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Tetherin restricts HSV-2 release and is counteracted by multiple viral glycoproteins

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ABSTRACT

Tetherin has been defined as a restriction factor of HIV-1 and several other enveloped viruses. However, the significance of tetherin in viral infection remains to be further addressed. Here, we investigated whether tetherin plays a role in HSV-2 infection. Our study revealed that overexpression of tetherin restricted the release of HSV-2 into the extracellular medium, while knockdown of tetherin by siRNA enhanced its release. We further demonstrated that HSV-2 infection and viral glycoproteins gB, gD, gH and gL but not gM significantly downregulated the endogenous expression of tetherin. Additional study indicated that tetherin likely physically interacted with gB, gD, gH and gL. This is the first time that tetherin has been shown to be counteracted by multiple viral components of a virus. Our findings inform the complexity of HSV-2-host interactions, providing basis for understanding the role of tetherin as a viral restriction factor and the mechanisms underlying viral countermeasures.

Introduction

Tetherin (BST2/CD317/HM1.24) is widely expressed in responses to type I interferons and constitutively expressed on several cell types, including bone marrow stromal cells, plasmacytoid dendritic cells, terminally differentiated B cells and the apical surface of polarized epithelial cells (Blasius et al., 2006; Rollason et al., 2009). Its gene expression in primary cells can be induced by cellular activation (Saraiva Raposo et al., 2013). Tetherin is a type II transmembrane protein (Ohtomo et al., 1999) with an unusual topology which contains an N-terminal cytoplasmic domain, a single membrane-spanning α-helices, an extracellular (ecto-) domain, and a C-terminal GPI anchor (Kupzig et al., 2003). Tetherin associates with cholesterol-enriched lipid rafts which are involved in both virus budding and cell-to-cell spread (Goffinet et al., 2009; Kupzig et al., 2003; Rollason et al., 2009, 2007).

Tetherin was identified to be involved in the host defense against viruses by a proteomic analysis of the plasma membrane treated with the K5/MIR2 protein of KSHV (Bartee et al., 2006). At that time, tetherin was thought to be involved in normal and malignant B cell differentiation (Goto et al., 1994) and had no other defined function. In 2008, tetherin was revealed to exert antiviral activity by the formation of tethers to capture the nascent HIV-1 viral particles to the cell surface, thereby inhibiting viral release (Neil et al., 2008; Van Damme et al., 2008). In addition to HIV-1, tetherin has been reported to restrict the release of a number of enveloped viruses, including the members of Retroviridae (HIV-2, SIV, etc), Filoviridae (Ebola virus and Marburgvirus), Arenaviridae (Lassafevervirus), Herpesviridae (KSHV), Rhabdoviridae (vesicularstomatitis virus), Orthomyxoviridae (influenza Avirus), Paramyxoviridae (Nipahvirus) and Flaviviridae (Dengue virus) (Douglas et al., 2010; Jouvenet et al., 2009; Kaletsky et al., 2009; Le Tortorec et al., 2011; Mansouri et al., 2009; Pan et al., 2012; Sakuma et al., 2009). The listed viruses restricted by tetherin share little or no homology with one another except a lipid envelope. To date, several viral proteins, including Vpu (HIV-1) (Neil et al., 2008; Van Damme et al., 2008), Nef (SIV) (Jia et al., 2009), Env (HIV-2 and SIV) (Gupta et al., 2009; Le Tortorec and Neil, 2009), K5 (KSHV) (Bartee et al., 2006; Mansouri et al., 2009), Ebola glycoprotein (Kaletsky et al., 2009), have been revealed to counteract the restrictive properties of tetherin.

Of interest, among the viruses restricted by tetherin, KSHV belongs to the γ-herpesvirus subfamily. In contrast, human cytomegalovirus (HCMV), a member of the β-herpesvirus subfamily, has been reported

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to be enhanced by tetherin at the stage of viral entry (Viswanathan et al., 2011). Herpesviridae is a large family subdivided into three subfamilies: α-herpesvirinae, β-herpesvirinae and γ-herpesvirinae. Given the opposite effects of tetherin on KSHV and HCMV, we asked whether tetherin plays a role in the infection of α-herpesviruses. Indeed, in the process of this manuscript submission, tetherin has been reported to restrict the release of HSV-1 with one report suggesting that virion host shutoff protein was important for tetherin counteraction (Zenner et al., 2013) and the other that tetherin was moderately antagonized by glycoprotein M (Blondeau et al., 2013), indicative of a greater complexity in herpesvirus–tetherin interactions.

HSV-2 is a typical member of the α-herpesvirus subfamily. It is the predominant cause of genital herpes and its infection increases the risk of HIV-1 acquisition and transmission (Baeten et al., 2004; Freeman et al., 2006). In the current study, we addressed whether tetherin plays a role in HSV-2 infection. We demonstrated that the release of mature infectious HSV-2 particles could be significantly impaired by tetherin overexpression, while knockdown of tetherin enhanced HSV-2 release. Unlike its role played in the entry of HCMV, tetherin did not enhance HSV-2 entry. We further revealed that HSV-2 infection and several viral glycoproteins, including gB, gD, gH and gL, significantly downregulated the expression of tetherin. Our data together demonstrate that tetherin restricts HSV-2 release. Such tetherin-mediated restrictions can be counteracted by HSV-2 gB, gD, gH, and gL.

Results

Overexpression of human tetherin decreases the release of HSV-2 progeny virions

In the current study, we attempted to address whether tetherin plays a role in HSV-2 infection. HSV-2 infects cervical epithelial cells. We therefore conducted our experiments by using HeLa cells. We first determined HSV-2 growth kinetics in HeLa cells (Fig. S1). Although tetherin was detected on the cell surface of untreated HeLa cells, the level of tetherin was increased ~2-fold upon treatment with IFN-α (Fig. 1A and B). Tetherin showed several isoforms upon SDS PAGE analysis, presumably due to heterogeneity of glycosylation during

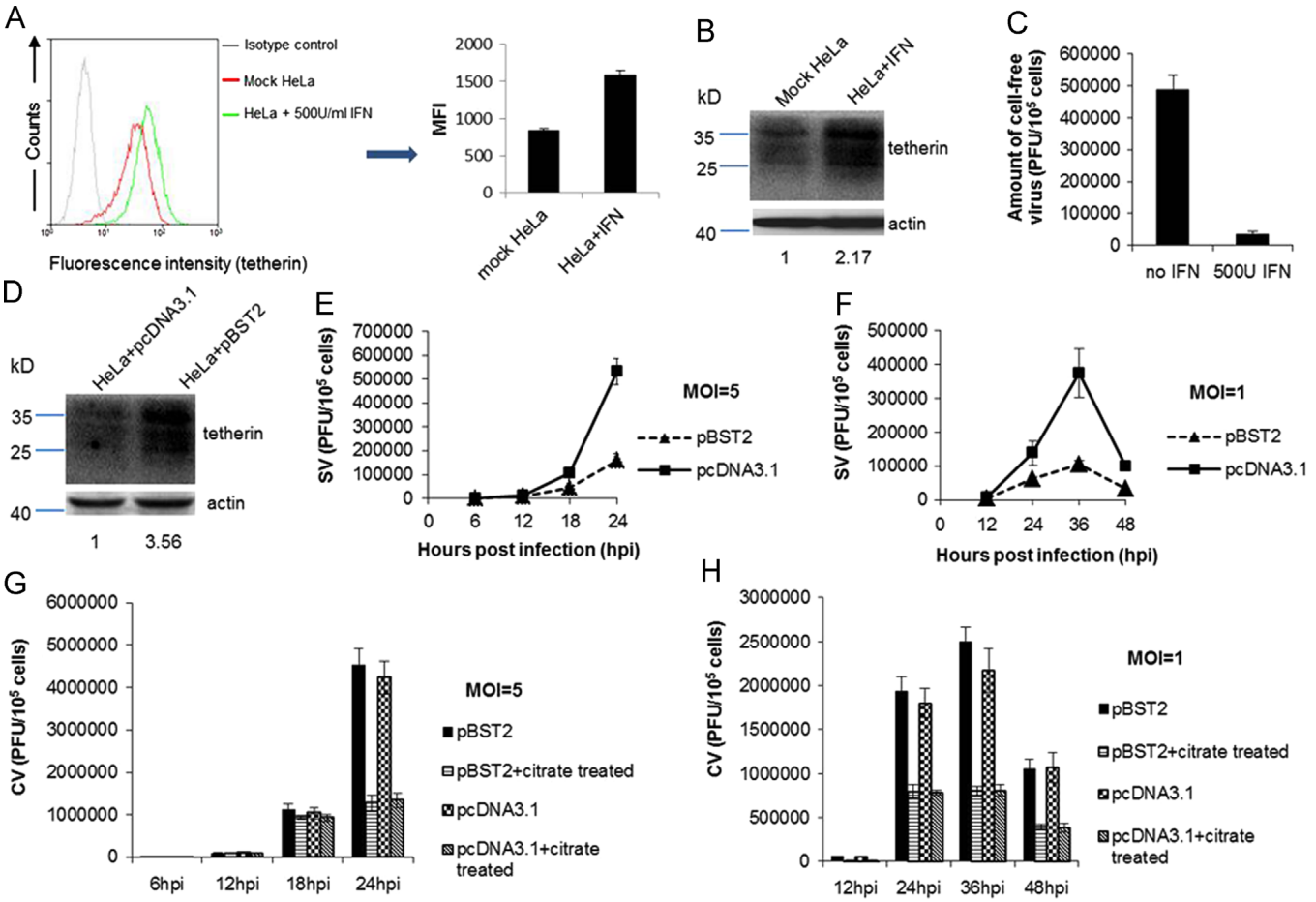
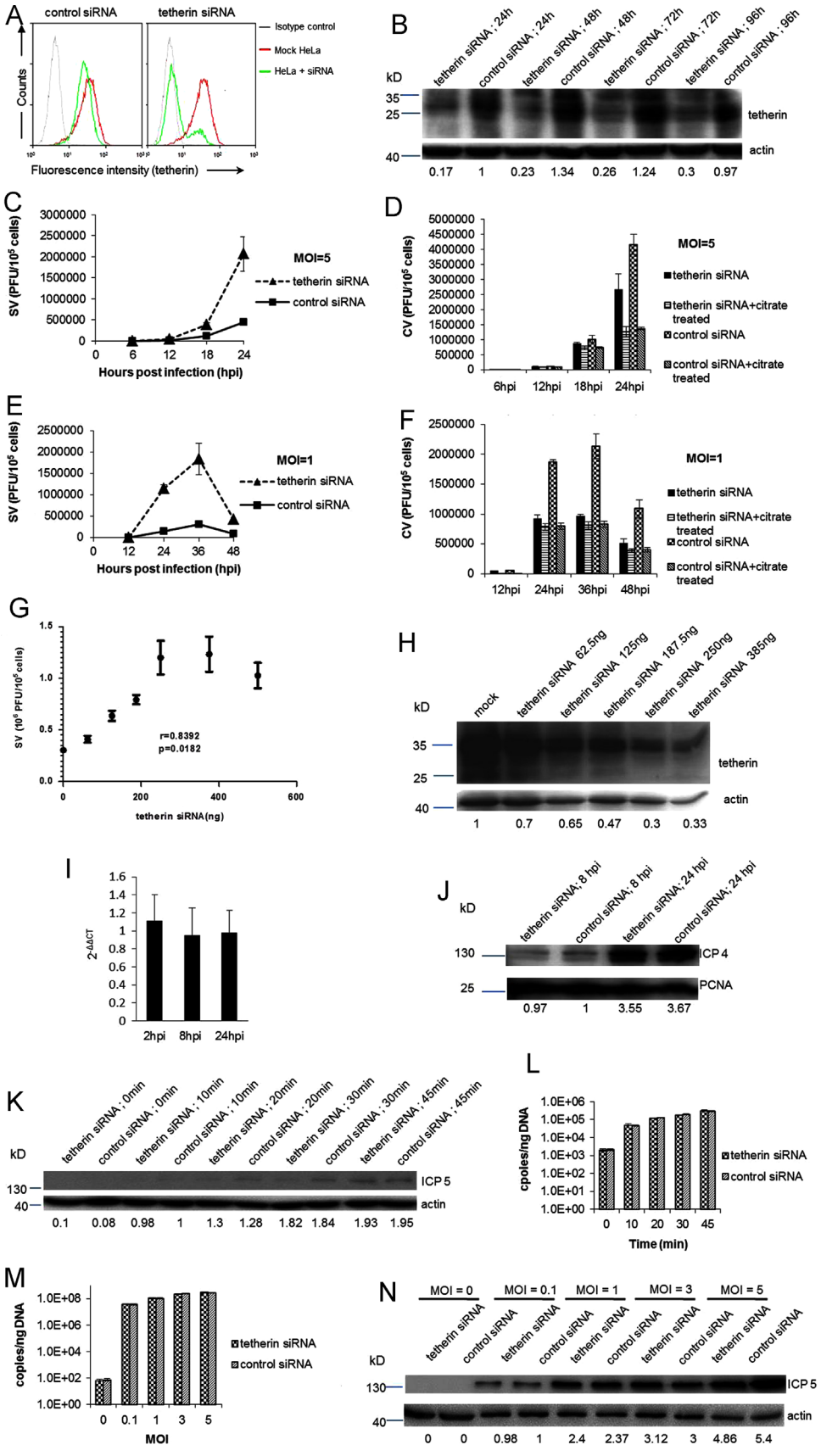


