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Human Herpesvirus 7 Glycoprotein B (gB), gH, gL, gO Can Mediate Cell Fusion*

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Abstract Human herpesvirus 7 (HHV-7) infection is dependent on the functions of structural glycoproteins at multiple stages of the viral life cycle. These proteins mediate the initial attachment and fusion events that occur between the viral envelope and a host cell membrane, as well as cell to cell spread of the virus. To characterize the HHV-7 glycoproteins that can mediate cell fusion, a cell-based fusion assay was used. 293T cells expressing the HHV-7 glycoproteins of interest along with a luciferase reporter gene under the control of the T7 promoter were cocultivated with SupT1 cells transfected with T7 RNA polymerase. HHV-7 glycoproteins gB, gH, gL and gO can mediate the fusion of 293T cells with SupT1 cells, and the fusion can be inhibited by anti-CD4 mAbs. Thus, the coexpression of HHV-7 gB, gO, gH and gL is sufficient and necessary for HHV-7 induced membrane fusion, and one of these glycoproteins or protein complex formed by these glycoproteins might be the ligand(s) of CD4 molecule.

Key words Human herpesvirus 7, glycoproteins, membrane fusion

Human herpesvirus 7 (HHV-7), a new type of lymphotropic human herpesvirus, was initially isolated from $CD4^+$ T cells of a healthy individual in 1990 [1]. The virus is highly seroprevalent, primary infection usually occurs during childhood, and it has been associated with cases of exanthem subitum, pityriasis rosea, neurological manifestations and transplant complications. HHV-7 was found to bind CD4 molecule of T lymphocytes competitively with human immunodeficiency virus (HIV) [2]. Some researchers suggested that HHV-7 could be used as a new vector for the gene therapy of AIDS[3]. However, very little is known about the precise mechanism by which HHV-7 enters into $CD4^+$ T cells.

Herpesvirus glycoproteins have prominent functions in several viral processes, including specific binding to the cellular surface, fusion of the viral and cellular membranes during viral entry, assembly of the virion, and egress of the virion from the infected cell. For herpesviruses, entry and virus-induced cell fusion are related processes in that penetration proceeds by fusion of the viral envelope with the cell membrane and requires many of the same viral glycoproteins as virus-induced cell fusion[4].

All herpesviruses encode glycoprotein B (gB), gH, and gL homologues[5,6]. Both gB and gH are highly conserved [7], and all three appear to be essential for herpesvirus infectivity [4]. HHV-7 gB consists of 822 amino acids and shows features characteristic of type Ⅰ integral membrane proteins. Computer analysis predicts that this protein has a signal peptide at the amino terminus, a large external domain which contains 11 potential N-glycosylation site, and a transmembrane domain followed by a shorter cytoplasmic tail. HHV-7 gB is able to bind cell surface heparan sulfate (HS), which functions as an essential co-receptor for a productive infection of $CD4^+$ T-lymphocytes[8].

In most herpesvirus, gH associates with gL to form a gH-gL complex. gH is thought to be important for fusion, whereas the role of gL is to severe as a chaperone, essential for the folding and transport of functional gH [9,10]. Similar to other herpesvirus, the

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HHV-7 gH and gL proteins form a complex in virus infected cells[5].

In some herpesviruses, a third viral glycoprotein associates with the gH-gL complex. Epstein-Barr virus gp42, HHV-6 gQ and human cytomegalovirus gO have been identified to combine to the gH-gL complex and are required for viral entry into susceptible cells[11~13]. HHV-7 gO also forms a complex with gH in infected cells[14].

Based on the evidence from other family members, we hypothesize that HHV-7 may use the similar gps to mediate cell fusion. To test our hypothesis, we describe an efficient and quantitative assay for studying the HHV-7 glycoproteins required for membrane fusion. We found that transient expression of gB, gH, gL and gO in 293T cells is necessary and sufficient to induce the fusion of the 293T cellular membranes with those of SupT1 cells susceptible to HHV-7, and the fusion can be inhibited by anti-CD4 mAbs.

