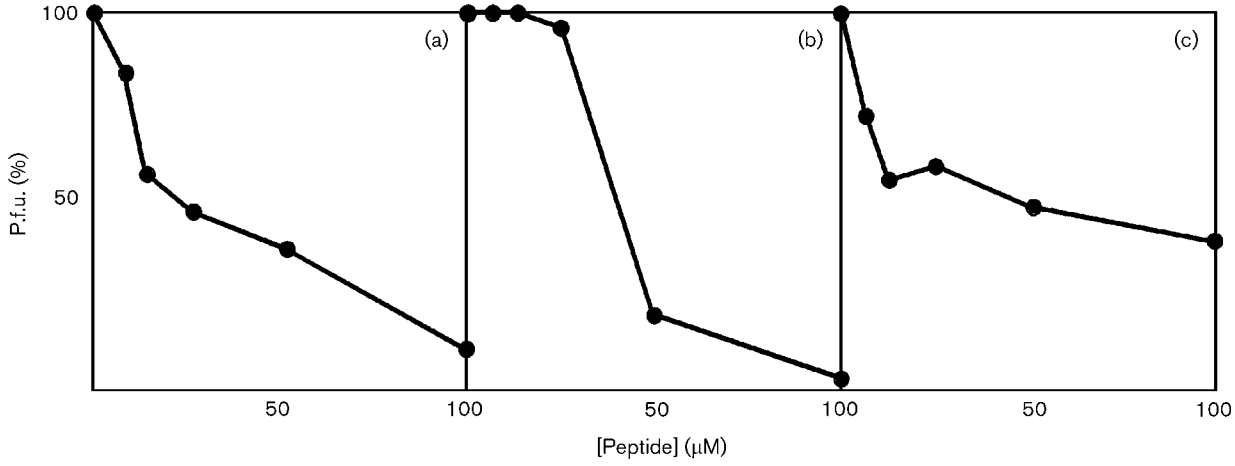

Fig. 3. Effect of peptide B477–510 on the replication of heterologous viruses. Monolayers of appropriate cells were infected with PRV (a), HSV-1 (b) and NDV (c) and inhibition of replication was assessed as described in the legend to Fig. 2.

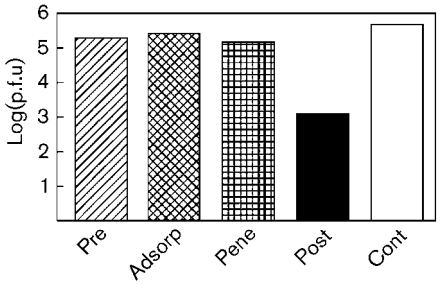

Fig. 5. Target stage of inhibition by peptide B477–510. Peptide (100 μM) was added to the cells at different stages of infection. Pre, the cells were incubated with the peptide at 37 °C for 1 h, washed extensively and infected with BoHV-1 at an m.o.i. of 0·01. Adsorp, the peptide was incubated with the cells during virus adsorption at 4 °C for 2 h. Pene, the peptide was added to the cells after adsorption at 4 °C and incubated at 37 °C for 1 h, followed by treatment with acid solution (0·1 M HCl/0·1 M sodium citrate, pH 2·5) for 5 min to inactivate extracellular virus. Post, the peptide was added to cells that had previously been incubated with the virus at 37 °C for 1 h. Cont, control (no peptide). At 48 h post-infection, virus titres of the supernatants were determined by plaque assays in duplicate.

with virus spread from the infected cell to the adjacent cells without affecting attachment or penetration of the virus.