Fig. 1. Overexpression of human tetherin decreases the release of HSV-2 progeny virions. (A) Flow cytometric analysis of cell-surface expression of tetherin on mock or IFN-α treated HeLa cells. One representative histogram is shown with mean fluorescence intensity (MFI) values obtained from three independent experiments ($P < 0.01$). (B) The expression of total tetherin in mock or IFN-α-treated HeLa cells was analyzed by western blot where actin was used as a loading control. Molecular weight standards in kilodaltons are shown on the left. The relative expression levels of tetherin to actin are shown. (C) Mock or IFN-α treated HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers were infected with HSV-2 at an MOI of 5 PFU/cell in which 0.35 ml virus suspension per well was added to 12-well plates followed by a 3 h of incubation and the supernatants were collected at 24 hpi. The amount of infectious cell-free virions was measured by plaque assay. (D) The expression of total tetherin in pcDNA3.1 or pBST2-transfected HeLa cells was analyzed by western blot where actin was used as a loading control. The relative expression levels of tetherin to actin are shown. (E–H) HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers transfected with pcDNA3.1 or pBST2 were infected with HSV-2 at an MOI of 5 or 1 PFU/cell in which 0.35 ml virus suspension per well was added to 12-well plates followed by a 3 h of incubation and the supernatants and infected cells were collected at different time points. The supernatant viruses (SV) and cell-associated viruses (CV) were measured by plaque assay. The titers of CV were from duplicate cultures in which the cells were treated or untreated with the pH3.0 citrate buffer before harvesting. One representative experiment out of three is shown (A, B and D). Data shown are mean ± SD of three independent experiments with each condition performed in triplicate (C and E–H).


A
control siRNA
tetherin siRNA
Isotype control
Mock HeLa
HeLa + siRNA
Counts
Fluorescence intensity (tetherin)
B
tetherin siRNA ; 24h
control siRNA ; 24h
tetherin siRNA ; 48h
control siRNA ; 48h
tetherin siRNA ; 72h
control siRNA ; 72h
tetherin siRNA ; 96h
control siRNA ; 96h
kD
35
25
40
tetherin
actin
0.17 1 0.23 1.34 0.26 1.24 0.3 0.97
C
SV (PFU/10^5 cells)
MOI=5
tetherin siRNA
control siRNA
Hours post infection (hpi)
D
CV (PFU/10^5 cells)
MOI=5
tetherin siRNA
tetherin siRNA+citrate treated
control siRNA
control siRNA+citrate treated
6hpi 12hpi 18hpi 24hpi
E
SV (PFU/10^5 cells)
MOI=1
tetherin siRNA
control siRNA
Hours post infection (hpi)
F
CV (PFU/10^5 cells)
MOI=1
tetherin siRNA
tetherin siRNA+citrate treated
control siRNA
control siRNA+citrate treated
12hpi 24hpi 36hpi 48hpi
G
SV (10^5 PFU/10^5 cells)
r=0.8392
p=0.0182
tetherin siRNA(ng)
H
mock
tetherin siRNA 62.5ng
tetherin siRNA 125ng
tetherin siRNA 187.5ng
tetherin siRNA 250ng
tetherin siRNA 385ng
kD
35
25
40
tetherin
actin
1 0.7 0.65 0.47 0.3 0.33
I
2^-ΔΔCT
2hpi 8hpi 24hpi
J
tetherin siRNA; 8 hpi
control siRNA; 8 hpi
tetherin siRNA; 24 hpi
control siRNA; 24 hpi
kD
130
25
ICP 4
PCNA
0.97 1 3.55 3.67
K
tetherin siRNA ; 0min
control siRNA ; 0min
tetherin siRNA ; 10min
control siRNA ; 10min
tetherin siRNA ; 20min
control siRNA ; 20min
tetherin siRNA ; 30min
control siRNA ; 30min
tetherin siRNA ; 45min
control siRNA ; 45min
kD
130
40
ICP 5
actin
0.1 0.08 0.98 1 1.3 1.28 1.82 1.84 1.93 1.95
L
cpoles/ng DNA
tetherin siRNA
control siRNA
Time (min)
M
copies/ng DNA
tetherin siRNA
control siRNA
MOI
N
MOI = 0
MOI = 0.1
MOI = 1
MOI = 3
MOI = 5
tetherin siRNA
control siRNA
kD
130
40
ICP 5
actin
0 0 0.98 1 2.4 2.37 3.12 3 4.86 5.4

post-translational modification (Ohtomo et al., 1999). These results were consistent with previous reports that the expression of tetherin could be enhanced by type I IFNs (Blasius et al., 2006). Following HSV-2 infection of IFN-α-treated or untreated HeLa cells at a multiplicity of infection (MOI) of 5 PFU/cell, supernatants were collected at 24 h post infection (hpi), and thereafter, the amount of infectious cell-free virions was analyzed using plaque assays. Compared to untreated cells, the release of HSV-2 progeny virions were severely reduced in the IFN-α-treated cells. In particular, treatment with 500 U/ml of IFN-α resulted in an approximate 14-fold reduction in the yield of infectious HSV-2 particles (Fig. 1C). Given that other cellular components induced by IFN-α may also inhibit the replication of HSV-2, we next determined whether overexpression of tetherin accounted for the reduced release of HSV-2 progeny virions. HeLa cells were transfected with tetherin expression plasmid (pBST2) or pcDNA3.1 prior to HSV-2 infection, and the expression level of tetherin was detected by western blot (Fig. 1D). Following HSV-2 infection at an MOI of 5 or 1 PFU/cell, the amount of infectious cell-free virions and cell-associated progeny virions was analyzed at different time points. Compared to pcDNA3.1-transfected cells, the production of supernatant viruses (SV) was decreased ~3.3-fold at an MOI of 5 PFU/cell (24 hpi) and ~3.6-fold at an MOI of 1 PFU/cell (36 hpi) in tetherin overexpressing cells (Fig. 1E and F). Considering that cell-associated viruses (CV) likely contained both intracellular viruses and extracellular viruses attached to the cell surface, we then used low pH citrate buffer which can inactivate virions on the cell surface but not intracellular ones. The titeres of CV in duplicate cultures in which the cells were treated or untreated with the pH3.0 citrate buffer before harvesting were tested. As shown in Fig. 1 G and H, the amount of infectious cell-associated viruses from citrate treated cultures decreased compared to that from citrate untreated cultures, in particular at 24 hpi with an MOI of 5 PFU/cell or at 36 hpi with an MOI of 1 PFU/cell, indicating that some of the viruses were extracellular but still attached to the cell surface. Moreover, such decrease was more apparent in the tetherin overexpressing cells. The total virus yields (SV+CV) were not significantly changed by overexpressing tetherin. Given the role of tetherin as a restriction factor in inhibiting the release of enveloped viruses, our data together suggest that overexpression of tetherin restricts the release of HSV-2.