1 Materials and methods

1.1 Virus and cells

YY5 strain, a Nanjing Local Strain of HHV-7 was propagated in SupT1 cells [15]. SupT1 cells, a T-lymphocyte-line susceptible to HHV-7 provided by Dr. Peiris of the University of Hong Kong, were passaged in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 0.1 g/L streptomycin (all from GIBCO, USA). 293T cells are human embryonic kidney cells expressing the SV40 large T antigen and were passaged in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and antibiotics.

1.2 Plasmids

The expression plasmid pCAGGS/MCS was kindly provided by Y. Kawaoka (University of Wisconsin, Madison). This plasmid was constructed by inserting a polylinker into pCAGGS [16]. The PCR products were inserted into pCAGGS and protein were expressed under control of the chicken beta-actin promoter and CMV IE enhancer[17]. Plasmid pCAGT7 expressing T7 RNA polymerase under the control of the chicken beta-actin promoter and the CMV-IE enhancer and plasmid pT7EMCLuc expressing the firefly luciferase gene under control of the T7 promoter were both gifts from Y. Matsuura (National Institute of Infectious Diseases, Tokyo, Japan)[18].

1.3 Construction of expression vectors

The HHV-7 (YY5) glycoprotein open reading frames of interest were inserted into pCAGGS/MCS as follows. HHV-7 DNA was isolated from HHV-7-infected SupT-1 cells by chloroform-phenol method. Amplification of the glycoprotein open reading frames of interest was done by PCR with Pyrobest polymerase (Takara, Japan). PCR products were desalted and concentrated with a gel extraction kit (Tiangen, China). The HHV-7 gH open reading frame was amplified by using an *Eco*R Ⅰ restriction endonuclease site-tagged sense primer(5′CCG**GAATTC**ATGTATTTTTACATAAATAG 3′) and a *Bam*H Ⅰ-tagged antisense primer (5′ CGC**GGATCC**TCAAAACAATCTAAATACAT 3′), introduced restriction endonuclease sites are shown in bold type. The amplified fragment was digested with *Eco*R Ⅰ and *Bam*H Ⅰ and then inserted between the *Eco*R Ⅰ and *Bgl* Ⅱ sites of pCAGGS/MCS, generating pCAGGS/H. The HHV-8 gL open reading frame was amplified by using an *Xho* Ⅰ restriction endonuclease site-tagged sense primer (5′ CCG**CTCGAG**ATGAAAACTAACATCTT TTT 3′) and a *Bgl* Ⅱ-tagged antisense primer (5′ GGA**AGATCT**TCAGTAAGTTCTAATGAGCA 3′). Afterdigestion with *Xho* Ⅰ and *Bgl* Ⅱ, the PCR fragment was inserted between the *Xho* Ⅰ and *Bgl* Ⅱ sites of pCAGGS/MCS, generating pCAGGS/L. The HHV-7 gB open reading frame was amplified by using an *Eco*R Ⅰ restriction endonuclease site-tagged sense primer (5′ CCG**GAATTC**ATGAAAATTCTATTCCT GAG 3′) and a *Bgl* Ⅱ-tagged antisense primer (5′ GGA**AGATCT**TCACAGTTCTTCTGTTGAAA 3′). The amplified fragment was digested with *Eco*R Ⅰ and *Bgl* Ⅱ and inserted between these sites of pCAGGS/MCS, generating pCAGGS/B. The HHV-7 gO open reading frame was amplified by using a *Sac* Ⅰ restriction endonuclease site-tagged sense primer (5′ GCGAGC**TCATGA**AAAACAAAATGTATTCAATGCT 3′) and a *Nhe* Ⅰ-tagged antisense primer (5′ CTA**GCTAGC**TTA TGTGCTTGTGTTACTATG 3′). The resulting PCR products were cloned between the *Sac* Ⅰ and *Nhe* Ⅰ sites of pCAGGS/MCS, generating pCAGGS/O and sequenced.

1.4 Antibodies production

Antibodies specific for HHV-7 glycoproteins were produced by immunizing BALB/c mice intramuscularly with HHV-7 glycoprotein expression vectors as previously described for HSV-1 gB and

gD[19]. For immunization, 50 μg of each expression vector was injected. Animal care and experiments were approved by an animal use committee at the Nanjing Medical University.