Interaction of the peptide with gB

To confirm that peptide B477–510 binds to BoHV-1 gB, SPR analysis was carried out (Fig. 8). mAb specific for the glycoprotein was immobilized onto the gold film in the SPR cell and the lysate containing gB was injected. Specific binding of gB to the mAb was demonstrated by successive

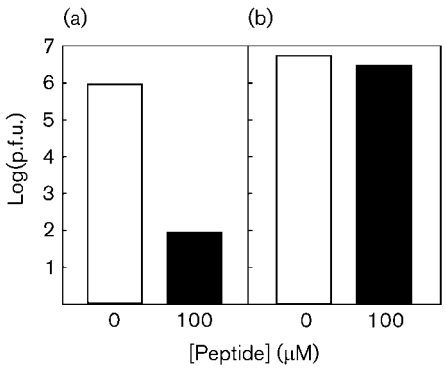

Fig. 6. Effect of m.o.i. on the inhibitory activity of the peptide. The cells were infected with BoHV-1 at an m.o.i. of 0·01 (a) or 10 (b) and incubated at 37 °C for 24 h in the presence (100 μM) or absence of the peptide. Progeny virus was titrated by plaque assays in duplicate.

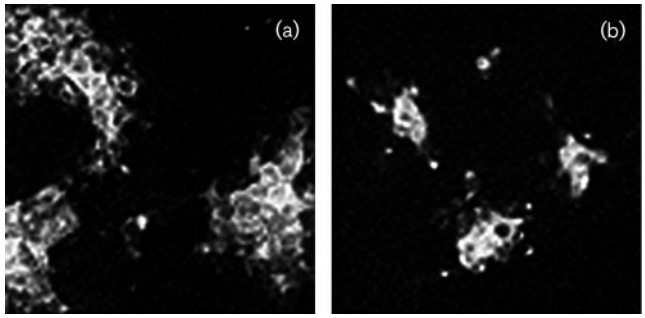

Fig. 7. Inhibition of BoHV-1 cell-to-cell spread by peptide B477–510. MDBK cells were infected with virus at an m.o.i. of 0·01 and incubated in the absence (a) or presence (100 μM) (b) of the peptide. After 18 h, the cells were fixed with methanol and reacted with mAb against BoHV-1 gD. After exposing to FITC-conjugated anti-mouse IgG, specific staining was examined under a fluorescent microscope.

increases in the SPR signal (data not shown). When peptide B477–510 was added on to the cell, the SPR signal increased with time, indicating accumulative binding of the peptide to gB. After washing with PBS, approximately 0·014 degrees of the change remained. In contrast, peptide B1–20 produced a shift in the baseline but no increase was observed during injection. After washing, the signal returned to the original baseline. These findings indicated that the peptide derived from the N-terminal end of BoHV-1 gB could not bind to the glycoprotein, whereas that from the HR1 region did bind to the glycoprotein.

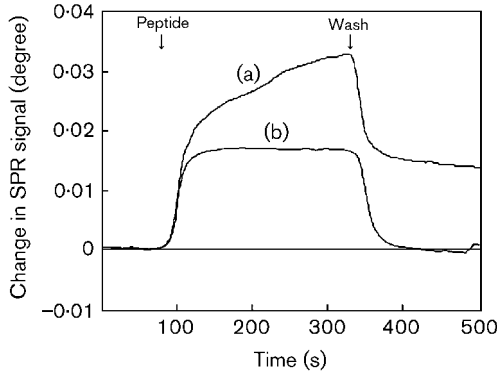

Fig. 8. Binding of peptide B477–510 to BoHV-1 gB. 293T cells were transfected with BoHV-1 gB-expressing plasmid and lysed with 1 % Triton X-100/1 % sodium deoxycholate 48–60 h later. The glycoprotein was immobilized using mAb against gB on the SPR cells, followed by washing with PBS. After baseline stabilization, 50 μl of peptide B477–510 (a) or B1–20 (b) was injected in parallel and washed again with PBS. The SPR signal was monitored at the flow rate of 15 μl min^{-1}.

DISCUSSION

In this study, we have demonstrated that the synthetic peptide corresponding to the HR1 region of BoHV-1 gB, which models a leucine zipper-like structure, inhibited the replication of the virus. The peptide had minimal influence on cell-free virions and host cells. Virus adsorption or penetration was also only slightly affected by the peptide. However, virus yield was markedly decreased when the peptide was added after penetration. Since the virus yield of cells infected with BoHV-1 at a high m.o.i. was minimally affected, the peptide did not seem to interfere with the synthesis or transport of viral components or release of virions. The foci of virus-infected cells were less developed in the presence of the peptide, suggesting that cell-to-cell spread was inhibited by the peptide. When the peptide was added to solid medium, no difference was observed in the plaque size at 3 days post-infection (data not shown). Since prolonged incubation reduced efficacy of the peptide, even in liquid medium, the peptide might be unstable in a weak solution. SPR analysis clearly demonstrated the interaction of the peptide with gB. The peptide derived from the HR1 region of gB must therefore bind to the glycoprotein at the cell surface and affect fusion activity of the glycoprotein to inhibit cell-to-cell spread of BoHV-1.