Downregulation of endogenous tetherin expression enhances the release of HSV-2 progeny virions

The decreased release of HSV-2 progeny virions appeared to be associated with the increased levels of tetherin expression, suggesting that tetherin is likely to be an effective restriction factor of HSV-2 release. To examine whether endogenous tetherin can reduce the release of HSV-2 progeny virions, HeLa cells were transfected with tetherin siRNA or control siRNA prior to HSV-2 infection. Flow cytometry analysis indicated that siRNA knockdown of tetherin resulted in a reduction of cell-surface expression of tetherin in ~80% cells (Fig. 2A). The total tetherin level was also significantly decreased as demonstrated by western blot analysis and the siRNA effect remained apparent up to 96 h post transfection (Fig. 2B). siRNA-pretreated HeLa cells were subsequently infected with HSV-2 at an MOI of 5 or 1 PFU/cell, and the amount of infectious cell-free viruses was determined at different time points. Compared to control siRNA-pretreated cells, the release of infectious HSV-2 progeny virions was increased ~4.6-fold at an MOI of 5 PFU/cell (24 hpi) and ~5.9-fold at an MOI of 1 PFU/cell (36 hpi) in tetherin siRNA pretreated cells (Fig. 2C and E). The titers of CV in duplicate cultures in which the cells were treated or untreated with the pH3.0 citrate buffer before harvesting were also tested. As shown in Fig. 2D and F, the amount of infectious cell-associated viruses from citrated treated cultures decreased compared to that from citrate untreated cultures, in particular at 24 hpi with an MOI of 5 PFU/cell or at 36 hpi with an MOI of 1 PFU/cell, indicating that some of the viruses were extracellular but still attached to the cell surface. Such decrease in the control siRNA pretreated cells was more apparent than that in the tetherin siRNA pretreated cells. And the total virus yields (SV+CV) were not significantly changed by downregulating tetherin expression. The correlation between the dose of tetherin siRNA, the expression level of tetherin and the production of HSV-2 progeny virions was further examined. As shown in Fig. 2G and H, the increased dose of tetherin siRNA positively correlated with the production of supernatant viruses and negatively correlated with the expression level of tetherin, indicating a negative association between tetherin expression and HSV-2 release.

Lower expression of tetherin associated with higher amount of infectious progeny virions, indicating that tetherin unlikely enhances HSV-2 entry. To further confirm the observation, we monitored whether the immediate early events of HSV-2 infection were influenced by tetherin. HeLa cells were transfected with tetherin siRNA or control siRNA followed by HSV-2 infection at an MOI of 5 PFU/cell. At different time points, cells were collected and the RNA expression of HSV-2 IE protein (ICP4) was detected by relative quantitative RT-PCR. According to $2^{-\Delta\Delta CT}$ method, the fold change in target gene expression is normalized to an endogenous reference gene. For the control sample, $2^{-\Delta\Delta CT}$ equals to one (Livak and Schmittgen, 2001). In terms of HSV-2 ICP4 RNA expression, the mean fold change of HeLa cells pretreated with tetherin siRNA relative to the control was around one (Fig. 2I), indicating that there was no

Fig. 2. Downregulation of endogenous tetherin expression enhances the release of HSV-2 progeny virions. (A) Flow cytometric analysis of tetherin cell-surface expression on HeLa cells pretreated with tetherin siRNA or control siRNA at 24 h post transfection. (B) The expression of total tetherin in HeLa cells pretreated with tetherin siRNA or control siRNA was analyzed at different time points by western blot where actin was used as a loading control. Molecular weight standards in kilodalton are shown on the left. (C–F) Tetherin siRNA or control siRNA pretreated HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers were infected with HSV-2 at an MOI of 5 or 1 PFU/cell in which 0.35 ml virus suspension per well was added to 12-well plates followed by a 3 h of incubation. Supernatants and infected cells were collected at different time points, and the supernatant viruses (SV) and cell-associated viruses (CV) were measured by plaque assay. The titers of CV were from duplicate cultures in which the cells were treated or untreated with the pH3.0 citrate buffer before harvesting. The dose of siRNA used in (A–F) was 250 ng/well. (G) Correlation between the production of HSV-2 progeny virions and the dose of tetherin siRNA. HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers pretreated with different doses of tetherin siRNA were infected with HSV-2 at an MOI of 5 PFU/cell in which 0.35 ml virus suspension per well was added to 12-well plates followed by a 3 h of incubation. Supernatants and infected cells were collected at 24 hpi, and the supernatant viruses (SV) were measured by plaque assay. Spearman test was applied and the spearman r and p value are shown. (H) Correlation between the expression level of tetherin and the dose of tetherin siRNA. (I) HeLa cells ($\sim 3.5 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers pretreated with tetherin siRNA or control siRNA for 24 h were infected with HSV-2 at an MOI of 5 PFU/cell in which 1 ml virus suspension per well was added to 12-well plates followed by a 1 h of incubation. At 2, 8 and 24 hpi, the RNA expression of HSV-2 IE protein (ICP4) in cells was measured by relative quantitative RT-PCR. The difference in ICP4 RNA expression was calculated on the basis of $2^{-\Delta\Delta CT}$ values. (J) The protein expression level of ICP4 (parallel samples in (I)) was measured by western blot. (K) HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers pretreated with tetherin siRNA or control siRNA for 24 h were infected with HSV-2 at an MOI of 5 PFU/cell (0.35 ml per well in 12-well plates). At 0, 10, 20, 30 and 45 min post infection, cells were harvested and the protein expression level of ICP5 was measured by western blot. (L) HeLa cells (parallel samples in (K)) were treated for 2 h with DNase-I and viral genomic DNA isolated and intracellular HSV-2 genome copy number enumerated by quantitative Taqman PCR specific for gB. The probe was labeled at the 5′ end with FAM and at the 3′ end with TAMRA. Values are presented as copies/ng DNA (HSV-2 episomes per nanogram of total cellular DNA). (M) HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers pretreated with tetherin siRNA or control siRNA for 24 h were infected with HSV-2 at different MOIs (0, 0.1, 1, 3, 5 PFU/cell) in which 0.35 ml virus suspension per well was added to 12-well plates followed by a 3 h of incubation. At 24 hpi, intracellular HSV-2 genome copy was measured as (L). (N) HeLa cells (parallel samples in (M)) were harvested and the protein expression level of ICP5 was measured by western blot. Data shown are mean ± SD of three independent experiments with each condition performed in triplicate (C–G, I, L and M). One representative experiment out of three is shown (A, B, H, J, K and N).

difference in ICP4 RNA expression between HeLa cells pretreated with tetherin siRNA and those pretreated with control siRNA. We also examined the protein expression of viral immediate early gene (ICP4) (at 8 and 24 hpi) in the infected cells by western blot. The expression level of ICP4 in HeLa cells pretreated with tetherin siRNA was similar to that pretreated with control siRNA (Fig. 2J). At the same time, the genomic DNA and protein from incoming viruses were detected at several time points (0, 10, 20, 30, 45 min) post infection. As shown in Fig. 2K and L, the copies of HSV-2 genomic DNA and the protein level of HSV-2 major capsid protein (ICP5) in the tetherin siRNA pretreated HeLa cells showed no difference compared to those in the control siRNA pretreated HeLa cells at 10, 20, 30, 45 min post infection. In addition, the intracellular copies of HSV-2 genome and the protein expression of late gene (ICP5) were determined at 24 hpi using different MOIs (0, 0.1, 1, 3, 5 PFU/cell). As shown in Fig. 2M and N, HSV-2 genome level remained constant and there was no difference in ICP5 expression in the cells pretreated with tetherin siRNA or control siRNA. These results further support the notion that tetherin has no effect on HSV-2 gene expression. Our data together imply that tetherin does not enhance HSV-2 entry and replication. In conclusion, downregulation of endogenous tetherin expression enhances the release of HSV-2 progeny virions.

Given our current knowledge of tetherin as an anti-viral protein that tethers virions to cell membranes, we hypothesized that tetherin might decrease the release of HSV-2 progeny virions through a similar mechanism. We therefore examined the association of HSV-2 virion particles with the cell membranes of HeLa cells pretreated with tetherin or control siRNA by electronic microscopy. Large-scale surface clustering of mature HSV-2 virions was seen in the control siRNA-pretreated cells (Fig. 3B and E), whereas single or small clusters of virions were observed in association with the cell membranes of tetherin siRNA-pretreated cells (Fig. 3A and D), indicating that more particles were released to medium in tetherin-knocked down cells as measured by infectivity assays (Fig. 2C and E). Statistically, there was an approximately 6–7 fold greater density of mature particles on the surface of control siRNA-pretreated cells compared to the tetherin siRNA-pretreated cells (Fig. 3C and F). To determine whether tetherin was incorporated into HSV-2 virions, we fractionated virion preparations via a sucrose gradient cushion to separate cellular membrane fragments and defective viral particles from intact infectious virions. The representative viral and cellular proteins were examined by western blot. As shown in Fig. 4A, HSV-2 major capsid protein VP5 (ICP5) was detected in fractions 5–11 while envelope glycoprotein gB was mainly presented in fractions 8–11. Among those fractions, only fractions 8–11 infected Vero cells and had plaque formation, suggesting that intact infectious HSV-2 virions mainly existed in fractions 8–11. We also examined the presentation of CD326, an epithelial cells marker, and tetherin. CD326 was detected in the fractions 5–7, indicating that cellular components mainly existed in low density fractions. In agreement, tetherin was also detected in fractions 4–7, and peaked in fractions 5 and 6. Of note, a small amount of tetherin was detected in fractions 8–11, suggesting that tetherin is likely to be present in HSV-2 virions. To further confirm the observation in fraction assays, harvested HSV-2 virions were applied to poly-L-lysine coated coverslips and analyzed by immunofluorescence microscopy. A representative field observed is shown in Fig. 4B. The FITC-positive dots that were positive for tetherin colocalized with the Cy3-positive dots that were positive for HSV-2 ICP5. The fluorescence colocalization of tetherin and ICP5 indicated that tetherin might be incorporated into HSV-2 virion.

HSV-2 infection downregulates the expression of tetherin

To investigate whether HSV-2 infection can influence the expression of tetherin, cell surface expression of tetherin in the presence or absence of HSV-2 infection was examined by flow cytometry at 24 hpi. Compared to that on uninfected cells, the expression of tetherin on HSV-2 infected HeLa and ME-180 cells was remarkably diminished (Fig. 5A). We also examined tetherin expression on primary mucosal epithelial cells isolated from cervical explants using previously established methods (Howell et al., 1997; Hu et al., 2004; Huang et al., 2012). As shown in Fig. 5A, the expression level of tetherin on primary epithelial cells was increased after treatment with IFN-α for 24 h. Following HSV-2 infection, the expression of tetherin was remarkably decreased in primary epithelial cells. In addition to flow cytometry analyses, we also examined total protein expression by western blot. As shown in Fig. 5B and C, cells infected with HSV-2 showed a significant decrease of total tetherin protein. As a cell membrane protein control, the epithelial marker CD326 was analyzed parallelly and its expression appeared to be the same in samples with or without HSV-2 infection. Collectively, we conclude that HSV-2 infection can efficiently downregulate the expression of tetherin.