1.5 Western blot

HHV-7 infected and uninfected Supt1 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [0.01 mol/L Tris/HCl (pH 7.4), 0.15 mol/L NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L PMSF]. The lysed proteins were resolved by SDS-PAGE and electrotransferred onto a PVDF membrane for immunoblotting. After the blocking step, the membranes were incubated for 1 h with blocking buffer (PBS, 5% skimmed milk, 0.1% Tween 20) containing the polyclonal antibodies. The reactive bands were visualized with a horseradish peroxidase-conjugated second antibody (Boster, China).

1.6 Cell enzyme-linked immunosorbent assay

Subconfluent 293-T cells were transfected with plasmids expressing HHV-7 glycoproteins using Lipofectamine 2000 (invitrogen,USA) in DMEM according to the manufacturer's protocol. Individual wells were transfected with 0.75 μg of each expression vector, keeping the total amount of DNA added at 3 μg per well by the addition of empty vector DNA. Twelve hours after transfection the Lipofectamine 2000 solution was removed from the 293T cells, and the cells were detached using with 0.25% trypsin and 1 mmol/L tetrasodium ethylenediaminetetraacetic acid (EDTA) and replated onto 96-well plates. After 18 h the cells were washed twice with phosphate-buffered saline (PBS) and incubated with the appropriate primary mouse polyclonal antibody (anti-gH, anti-gL, anti-gB, and the anti-gO) in PBS with 3% bovine serum albumin (BSA) for 30 min . Subsequently, cell were fixed in PBS plus 2% formaldehyde and 0.2% gluteraldehyde, and incubated sequentially with biotinylated anti-mouse IgG conjugate and streptavidin conjugated-horseradish peroxidase. After adding TMB substrate, optical absorption (A) readings at 450 nm were obtained using a plate reader (Clinibio, Austria).

1.7 Cell fusion assays

Subconfluent 6-well plates containing 293-T cells were transfected with 0.75 μg of pCAGGS/H, pCAGGS/L, pCAGGS/B and pCAGGS/O, and 1 μg of pT7ENCLuc. For experiments in which specific glycoproteins were serially subtracted, the amount of DNA per transfection was kept constant by the addition of empty vector DNA. SupT1 cells were electroporated with 50 μg of pCAGT7 using a Gene Pulser (Bio-Rad, USA). A total of 10^7 cells were electroporated in 0.4 cm gap cuvettes (Bio-Rad) at 0.250 kV and 960-μF capacitance. Twelve hours after transfection, the 293T cells were washed with PBS, and 1.5×10^6 viable SupT1 cells were plated on top. Luciferase activity was quantified by using a luciferase reporter assay system (Promega, USA)12 to 48 h after cocultivation. Cells were washed with PBS and then lysed with passive lysis buffer. After the supernatant was collected, beetle luciferin substrate was added, and luminosity readings were obtained by using a TD-20/20 luminometer (Promega, USA).

1.8 Statistical analysis

Comparison of means was done by two-tailed *t* tests. Statistical analyses were performed with SPSS version 10.0.

2 Results

2.1 Construction of expression vectors

The HHV-7 (YY5) glycoprotein open reading frames of interest were inserted into pCAGGS/MCS as follows. The successful construction of the expression vectors was identified by restriction enzyme digestion and sequencing (Figure 1). Nucleotide sequencing revealed that no base mutation occurred.

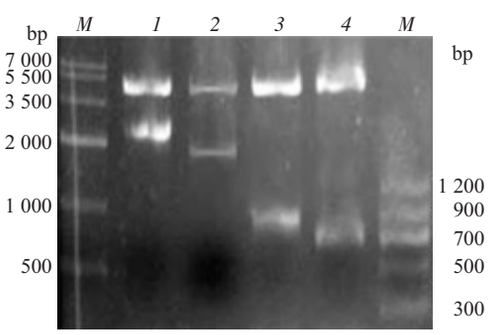

Fig. 1 Restriction enzyme analysis of recombinant plasmids

M: Molecular mass markers. *1* ~*4*: pCAGGS/B, pCAGGS/H, pCAGGS/O and pCAGGS/L were digested respectively.

