LETTER

Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1

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Herpes simplex virus-1 (HSV-1), the prototype of the α-herpesvirus family, causes life-long infections in humans. Although generally associated with various mucocutaneous diseases, HSV-1 is also involved in lethal encephalitis1. HSV-1 entry into host cells requires cellular receptors for both envelope glycoproteins B (gB) and D (gD)2–4. However, the gB receptors responsible for its broad host range in vitro and infection of critical targets in vivo5 remain unknown. Here we show that non-muscle myosin heavy chain IIA (NMHC-IIA), a subunit of non-muscle myosin II (NM-IIA), functions as an HSV-1 entry receptor by interacting with gB. A cell line that is relatively resistant to HSV-1 infection6 became highly susceptible to infection by this virus when NMHC-IIA was over-expressed. Antibody to NMHC-IIA blocked HSV-1 infection in naturally permissive target cells. Furthermore, knockdown of NMHC-IIA in the permissive cells inhibited HSV-1 infection as well as cell–cell fusion when gB, gD, gh and gl were co-expressed. Cell-surface expression of NMHC-IIA was markedly and rapidly induced during the initiation of HSV-1 entry. A specific inhibitor of myosin light chain kinase, which regulates NM-IIA by phosphorylation, reduced the redistribution of NMHC-IIA as well as HSV-1 infection in cell culture and in a murine model for herpes stromal keratitis. NMHC-IIA is ubiquitously expressed in various human tissues and cell types2 and, therefore, is implicated as a functional gB receptor that mediates broad HSV-1 infectivity both in vitro and in vivo. The identification of NMHC-IIA as an HSV-1 entry receptor and the involvement of NM-IIA regulation in HSV-1 infection provide an insight into HSV-1 entry and identify new targets for antiviral drug development.

To identify new HSV-1 entry receptor(s), we adopted a tandem affinity-purification approach with a membrane-impermeable cross-linker coupled with mass-spectrometry-based proteomics technology6 (Supplementary Figs 1 and 2). This approach identified NMHC-IIA as a potential gB receptor (Supplementary Fig. 2). To verify the specific association of HSV-1 gB with NMHC-IIA, co-immunoprecipitation studies were performed. In Vero cells exposed to HSV-1 expressing MEF–gB (a Myc-TEV-Flag tag fused to gB) or wild-type gB, NMHC-IIA co-precipitated with MEF–gB or gB (Fig. 1a and Supplementary Fig. 3a). However, NMHC-IIA did not co-precipitate with gH in Vero cells exposed to HSV-1 expressing MEF–gH (Fig. 1a). Furthermore, the rod domain of NMHC-IIA fused to glutathione S-transferase (GST) pulled down gB from lysates of cells infected with wild-type HSV-1, whereas GST alone did not (Supplementary Fig. 3b). To examine whether gB interacts with NMHC-IIA on the cell surface, cell surface proteins of Vero cells exposed to HSV-1 expressing MEF–gB were biotinylated and the immunoprecipitated complex of NMHC-IIA and MEF–gB was then detected by immunoblotting (Fig. 1b). NMHC-IIA co-precipitated with MEF–gB was biotinylated (Fig. 1b), whereas no biotinylated protein with a molecular mass similar to NMHC-IIA was detectable in cells exposed to HSV-1 expressing MEF–gH (Fig. 1b). To demonstrate further the gB–NMHC-IIA interaction on the cell surface, 293T cells transfected with a plasmid expressing HSV-1 gB or gD were analysed by flow cytometry with the rod domain of NMHC-IIA fused to the immunoglobulin G (IgG) Fc fragment (NMHC-IIA–Fc). Cells

Figure 1 | NMHC-IIA associates with HSV-1 gB and mediates HSV-1 infection. a, Vero cells exposed to HSV-1 expressing MEF–gB (MEF–gB) or MEF–gH (MEF–gH) at a multiplicity of infection (MOI) of 50 at 4 °C for 2 h to allow for viral adsorption, followed by a temperature shift to 37 °C (a permissive temperature for viral penetration) for 2 min, were immunoprecipitated (IP) with an anti-Flag antibody and analysed by immunoblotting (IB) with an anti-NMHC-IIA antibody. WCE, whole cell extract control. b, Cell surface proteins of Vero cells exposed to HSV-1 expressing MEF–gB or MEF–gH at 4 °C for 2 h, followed by a temperature shift to 37 °C for 15 min, were biotinylated, immunoprecipitated with an anti-Flag antibody, and analysed by immunoblotting with streptavidin or an anti-NMHC-IIA antibody. c, Mock-transfected 293T cells (red line) and 293T cells transfected with the gB or gD expression vector (green line) were stained with NMHC-IIA–Fc or control–Fc at 18 h after transfection and analysed by flow cytometry. d, Expression of NMHC-IIA, α-tubulin, or nectin 1 in HL60/puro and HL60/NMHC-IIA cells was analysed by immunoblotting. e, HL60/puro and HL60/NMHC-IIA cells were infected with HSV-1 (GFP) at an MOI of 1 and analysed by fluorescence and phase microscopy at 12 h after infection.
transfected with the gB expression plasmid, but not the gD expression plasmid, reacted with NMHC-IIA–Fc (Fig. 1c). Furthermore, NMHC-IIA–Fc staining was detected in cells infected with wild-type HSV-1 or a revertant gB-deficient HSV-1 in which the gB deficiency was restored, but not in those infected with an HSV-1 gB-deficient virus (Supplementary Fig. 3c). No transfected or infected cells stained with control–Fc (Fig. 1c and Supplementary Fig. 3c). Although we were unable to show binding of purified soluble gB to NMHC-IIA on the cell surface because gB also binds to glycosaminoglycans, which are abundant on the cell surface (data not shown), the series of findings described earlier prove a specific interaction between HSV-1 gB and NMHC-IIA.