Multiple HSV-2 glycoproteins contribute to the downregulation of tetherin expression

The “tethered” virions observed in electron microscopy images were fully mature and had complete and intact outer membranes, implying interactions between the molecules on cell and virion exteriors. We hypothesized that some of HSV-2 viral envelope glycoproteins might mediate an interaction between virion and tetherin. Considering that envelope glycoproteins (gB, gD, gH, and gL) are essential for HSV entry (Caiet al., 1988; Cheshenko and Herold, 2002; Desai et al., 1988; Hutchinson et al., 1992; Ligas and Johnson, 1988; Muggeridge, 2000), we focused on gB, gD, gH, and gL to address their potential roles played in the counteraction of tetherin. We examined HSV-2 gM because the gM of HSV-1 has recently been shown to moderately antagonize tetherin. We also examined glycoproteins gE and gG. To define the mechanisms by which HSV-2 downmodulates tetherin expression, HeLa cells were transfected with plasmid expressing gB-flag, gD-flag, gH-flag, gL-flag, gE-flag, gG-flag gM-flag, or control plasmid pcDNA3.1. At 48 h post transfection, cells were harvested and the cell-surface expression of tetherin was examined by flow cytometry. Compared to pcDNA3.1-transfected cells, the cell-surface expression of tetherin was significantly decreased in gB, gD, gH, or gL-transfected cells whereas the expression of tetherin in gM, gE or gG transfected cells remain unchanged (Fig. 6A). The parallel cell lysates were analyzed by western blot. In accordance with the flow cytometry assay, tetherin was significantly decreased in cells transfected with plasmid expressing gB-flag, gD-flag, gH-flag or gL-flag, but not in those transfected with gM-flag (Fig. 6B), suggesting that gB, gD, gH and gL could specifically downmodulate tetherin. The expression of gB-flag, gD-flag, gH-flag, gL-flag and gM-flag was confirmed (Fig. 6C). The epithelial marker CD326 was analyzed parallelly as a cell membrane protein control, and was unaffected by viral glycoproteins compared to tetherin (Fig. 6B and C). These data collectively suggest that HSV-2 envelope glycoproteins highly likely account for the downmodulation of tetherin during HSV-2 infection.

gB, gD, gH, and gL physically interact with tetherin

To further confirm the specificity, we next investigated whether there was a physical interaction between tetherin and gB, gD, gH or gL. The cellular localization of tetherin and HSV-2 glycoproteins was detected by immunofluorescence assay. The cellular localization of a protein can be altered by its interacting proteins (Liu et al., 2009). Therefore, we examined the localization of HSV-2 glycoproteins in 293T cells which have no endogenous tetherin expression (Fig. S3). Images showed that gB-flag and gD-flag were detected on the cell

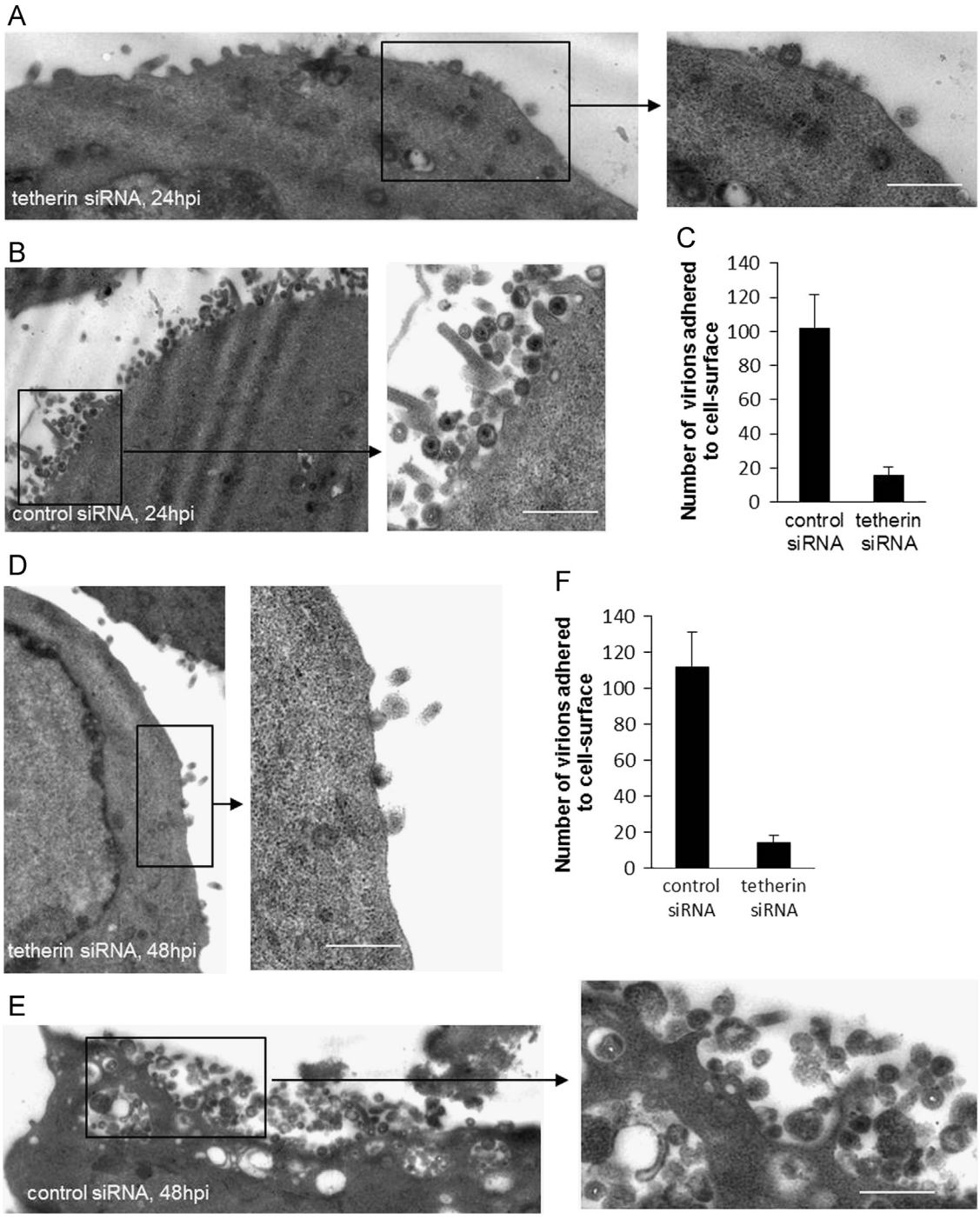


Fig. 3. Association of HSV-2 virion particles with cell membranes. Tetherin siRNA or control siRNA pretreated HeLa cells ($\sim 3.5 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers were infected with HSV-2 at an MOI of 3 PFU/cell in which 1 ml virus suspension per well was added to 12-well plates followed by a 1 h of incubation. At 24 or 48 hpi, infected cells were harvested and cell sections were prepared for electron microscopy. Images show the accumulation of mature HSV-2 virions adhered to the surface of HeLa cells pretreated with control siRNA (B and E) or tetherin siRNA (A and D). Scale bars in all panels represent 500 nm. (C) Quantitative analysis of mature particles on the surface of tetherin or control siRNA-pretreated cells at 24 hpi. (F) Quantitative analysis of mature particles on the surface of tetherin or control siRNA-pretreated cells at 48 hpi. The images were chosen randomly, and 10 images were obtained in each condition. One representative image out of 10 is shown (A, B, D and E). The number of particles was obtained from 10 individual cells. Data shown are mean ± SD of three independent experiments with each condition performed in triplicate (C and F).

surface of permeabilized or non-permeabilized cells. In addition, the cellular localization of untagged HSV-2 glycoproteins gB and gD in transfected cells was consistent with that of flag-tagged gB and gD (Fig. S2). In contrast, gH-flag and gL-flag were only detected in permeabilized cells (Fig. S3), informing that gH-flag and gL-flag expressed independently failed to be transported to the cell surface. Nevertheless, similar to gB-flag and gD-flag, both gH-flag and gL-flag demonstrated colocalization with tetherin in the transfected cells (Fig. S3). The colocalization of tetherin and the viral glycoproteins in HeLa cells was also examined. As shown in Fig. 7, the colocalization of the viral glycoproteins and tetherin in HeLa cells was consistent with that in 293T cells. We further cotransfected plasmids expressing gH-flag and gL-flag (or gH and gL) into HeLa cells and assessed their cellular localization. As shown in Fig. S4A, the gH-flag-gL-flag complex and gH-gL complex had similar cell surface localization. The surface expression of tetherin was also determined in cells