2.2 Antibody identification

The recognition of protein antigens in Western blots by antibodies from DNA immunized mice was analyzed. As shown in Figure 2, the antibodies for gH, gL and gO could specifically react with proteins MWs of 85 ku, 35 ku and 49 ku, respectively. But two molecular-mass bands, a precursor form of 112 ku and

a cleaved form of 51 ku , were detected by serum of gB immunized mice.

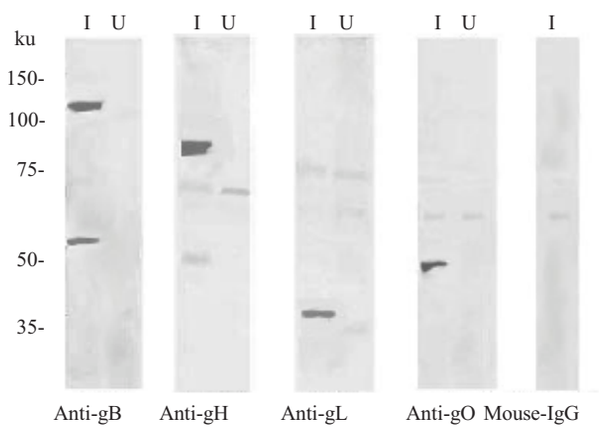

Fig. 2 Identification of the specificity of the antibodies against HHV-7 glycoproteins by Western blot

HHV-7 infected (I) and uninfected (U) Supt1 cells were lysed and seperated on SDS-PAGE gel, followed by immublotting with indicated antibodies. Mouse IgG was used as a negative control.

2.3 Cell surface expression of HHV-7 glycoproteins

To investigate if HHV-7 glycoproteins could induce cell-cell fusion, the open reading frames for several HHV-7 glycoproteins, including gB, gO, gH, and gL, were inserted into plasmid pCAGGS/MCS which has been demonstrated to be capable of driving the efficient expression of multiple HHV-7 glycoproteins when transfected in 293T cells [17]. The expression of each glycoprotein in 293T cells was then confirmed by cell enzyme-linked immunosorbent assay (CELISA). As shown in Figure 3, gB, gO, gH and gL were detected on the cell surface in 293T cells transfected with pCAGGS/MCS containing gB, gO, gH and gL ORF. Furthermore, we found low levels of gL or gO expression on 293T cell in the absence of gH, indicating that correct processing and cell surface presentation of gL or gO were dependent on gH coexpression.

2.4 Efficient HHV-7 induced membrane fusion requires gB,gO, gH and gL

Because the efficient fusion of cells by herpesvirus glycoproteins requires the expression of entry receptors, it was reasoned that SupT1 cells susceptible to HHV-7 entry may also be able to fuse with 293T cells expressing the correct set of HHV-7 glycoproteins. To detect cell fusion, a luciferase reporter gene activation assay was used. 293T cells transfected with the luciferase reporter gene under the control of the T7 promoter and the HHV-7 glycoproteins of interest were