Antiviral activity of a synthetic peptide corresponding to the HR region of a glycoprotein was first described for HIV (Wild *et al.*, 1992). DP-107 is a 38 aa peptide representing the HR1 region of HIV gp41 and contains a leucine zipper motif. The peptide inhibits HIV infection by affecting both the cell-free virus and cell-to-cell fusion, although it has little or no effect on cell-free virions. DP-178, an amphipathic 36 aa peptide corresponding to the HR2 region of HIV gp41, inhibits viral entry and cell-to-cell fusion *in vitro*, with an EC_{50} that is much lower than that of DP-107 (Wild *et al.*, 1994), and virus replication *in vivo* (Kilby *et al.*, 1998). Although peptides derived from the HR1 region of measles virus and Sendai virus F proteins show no inhibition (Rapaport *et al.*, 1995; Wild & Buckland, 1997), those derived from the HR1 region of NDV F protein are as inhibitory as, or more inhibitory than, peptides from the HR2 region (San Roman *et al.*, 2002; Young *et al.*, 1997, 1999). These HR regions are located near the fusion peptide and the TM domains of the glycoproteins. During fusion, they interact with each other and refold into helical bundles to place the fusion peptide near the TM domains, resulting in the close proximity of the attaching and target membranes (Kliger *et al.*, 2001; Chen *et al.*, 2001). Peptides with sequences from either region are supposed to block this interaction (Rimsky *et al.*, 1998). Peptide B477–510 may also bind to the HR2 region of gB of BoHV-1, PRV or HSV-1 and prevent it from interacting with the corresponding HR1 region. Despite there being no fusion peptide within the sequence of gB, the approach of the virus-infected cell membrane to the adjacent cell membrane may be inhibited. Interestingly, the peptide corresponding to the HR1 region of BoHV-1 gB partly inhibited the replication of NDV. It is postulated that not only the sequence but also the secondary structure or hydrophobicity profile of the peptide is important for the interaction. Hecate, a 23 aa amphipathic α-helical peptide analogue of melittin, has been reported to inhibit HSV-1-induced cell fusion and virus spread, although the mechanism is unknown (Baghian *et al.*, 1997).

BoHV-1 gB has three continuous hydrophobic segments in its C terminus. It has been demonstrated that the second segment, aa 717–736, is involved in the fusogenic activity of the glycoprotein and that the third functions as the membrane anchor (Li *et al.*, 1997). The HR2 region of BoHV-1 gB overlaps the first and second segments. A hydrophobic stretch of 34 aa near the TM domain of human cytomegalovirus gB, containing the entire HR2 region of the glycoprotein, has been proposed as a fusogenic domain (Bold *et al.*, 1996). Some residues within the HR2 region of HSV-1 gB, which are conserved among herpesviruses, have also been shown to be important for virus penetration (Wanas *et al.*, 1999). The putative interaction between the HR1 and HR2 regions may be essential for fusion by herpesvirus gBs.

Co-expression of gB, gD, gH and gL is necessary for HSV-1 to induce sufficient membrane fusion (Turner *et al.*, 1998), whereas gB, gH and gL are sufficient for PRV (Klupp *et al.*, 2000). In the case of BoHV-1, expression of gB alone can induce membrane fusion (Fitzpatrick *et al.*, 1990; van Drunen Little-van den Hurk *et al.*, 1992). Although the fusion process by herpesviruses is complex and may be modulated by different viral components, it is certain that gB plays a central role in the process. Further studies should give insight into the mechanism of infection by herpesviruses.

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