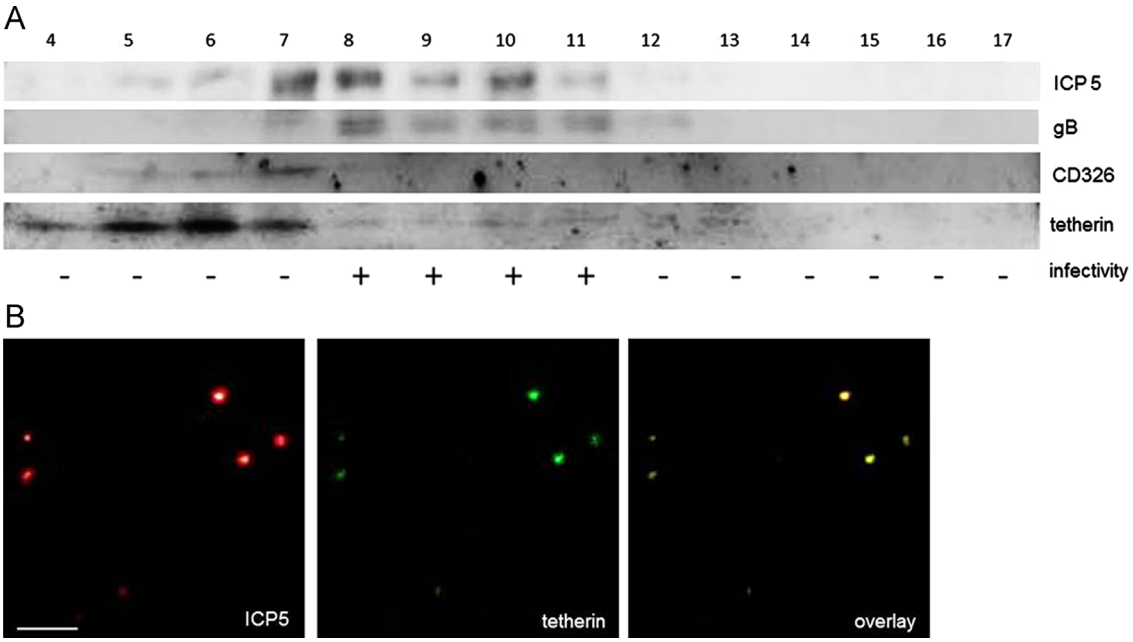


Fig. 4. Tetherin is highly likely to be incorporated into HSV-2 virions. (A) HSV-2 virion preparations were fractioned via a sucrose gradient cushion (10–50%) to separate cellular membrane fragments and defective viral particles from intact infectious virions. The representative viral and cellular proteins were assessed by western blot. One representative experiment out of three is shown. (B) Immunofluorescence analysis of HSV-2 virions. Virions harvested from HSV-2 infected HeLa cells were pelleted and applied to poly-L-lysine-coated coverslips prior to immunofluorescence analysis using anti-ICP5 and anti-tetherin antibodies. The FITC-positive dots were positive for tetherin and the Cy3-positive dots were positive for HSV-2 ICP5. Representative fields observed in two experiments are shown. Scale bars represent 500 nm.

expressing gH-flag-gL-flag or gH-gL complexes by flow cytometry. In both cases, a similar down-regulation of tetherin was observed and such down-regulation was comparable to that seen in cells transfected with gH or gL alone (see Fig. S4B). These data together suggest a physical interaction between tetherin and gB, gD, gH or gL. Co-immunoprecipitation assay was performed to further confirm these findings. HeLa cells were transfected with control pcDNA3.1 or plasmid expressing gB-flag, gD-flag, gH-flag, gL-flag or gM-flag. 48 h post transfection, precleared cell lysates from the transfected cells were incubated with an isotype control antibody and an anti-flag antibody. The precipitates were analyzed by western blot using an antibody against tetherin, while the precleared cell lysates were analyzed by western blot using the anti-flag antibody. As shown in Fig. 8A, the antibody against flag was able to specifically precipitate the immune complex that contained tetherin and gB-flag, gD-flag, gH-flag or gL-flag. Co-IP experiments were also performed by pulling down with the anti-tetherin antibody, followed by western blot with the anti-flag or anti-tetherin antibody. The antibody against tetherin was able to specifically precipitate the immune complex that contained tetherin and gB-flag, gD-flag, gH-flag or gL-flag (Fig. 8B). The precipitates treated with the isotype control antibody were also analyzed by western blot with the anti-flag or anti-tetherin antibody (Fig. 8C) and there was no corresponding bands observed. The expression of tetherin in transfected cells was analyzed by western blot (Fig. 8D) and immunofluorescence (Fig. 7), confirming its correct expression in terms of protein size and subcellular localization. The results together confirmed that there was a physical interaction between tetherin and gB, gD, gH and gL, respectively.

Discussion

The significance of tetherin in viral infection remains to be further addressed. In the current study, we investigated whether tetherin plays a role in the infection of α-herpesvirus HSV-2. Like the γ-herpesvirus KSHV, HSV-2 progeny viral particles can be trapped by tetherin. Unlike its role in β-herpesvirus HCMV infection, tetherin does not enhance HSV-2 entry. Of note, HSV-2 infection significantly downregulates the expression of tetherin and multiple HSV-2 glycoproteins account for such downregulation. Our findings that tetherin plays a key role in inhibiting the release of HSV-2 virions support the notion that tetherin is a broad-spectrum restriction factor of enveloped viruses (Douglas et al., 2010). To date, viruses found to be restricted by tetherin share little or no homology with one another except for a lipid envelope, making it difficult to forecast the effect of tetherin on different viruses. The results in this study together provide an initial understanding on the inhibitory effect of tetherin against HSV-2. It is probable that tetherin may have multifaceted function on different viruses, and this notion will remain uncertain until more enveloped viruses have been investigated to address the roles of tetherin in viral infection and spread.

Tetherin has an unusual topology with both ends embedded in the cellular membrane by two different types of membrane anchor: a transmembrane domain proximal to the N-terminus and a C-terminal GPI anchor (Kupzig et al., 2003). It is hypothesized that tetherin tethers virions to cell membranes, and to each other by linking the viral envelope with the cell membrane through each of its membrane domains or through dimers that formed between virion-associated tetherin and tetherin in the cell membrane (Perez-Caballero et al., 2009). Recently, Venkatesh et al. have shown that HIV-1 virion tethers are composed of the tetherin protein itself and that the axially configured tetherin homodimers are directly responsible for trapping virions at the cell surface (Venkatesh and Bieniasz, 2013). In favor of the above model, our data suggest that tetherin is likely to be incorporated into HSV-2 virions. Electron microscopy showed that HSV-2 virion particles associated with the cell membranes of HeLa cells and that more virions were seen in the control siRNA-pretreated cells than the tetherin siRNA-pretreated cells, while infectivity assays demonstrated that more virions were released into the medium in tetherin-knocked down cells. These data together suggest that cell surface expression of tetherin appears to inhibit HSV-2 release. Flow cytometry assay showed that HSV-2 infection downregulated tetherin expression on

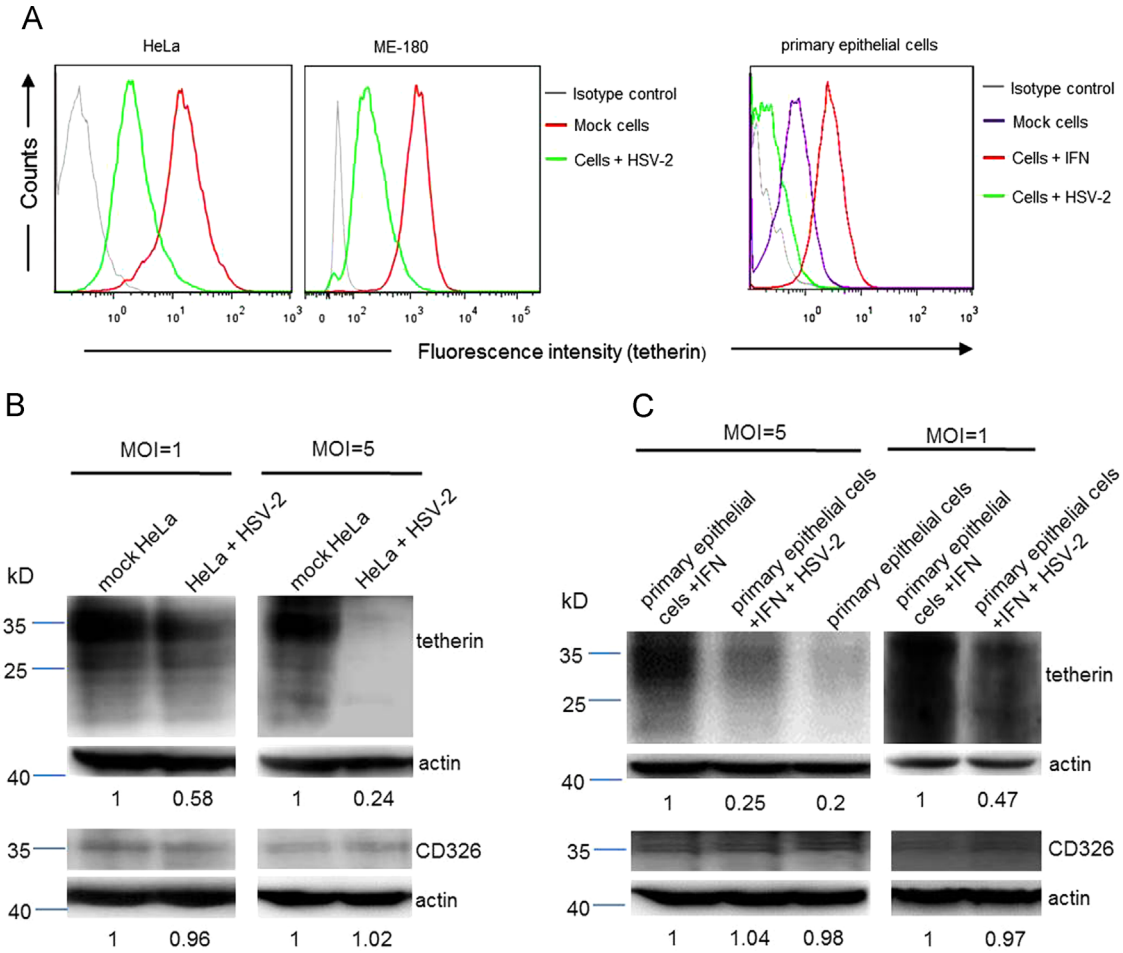


Fig. 5. HSV-2 infection downregulates the expression of tetherin. HeLa cells ($\sim 3.5 \times 10^5$) were plated onto 12-well plates overnight, and then the monolayers were infected with HSV-2 at an MOI of 5 or 1 PFU/cell in which 1 ml virus suspension per well was added to 12-well plates followed by a 1 h of incubation. (A) Flow cytometric analysis of tetherincell-surface expression on HeLa, ME-180 and IFN-α pretreated primary epithelial cells in the presence or absence of HSV-2 infection. Cells were stained with anti-tetherin antibody (red, purple or green line) or isotype-matched control IgG (gray line). (B) The expression of total tetherin and CD326 in mock, HSV-2 infected HeLa cells at 24 hpi was analyzed by western blot where actin was used as a loading control. The relative protein level of tetherin was normalized by that of actin. One representative experiment out of three is shown. (C) The expression of total tetherin and CD326 in mock, IFN-α pretreated or IFN-α pretreated plus HSV-2 treated primary epithelial cells at 24 hpi was analyzed by western blot where actin was used as a loading control. The relative protein level of tetherin was normalized by that of actin. One representative experiment out of three is shown.

the cell surface. On the one hand, the cell surface expression of tetherin inhibits HSV-2 release. On the other hand, HSV-2 antoganizes the restriction of host cell by decreasing the expression of tetherin. Since tetherin is a protein that recycles between the cell surface and intracellular compartments (Kupzig et al., 2003), tetherin downregulated by HSV-2 infection can be supplied from the intracellular compartments. A balance may exist between HSV-2 countermeasures and tetherin restriction. This is likely to be one of the reasons that we can still detect a large amount of cell-associated viruses by electron microscopy.