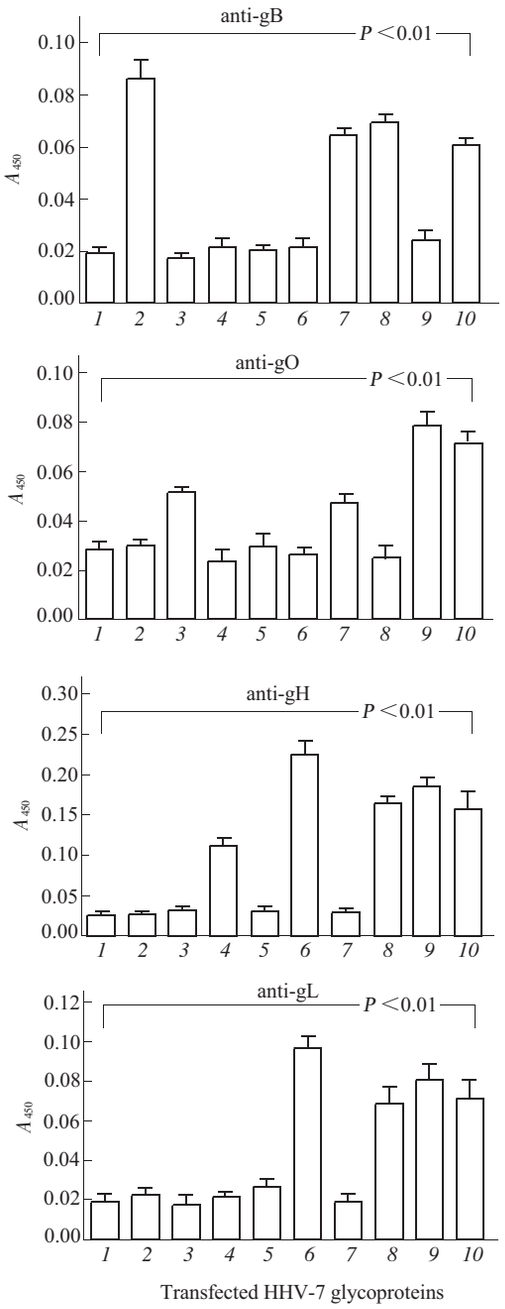

Fig. 3 Cell surface expression of HHV-7 glycoproteins detected by binding of specific antibodies

293T cells were transfected with plasmids expressing the indicated HHV-7 glycoproteins. After replating onto 96-well dishes and overnight incubation, cells were incubated with primary antibody diluted in PBS with 3% BSA. Cells were subsequently fixed and then sequentially incubated with species-specific biotinylated anti-IgG conjugate and streptavidin-horseradish peroxidase conjugate. After addition of TMB substrate, OD readings were obtained. Shown are the mean values and standard deviations for three replicate samples. The differences between readings obtained with the vector and those obtained with a combination of gB,gO,gH and gL were all statistically significant. *1*: Vector, *2*: gB, *3*: gO, *4*: gH, *5*: gL, *6*: gH+gL, *7*: gB+gO, *8*: gB+gH+gL, *9*: gO+gH+gL, *10*: gB+gO+gH+gL.

co-cultured with SupT1 cells transfected with T7 RNA polymerase. Because the contents of the 293T and SupT1 cells must mix in order for the T7 RNA polymerase to transcribe the luciferase gene, the level of luciferase activity represents the extent of cellular fusion.

To determine which glycoprotein (s) is required for HHV-7-induced membrane fusion, 293T cells were transfected with gB, gO, gH, and gL respectively or in combination and mixed with SupT1 cells. Although transfection of gB or gH/gL complex both resulted in about twofold increase in mean luciferase activity respectively, efficient luciferase activity required the expression of all four glycoproteins. Luciferase activity increased about 65-fold when 293T cells were transfected with gB,gO, gH and gL (Figure 4).

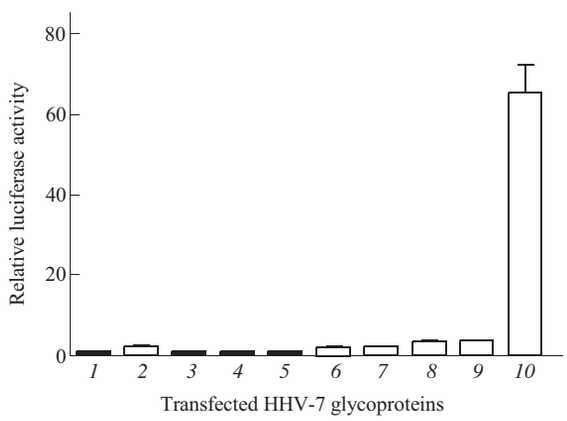

Fig. 4 Coexpression of gB, gO, gH, and gL is necessary and sufficient for HHV-7-induced membrane fusion

293T cells were transfected with the indicated HHV-7 glycoproteins and/or control vector as well as the luciferase reporter plasmid. SupT1 cells were transfected with T7 RNA polymerase. Twelve hours after tranfection SupT1 cells were plated over the 293T cells. Luciferase activity was measured 24 h after cocultivation. The mean relative luminosity units from three separate experiments run in duplicate are illustrated. The relative luciferase activity of the vector transfected 293T cells is normalized to one luciferase unit. Other values indicate fold induction above that of the vector control. *1*: Vector, *2*: gB, *3*: gO, *4*: gH, *5*: gL, *6*: gH–gL, *7*: gBgO, *8*: gBgH–gL, *9*: gOgH–gL, *10*: gBgOgH–gL.