The "tethered" HSV-2 virions were fully mature and closely to the cell surface or to each other, suggesting that the interactions were mediated by tetherin and the molecules on the virion exterior. We investigated several proteins in the outermost part, including glycoproteins gB, gD, gH, gL and gM. These viral proteins are transmembrane proteins and required for the interaction between virus and the host cell. Our flow cytometry data showed that several glycoproteins (gB, gD, gH and gL) distinctly downregulated tetherin from cell surface, indicating that tetherin is likely counteracted by multiple HSV-2 proteins. This notion was further strengthened by the results that several HSV-2 glycoproteins, including gB, gD, gH and gL, physically interacts with tetherin. Of interest, although gH and gL expressed independently failed to be transported to the cell surface, each of them alone colocalized with tetherin and downregulated the expression of tetherin. Moreover, coexpression of gH and gL resulted in the downregulation of tetherin similar to that of gH or gL alone. To date, most of the reported tetherin antagonists, including Vpu (HIV-1), Nef (SIV), K5 (KSHV), Env (HIV-2 and SIV), Ebola glycoprotein (Ebola virus) are membrane-associated proteins or phosphoproteins (Bartee et al., 2004; Chan and Kim, 1998; Cullen, 1994; Strebel et al., 1988). It seems that different viruses have evolved different strategies to counteract tetherin restriction. Of interest, among the reported envelope viruses restricted by tetherin, only one tetherin antagonist has been revealed in each virus. Given the size and proportion of HSV-2 genome likely accounted for immune evasion, it is probable that the interaction between tetherin and HSV-2 involves a more complex procedure. More recently, two groups report the effect of tetherin on HSV-1 release. Zenner et al. reported that virion host shutoff protein was important for tetherin counteraction (Blondeau et al., 2013), whereas Blondea et al. showed that tetherin was moderately antagonized by glycoprotein M (Blondeau et al., 2013), implicating that tetherin is likely counteracted by more than one HSV-1 component. Although HSV-2 is different from HSV-1, their data together reinforce our conclusion that tetherin is counteracted by multiple viral components of HSV-2. Likewise, HIV-1 Vpu has been shown to downregulate tetherin

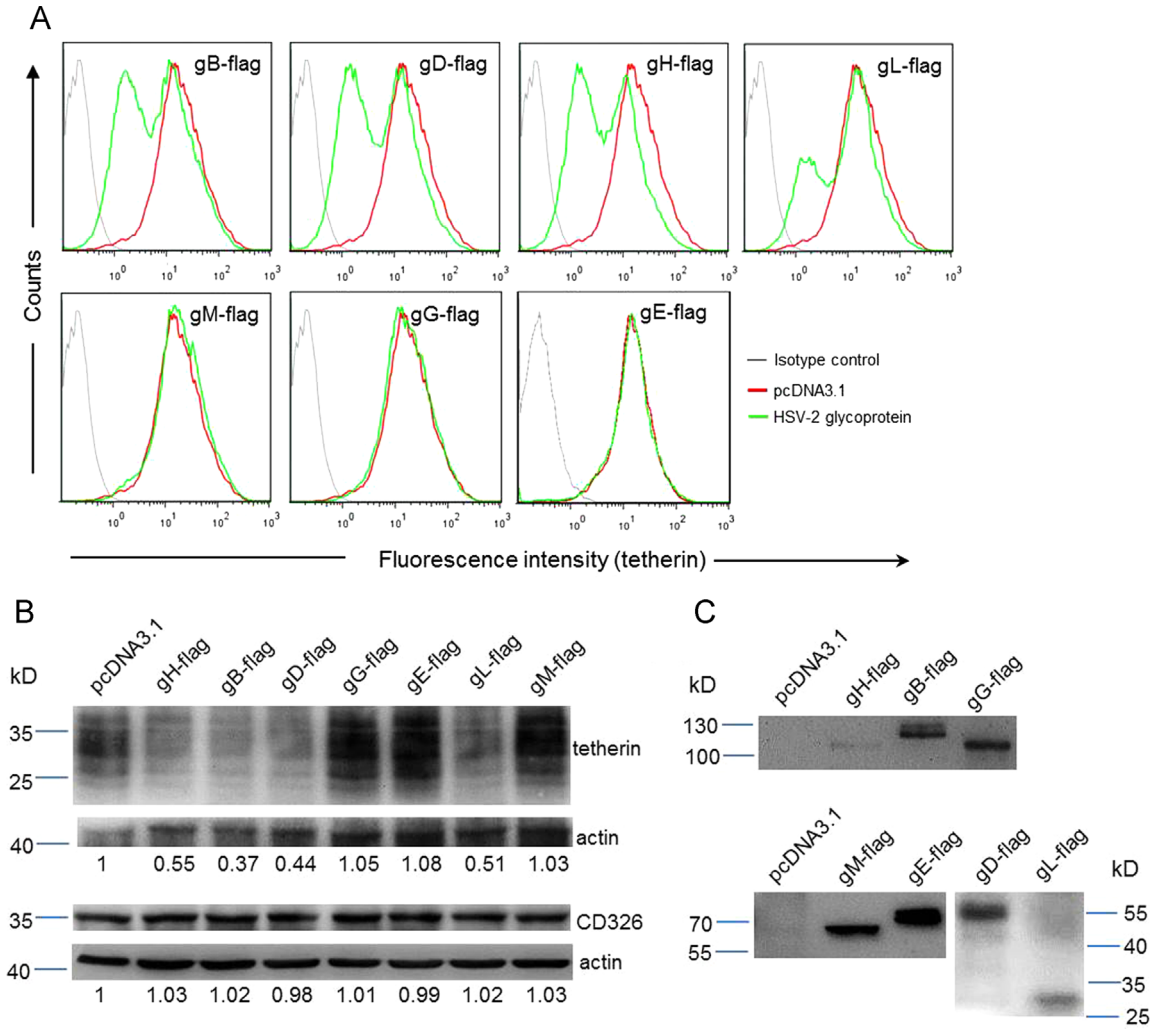


Fig. 6. Multiple HSV-2 glycoproteins downregulate the expression of tetherin. (A) Flow cytometric analysis of tetherin cell-surface expression on HeLa cells transfected with pcDNA3.1 or plasmid expressing HSV-2 glycoprotein gB, gD, gH, gL, gE, gG or gM. Cells were stained with an anti-tetherin antibody (red or green line) or isotype-matched control IgG (gray line). (B) Western blot was used to analyze the expression of total tetherin and CD326 in HeLa cells (parallel samples in (A)), where actin was used as a loading control. The relative protein level of tetherin and CD326 were normalized by that of actin. (C) The expression of each glycoprotein in the transfected cells (parallel samples in (A)) was analyzed by WB using an anti-flag monoclonal antibody. One representative experiment out of three is shown.

expression whereas Ebola GP seems to counteract tetherin without removing it from the cell surface (Kuhl et al., 2011), suggesting that different viral antagonists employ different mechanisms to counteract tetherin restriction.

In addition to gM, we have identified other glycoproteins (gE and gG) that do not affect tetherin expression. Given the complexity of HSV-2 genome containing over 70 genes, we cannot completely rule out the involvement of other HSV-2 components that may also contribute to the degradation of tetherin. Considering that HSV-2 infection is associated with an increased risk of HIV-1 acquisition and that both viral infections can downregulate the expression of tetherin, while beyond the scope of this study, it will be interesting to address the biological significance of tetherin in mucosal infection when an appropriate co-infection model becomes available. Nevertheless, our study has demonstrated that tetherin restricts the release of cell-free HSV-2 progeny viral particles, whereas HSV-2 infection in turn downmodulates the endogenous expression of tetherin to facilitate viral release. Unlike other tetherin antagonists, multiple HSV-2 glycoproteins, in particular gB, gD, gH, and gL, physically interact with tetherin. Our findings inform the complexity of HSV-2-host interactions, providing basis for further understanding the role of tetherin as a vrial restriction factor and the mechanisms underlying viral countermeasures.

Conclusions

We have demonstrated that tetherin can restrict the release of infectious HSV-2 virus particles into the extracellular medium and that HSV-2 infection and viral glycoproteins gB, gD, gH and gL but not gM downregulate the endogenous expression of tetherin. This is the first time that tetherin has been shown to be counteracted by multiple viral components of a virus. Our findings inform the complexity of HSV-2-host interactions, providing basis for further understanding the role of tetherin as a viral restriction factor and the mechanisms underlying viral countermeasures.

Materials and methods

Virus and cells

HSV-2 (strain G) was obtained from LGC standards and propagated in African green monkey kidney cells (Vero). Virus stock was stored at −80 °C before used for infection. Human cervical epithelial cell lines HeLa and ME-180, and embryonic kidney cell line 293T were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in 5% CO_2. Human cervical tissues were obtained from the

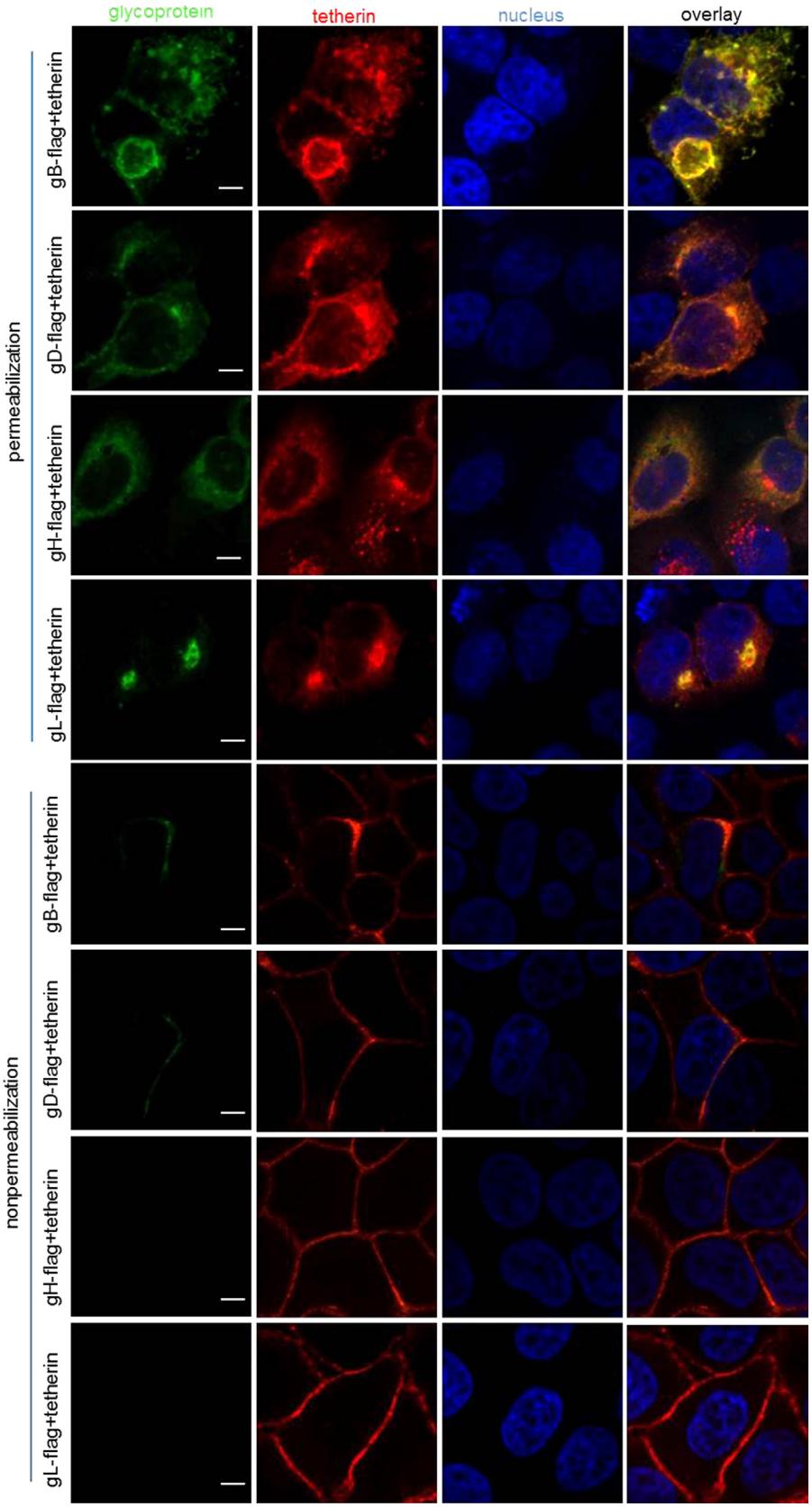


Fig. 7. The cellular localization of tetherin and HSV-2 glycoproteins gB, gD, gH and gL in transfected cells. HeLa cells transfected with plasmid expressing gB-flag, gD-flag, gH-flag or gL-flag were costained with anti-flag (green) and anti-tetherin (red) antibodies. Nuclei were counterstained with Hoechst 33258 (blue). Representative confocal images from three independent experiments are shown. Scale bars in all panels represent 4 μm.

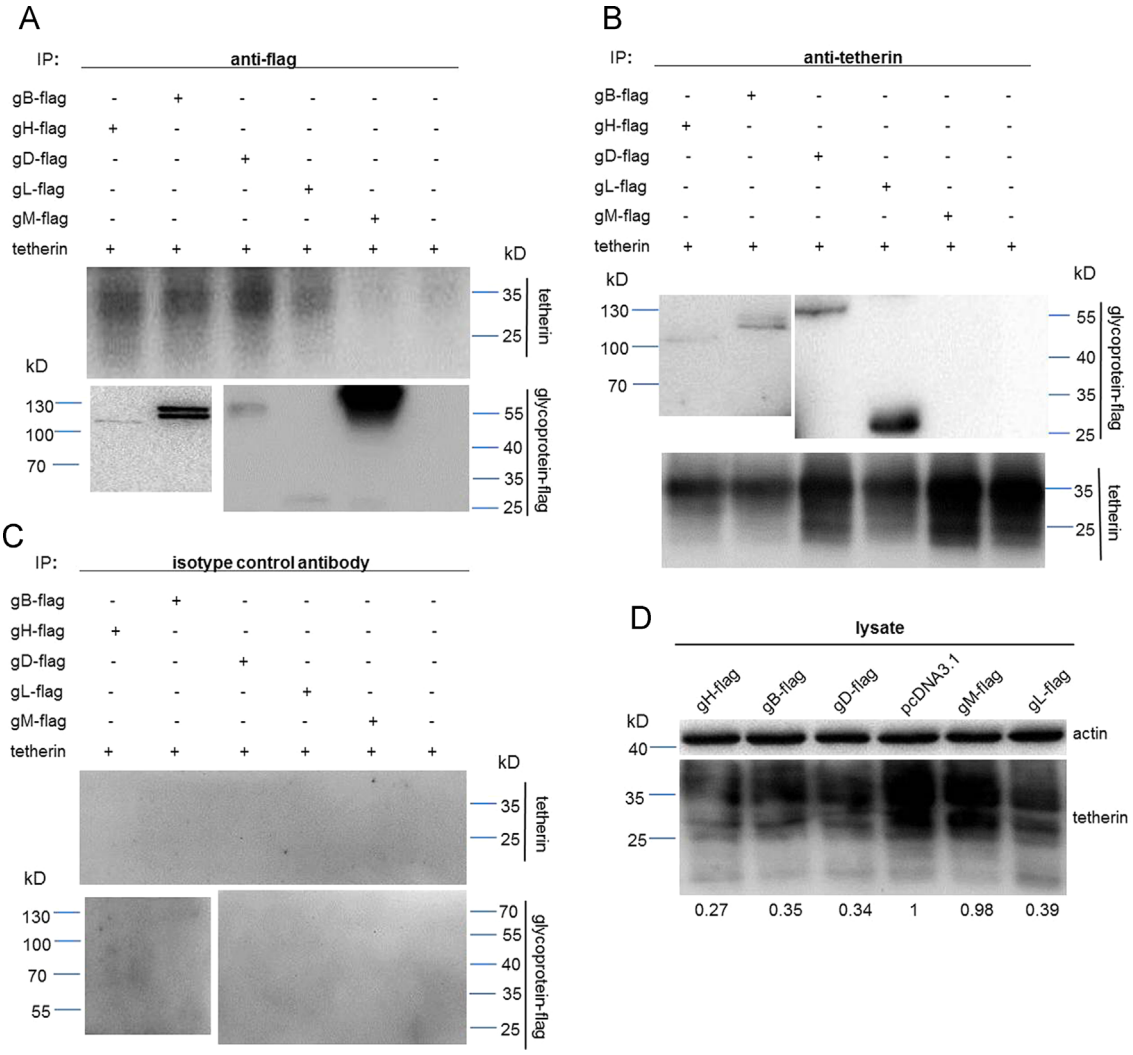


Fig. 8. gB, gD, gH, and gL physically interact with tetherin. HeLa cells were cotransfected with pBST2 and plasmid expressing gB-flag, gD-flag, gH-flag, gL-flag or gM-flag. 48 h post transfection, cell lysates were analyzed by co-IP. gB-flag, gD-flag, gH-flag, gL-flag or gM-flag coimmunoprecipitated with tetherin was pulled down using the anti-tetherin or anti-flag antibody. (A) Immunoblot probed with an anti-flag monoclonal antibody in pull-down assay between each flag-glycoprotein fusion protein and tetherin. (B) Immunoblot probed with an anti-tetherin polyclonal antibody in pull-down assay between each flag-glycoprotein fusion protein and tetherin. (C) Immunoblot probed with an isotype control antibody in pull-down assay between each flag-glycoprotein fusion protein and tetherin. (D) The expression of tetherin in the transfected cells (parallel lysates in (A) and (B)) was analyzed by WB. The relative protein level of tetherin was normalized by that of actin. One representative experiment out of three is shown.

Obstetrics and Gynecology Department at Hubei Hospital of Chinese Medicine. Primary human cervical epithelial cells were isolated as described previously (Howell et al., 1997; Hu et al., 2004; Huang et al., 2012). Briefly, tissues were cut into 3-mm × 3-mm size, and digested by collagenase for 30 min. The separated cells were filtered through a stainless steel strainer (0.5–1.0 mm) and cultured in complete medium for 3 h, and then the inflammatory cells were discarded. Human cervical epithelial cells were cultured in 12-well plates to 90–95% subconfluent before use.

All protocols involving human subjects were reviewed and approved by the Local Research Ethics Committee. Informed written consents from the human subjects were obtained in this study.

Plasmids

Tetherin/BST2-expressing plasmid (pBST2) was from Origene. The ORFs of glycoproteins gB, gD, gH, gL were cloned into pcDNA3.1(+) (named pgB, pgD, pgH and pgL, respectively). The ORFs of glycoproteins gB, gD, gH, gL, gM, gE and gG with flag tag fused at the C-terminal were cloned into pcDNA3.1(+) (named pgB-flag, pgD-flag, pgH-flag, pgL-flag, pgM-flag, pgE-flag and pgG-flag), respectively. The construction, cloning and propagation of plasmids were carried out using standard techniques (Sambrook and Russell, 2001). All constructs were verified by DNA sequencing (Sunny Biotechnology Co. Ltd, Shanghai, China).

Flow cytometry

For flow cytometric analysis of surface tetherin, cells were trypsinized and resuspended in flow cytometry buffer (1 × PBS-3% fetal bovine serum). Cells were stained for surface tetherin expression using a phycoerythrin (PE)-conjugated antibody against tetherin (12-3179; eBioscience). Control antibody was isotype-matched PE conjugated IgG1 (eBiosciences). Stained cells were analyzed on a Guava Easy-Cyte Mini (Millipore) flow cytometer and data were analyzed using the Flowjo and Summit 5.2 (Beckman Coulter) software.

siRNA mediated knockdown of tetherin

Tetherin siRNA (SI02777054) and control siRNA (1027281) were purchased from Qiagen. HeLa cells (~2×10^5) were plated onto 12-well plates overnight. Hela monolayers were transfected with

tetherin siRNA or control siRNA using HiPerFect Transfection Reagent (301704; Qiagen) according to the manufacturer's instructions.