2.5 Kinetics of HHV-7 glycoprotein-mediated cell fusion

To study the kinetics of fusion, the expression of luciferase activity was monitored over time. As shown in Figure 5, maximal luciferase activity occurred approximately 18 to 24 h after co-culture and persisted for 48 h.

2.6 Dose-dependent inhibition of cell fusion by anti-CD4 mAbs

As CD4 molecule is a cell receptor of HHV-7, to confirm that CD4 is indeed responsible for the cell-cell fusion, we performed antibody-mediated inhibition experiments of cell fusion. After preincubated with anti-CD4 mAbs M-T441 (ANCELL, USA), SupT1 cells were plated on the 293T cells, the luciferase activity was quantified, and then a dose-dependent inhibition of cell fusion was observed. When the concentration of anti-CD4 mAbs reached 10 mg/L, the luciferase activity decreased more than 75% (Figure 6).

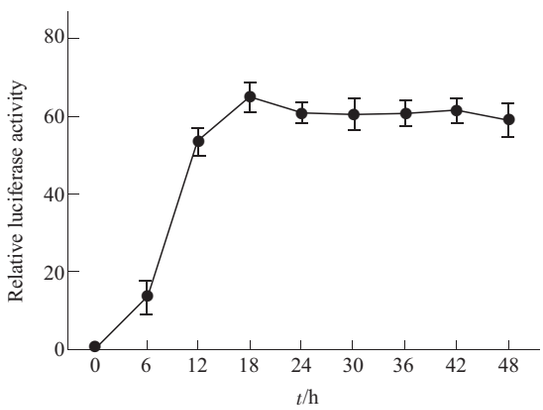

Fig. 5 Kinetics of cell fusion as measured by luciferase production

Luciferase activity was measured at the indicated times after cocultivation of 293T and SupT1 cells. 293T cells were transfected with plasmids expressing the indicated glycoproteins or a control vector and the luciferase reporter plasmid. SupT1 cells were transfected with T7 RNA polymerase. Shown are mean relative luminosity units (RLU) and standard deviations for duplicate samples obtained on three separate occasions. The relative luciferase activity of the vector transfected 293T cells is normalized to one luciferase unit. These values indicate fold induction above that of the vector control.

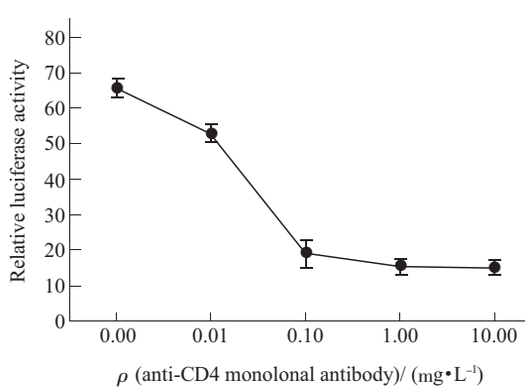

Fig. 6 Dose-dependent inhibition of cell fusion by anti-CD4 mAbs

After preincubated with anti-CD4 mAbs, SupT1 cells were plated on the 293T cells , the luciferase activity was quantified. Data represent mean values from three experiments. The relative luciferase activity of the vector transfected 293T cells is normalized to one luciferase unit.These values indicate fold induction above that of the vector control.

3 Discussion

HHV-7 mainly infects CD4+T lymphocytes and the CD4 protein is an essential component of the cellular receptor for HHV-7. Infection of CD4+T cells by either HHV-7 or HIV leads to the down-regulation of the CD4 receptor and inhibition of infection with the reciprocal virus[3]. HHV-7 has been shown to be a powerful inhibitor of HIV-1 infection also in mononuclear phagocytes[20]. In addition, it was found that HHV-7 suppresses the replication of CCR5-tropic (R5) HIV-1 but only mildly inhibits replication of CXCR4-tropic (X4) HIV-1 in HHV-7 and HIV-1 co-infected lymphoid tissue[21]. The highly selective tropism of HHV-7 for CD4+ T cells and the antagonism between HHV-7 and HIV suggests a possible future application of the virus. However, the precise mechanism by which HHV-7 enters CD4+ T cells is still unknown.

For herpesviruses, entry and virus-induced cell fusion are related processes. Penetration proceeds by fusion of the viral envelope with the cell membrane. Entry and cell-cell fusion require many of the same viral glycoproteins. Cell-cell fusion assays have been used as a tool to identify the fusion machinery of several herpesviruses[22~27]. In this study, we also used a virus-free cell fusion assay to analyze HHV-7 entry. The fusion assay utilized a luciferase gene activation system to quantify fusion. In 293T cells, which mimic the virion, HHV-7 glycoproteins of intrest (gB,gH,gL and gO) were transiently transfected. We found that the co-expression of gB, gO, gH and gL is sufficient and necessary for HHV-7 induced membrane fusion. Consistent with this result, the three proteins gB,gH and gL are required in herpes simplex virus type 1 (HSV-1) and HSV-2, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated virus (KSHV) mediated cell-cell fusion. It is thought that the basic fusion machinery for herpesvirus entry includes gB, gH, and gL, however, additional receptor-binding viral glycoproteins may also be required. For example, HSV requires gD as a ligand for entry receptors. EBV requires gp42, a component of the gH-gL-gp42 complex, as a ligand for human leukocyte antigen (HLA) class Ⅱ molecules on B lymphocytes. Betaherpesviruses do not encode a homolog of gD, but they encode gO or/and gQ which form(s) a complex with gH-gL as EBV gp42. HHV6 gH-gL-gQ complex can bind CD46 receptor and it is speculated that gH-gL-gO might act with another cell receptor[28,29]. HHV-7 gO also forms a complex with gH and we found that it was required in HHV7 mediated cell-cell fusion.

For herpesviruses may engage multiple receptors, we performed antibody-mediated inhibition experiments of cell fusion to confirm that CD4 was indeed responsible for the cell-cell fusion. And we observed a dose-dependent inhibition by anti-CD4 mAbs. The result indicated the CD4 was a key requirement for HHV-7 mediated cell-cell fusion. As herpesvirus fusion machines do not required a low pH activation step, it has been suggested that receptor binding initiates membrane fusion. HSV requires expression of receptor binding gD， and receptor binding can be attributed to gH-gL-gp42 complex for EBV. One glycoprotein from HHV-7 gB, gH , gL and gO or protein complex formed by these proteins might be the ligand of CD4 molecule. Further work to study the potential interaction is needed.

This study provides the groundwork for an in-depth analysis of the entry machine of HHV-7. Understanding the molecular mechanisms by which HHV-7 enters CD4+T cells will lead to the development of a new HHV-7-based viral vector. This vector can be developed to deliver exogenous DNA to CD4+T cells, or selective replicate within such cells efficiently. Such vector systems might allow for the introduction of therapeutic genes into HIV-1 target cells (gene therapy) and the selective manipulation of T-cell immunity (immunotherapy).

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人类疱疹病毒 7 型糖蛋白 gB、gH、gL、gO 介导细胞融合 *

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摘要　人类疱疹病毒 7 型(HHV-7)的感染依赖于包膜糖蛋白在病毒生命周期的多个阶段发挥功能．这些蛋白质可以介导病毒吸附，病毒包膜和宿主细胞膜融合以及病毒在细胞间的接触传播．将表达 HHV-7 糖蛋白的 293T 细胞与 HHV-7 易感的 SupT1 细胞共培养，检测虫荧光素酶报告基因的表达，以鉴定介导膜融合的 HHV-7 糖蛋白．研究发现，HHV-7 糖蛋白 gB、gH、gL、gO 能介导 293T 细胞与 SupT1 细胞的融合，且融合可被抗 CD4 单抗所抑制．结果表明，糖蛋白 gB、gH、gL、gO 对于 HHV-7 引发的膜融合是必需的，其中某个蛋白质或所形成的蛋白质复合物可能是 CD4 的配体．

关键词　人类疱疹病毒 7 型，糖蛋白，膜融合
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