Viral release assay

HeLa cells were treated with or without recombinant human IFN-α (Sigma) and cultured for a further 24 h before infection with HSV-2 at an MOI of 5 PFU/cell. To assess the effect of tetherin on the release of HSV-2 progeny virions, HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers were transfected with pBST2 or pcDNA3.1 or pretreated with tetherin or control siRNA. At 6 h post transfection, cells were infected with HSV-2 at an MOI of 5 or 1 PFU/cell. At different time points post infection, supernatants were harvested and filtered. The cell-associated viruses were harvested by freeze-thaw once followed by sonicate in ice bucket 20×2 S. The virus containing supernatants were stored at -80 °C.

To measure infectious virions released from HeLa cells, virus containing supernatants were serially diluted and used to inoculate Vero cells. Confluent monolayers of Vero cells in 12-well plates were infected with serially diluted HSV-2 for 1 h at 37 °C. After removal of the inoculum and washes, 1 ml fresh medium was added, and the cultures were incubated at 37 °C. At 48 hpi, cells were stained with 0.1% crystal violet for 5 min. Cells were then washed three times with nanopure water, and the numbers of plaques were counted to measure the release of infectious viruses. In some cases, the titers were also determined on HeLa cells. ~60% confluent monolayers of HeLa cells in 12-well plates were infected with 0.35 mL serially diluted virus-containing supernatants for 3 h at 37 °C (Farnham, 1958). After removal of the inoculum and washes, 1 ml fresh medium was added, and the cultures were incubated at 37 °C. At 72 hpi, cells were stained with 0.1% crystal violet for 5 min. Cells were then washed three times with nanopure water, and the numbers of plaques were counted to measure the release of infectious viruses.

Viral entry assay

To assess the effect of tetherin on the entry of HSV-2 progeny virions, HeLa cells ($\sim 2 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers were pretreated with tetherin or control siRNA for 24 h. The cultures were subsequently replaced with cold (4 °C) medium and placed on ice for 10 min, followed by the addition of precooled HSV-2 (MOI=5 PFU/cell) and an incubation at 4 °C for 60 min. After the removal of viruses, cells were washed twice with cold PBS and prewarmed medium (37 °C) was added. The cultures were then shifted to 37 °C. At various time points (0, 10, 20, 30, 45 min), the monolayers were treated with pH3.0 citrate buffer for 1 min at room temperature followed by twice washes with PBS. Cells were harvested and the copies of HSV-2 genomic DNA and the levels of viral protein expression were detected by quantitative PCR and western blot, respectively.

Isolation of extracellular virions

For the preparation of viral particles, HeLa cells were infected with HSV-2 at an MOI of 1 PFU/cell for 2 days. Culture supernatants were harvested and cleared by centrifugation at $1000 \times g$ for 5 min, and then the membrane contaminants were removed through filtration using a 0.22-μm filter. The partially cleaned virions were further purified by ultracentrifugation using sucrose gradient cushion (10–50% in PBS) at $35{,}000 \times g$ for 16 h at 4 °C in a Beckman SW40 rotor to separate viral particles from cellular medium components. Seventeen gradient fractions were collected for subsequent detection of cellular and viral proteins by western blot and immunofluorescence analysis.

Electron microscopy

HeLa cells ($\sim 3.5 \times 10^5$ per well) were plated onto 12-well plates overnight. HeLa monolayers were transfected with tetherin or control siRNA. At 6 h post siRNA transfection, cells were infected with HSV-2 at an MOI of 3 PFU/cell, and the inoculum was removed 1 h later. Following 24 or 48 h infection, cells were harvested for electron microscopy. Following fixation with 2.5% glutaraldehyde/1% paraformaldehyde in cacodylate buffer (0.1 M sodium cacodylate [pH 7.4], 35 mM sucrose, 4 mM $CaCl_2$), cells were stained in 1% OsO4 and 4% uranyl acetate for 2 h, dehydrated in a graded ethanol series (50–100%) and subsequently embedded in epoxy resin Embed-812 (Electron Microscopy Sciences). Thin sections (80 nm) were stained with 2% saturated uranyl acetate for 15 min, rinsed with water, and then stained with Reynolds' lead citrate for 15 min. Electron micrographs were taken on a Tecnai transmission electron microscope (FEI Tecnai G^2 20 TWIN) at an accelerating voltage of 200 kV.

Quantitative PCR

For relative quantitative RT-PCR, cells were collected and the total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. RNase-free DNase I (Fermentas) was used to eliminate the contamination of genomic DNA. cDNA was then synthesized by moloney murine leukemia virus transcriptase (Promega). The newly synthesized cDNA was used as the template for the amplification of a highly specific nucleotide region of ICP4 gene. The primers 5′-GCGAGCTGCGGTTCGT-3′ and 5′-GCCACGCGCAGGTC-3′ were used for ICP4 amplification. GAPDH was used as an internal control amplified with primers 5′-GGGAAGCTCACTGGCATGG-3′ and 5′-TTACTCCTTGGAGGCCATGT-3′. Relative quantitative PCR was performed using a SYBR Green Real-Time PCR Master Mix (Toyobo) Dye and an ABI stepone real-time PCR system (Applied Biosystems) as previously described (Chen et al., 2013). The final reaction conditions were as follow: 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The difference in gene expression was calculated on the basis of $2^{-\Delta\Delta_{CT}}$ values.

For absolute quantitative PCR, cells were collected and treated for 2 h with DNase-I, and viral genomic DNA was isolated. The intracelluler HSV-2 genome copy number was enumerated by quantitative Taqman PCR specific for gB. The probe was labeled at the 5′ end with FAM and at the 3′end with TAMRA. The TaqMan Universal Master Mix II (4427983; Applied Biosystems, Foster City, CA) was used according to the manufacturer's instructions. PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The final 50 μl PCR system and reaction conditions were as described previously (Aumakhan et al., 2010).

Western blot

Prepared cell lysates were resolved by 12% SDS-PAGE and transferred to 0.45 μm polyvinylidenedifluoride membranes (Millipore). Nonspecific binding was blocked using 5% non-fat milk in PBS overnight at 4 °C. The membrane was incubated with primary antibody against tetherin (rabbit polyclonal antibody; 11721; NIH AIDS Research and Reference Reagent Program) at a dilution of 1:5000, ICP4 (rabbit polyclonal antibody; ab96431; Abcam) at a dilution of 1:3000, ICP5 (mouse monoclonal antibody; ab6508; Abcam) at a dilution of 1:3000, gB (mouse monoclonal antibody; sc-52425; Santa Cruz) at a dilution of 1:1000, Ep-CAM (mouse monoclonal antibody; ZM-0359; ORIGENE), HSV-2 (sheep polyclonal antibody; PAB13979; Abnova) at a dilution of 1:2000, FLAG (mouse monoclonal antibody; F1804; Sigma) at a dilution of 1:3000, PCNA (rabbit polyclonal antibody; 10205-2-AP; PROTEINTECH) or β-actin (mouse monoclonal antibody; sc-81178; Santa

Cruz) at a dilution of 1:500, for 1 h at 37 °C. The membrane was washed five times with 0.1% Tween 20/PBS, followed by an incubation for 1 h with HRP conjugated goat anti-rabbit secondary antibody (1:10,000; BA1054, Boster) or HRP conjugated goat anti-mouse secondary antibody (1:10,000; BA1050, Boster). Following five washes with 0.1% Tween 20/PBS, the bands were visualized by exposure to FluorChem HD2 Imaging System (Alpha Innotech) after the addition of chemiluminescent substrate (SuperSignal® West Dura Extended Duration Substrate; 34075; Thermo Scientific Pierce).The grayscale values of WB bands were analyzed using software Image J.

Immunofluorescence

To detect the cellular localization of tetherin and HSV-2 glycoproteins, transfected cells on 35 mm glass bottom culture dishes were washed twice with PBS, followed by fixation with 4% (w/v) cold paraformaldehyde for 30 min at room temperature. Cells were permeabilized with PBST (PBS-0.2% (v/v) Triton X-100) for 10 min at room temperature and then blocked with PBS-2% (w/v) BSA for 1 h at room temperature. Cells were incubated for 1 h at 37 °C with a mouse monoclonal antibody against FLAG (F1804; Sigma) at a dilution of 1:200, gB (mouse monoclonal antibody; ab6506; Abcam) at a dilution of 1:200, gD (mouse monoclonal antibody; sc-58154; Santa Cruz) at a dilution of 1:50 or HSV-2 (sheep polyclonal antibody; PAB13979; Abnova) at a dilution of 1:200, followed by an incubation for 1 h at 37 °C with a FITC-conjugated goat anti-mouse secondary antibody (Boster, China) at a dilution of 1:100, a Cy3-conjugated goat anti-rabbit secondary antibody (Boster, China) at a dilution of 1:100 or a FITC-conjugated rabbit anti-goat secondary antibody (Boster, China) at a dilution of 1:100 in PBS-2% (w/v) BSA. To assess the localization of HSV-2 glycoprotein on cell surface, transfected cells on 35 mm glass bottom culture dishes were washed twice with PBS, followed by fixation with 4% (w/v) cold paraformaldehyde without permeabilization for 30 min at room temperature, and then incubated for 1 h at 4 °C with a mouse monoclonal antibody against FLAG (F1804; Sigma), gB (ab6508; Abcam), gD (sc-52425; Santa Cruz) or HSV-2 (PAB13979; Abnova), followed by an incubation for 1 h at 4 °C with a FITC-conjugated goat anti-mouse secondary antibody, Cy3-conjugated goat anti-rabbit secondary antibody or FITC-conjugated rabbit anti-goat secondary antibody (Boster, China). Cells were washed three times after each incubation with PBS and then twice with distilled water. Nuclei were dyed by Hoechst 33258 (Invitrogen). Stained cells were analyzed using confocal microscopy (PerkinElmer UltraViewVoX) using a 60× oil objective with 1.5-fold optical zoom and 2.5-fold digital zoom.

Co-immunoprecipitation

Co-immunoprecipitation assay was carried out using Pierce Cross-link Immunoprecipitation Kit (26147; Thermo scientific) according to the manufacturer's instructions. For co-immunoprecipitations of tetherin and HSV-2 glycoproteins, mouse monoclonal antibody against FLAG (F1804; Sigma) and rabbit polyclonal antibody against tetherin (11721; NIH AIDS Research and Reference Reagent Program) were used for pull-down experiments. These two antibodies were also used for western blot analysis of the immunoprecipitates and the lysates.

Statistical analysis

All experiments were repeated for at least three times, and the data are presented as mean ± SD unless otherwise specified. The difference of mean value was analyzed by a paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Acknowledgments

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Appendix A. Supporting information

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