To examine whether NMHC-IIA is involved in HSV-1 entry, we established human promyelocytic HL60 cells stably expressing high levels of NMHC-IIA (HL60/NMHC-IIA cells) (Fig. 1d); HL60 cells express NMHC-IIA at a low level and are relatively resistant to HSV-1 infection. Infection of HL60/NMHC-IIA cells with HSV-1 expressing a GFP marker (HSV-1(GFP)) resulted in a remarkable increase in the percentage of virus-infected cells compared to control HL60/puro cells (Fig. 1e, Supplementary Fig. 4a and Supplementary Discussion 1). Infection of HL60/NMHC-IIA cells with HSV-1(GFP) was blocked by anti-NMHC-IIA serum in a dose-dependent manner, whereas control serum had little effect on infection (Supplementary Fig. 4b). Overexpression of NMHC-IIA also enhanced susceptibility of HL60 cells to infection by pseudorabies virus (a porcine α-herpesvirus) expressing a GFP marker, and this infection was inhibited by anti-NMHC-IIA serum (Supplementary Fig. 5). These observations indicate that NMHCII-A mediates HSV-1 and pseudorabies virus infections and suggest that NMHC-IIA is commonly used by α-herpesviruses for their entry.

To verify further the role of endogenously expressed NMHC-IIA in HSV-1 infection, we analysed the effect of anti-NMHC-IIA serum on HSV-1 infection in Vero cells that express NMHC-IIA endogenously. Anti-NMHC-IIA serum inhibited infection of Vero cells with HSV-1(GFP) in a dose-dependent manner, whereas control serum had little effect on infection (Fig. 2a and Supplementary Fig. 6a). By contrast, anti-NMHC-IIA serum had no effect on influenza virus infection (Fig. 2b). Inhibition of HSV-1 infection by anti-NMHC-IIA serum was also observed in human epithelial cells, HaCaT, HCE-T and NCI-H292 cells (Supplementary Fig. 7) and in CHO-K1 cells over-expressing the gD receptor nectin 1 (Supplementary Fig. 8a) but not in CHO-K1 cells overexpressing the gB receptor PIRLrz (Supplementary Fig. 8b).

To examine the role of NMHC-IIA in HSV-1-mediated membrane fusion, we tested first whether the inhibition of HSV-1 infection by the anti-NMHC-IIA antibody was restored by treatment with polyethylene glycol (PEG), which can chemically induce fusion by dehydrating the surfaces of juxtaposed membranes and can therefore promote the entry of entry-defective or entry-blocked herpesviruses when adsorbed onto the cell surface. PEG treatment significantly increased the number of virus-infected cells in the presence of the anti-NMHC-IIA antibody compared to cells that were not treated with PEG (Supplementary Fig. 6b). Second, we used a cell–cell fusion assay, which allows the measurement of HSV-1-induced membrane fusion, relying on transient expression of HSV-1 glycoproteins. When NMHC-IIA knockdown Vero cells (that is, cells stably expressing short hairpin RNA against NMHC-IIA) were co-cultured with Vero cells transiently expressing HSV-1 gB, gD, gH and gL, the efficiency of cell–cell fusion significantly decreased compared to cell–cell fusion when Vero cells stably expressing control shRNA were co-cultured with Vero cells expressing the viral glycoproteins (Fig. 2c, d). In contrast, NMHC-IIA knockdown had little effect on vesicular stomatitis virus envelope-G-protein-mediated cell–cell fusion (Fig. 2e). NMHC-IIA knockdown also resulted in a significant decrease in HSV-1 infection, but had no effect on influenza virus infection (Fig. 2f, g). These results indicate that HSV-1 uses NMHC-IIA as a functional cellular receptor in naturally permissive cells that express NMHC-IIA endogenously and that NMHC-IIA is required for efficient cell–cell fusion mediated by HSV-1 envelope glycoproteins.

It is known that NMHC-IIA mainly functions in the cytoplasm but not on the cell surface. Yet here we demonstrated that NMHC-IIA associates with gB on the cell surface upon viral entry and functions as an HSV-1 entry receptor. This discrepancy prompted us to examine whether NMHC-IIA is redistributed during HSV-1 entry. In Vero cells mock-incubated at 37°C or exposed to HSV-1 at 4°C to allow for viral adsorption, NMHC-IIA localized throughout the cytoplasm (Fig. 3a). However, at 2 and 15 min after the temperature shift to 37°C (a permissive temperature for viral penetration), marked enrichment of NMHC-IIA at the plasma membrane was observed (Fig. 3a). Biotinylation of surface proteins of Vero cells mock-incubated or exposed to wild-type HSV-1 followed by precipitation with avidin beads and immunoblotting with an anti-NMHC-IIA antibody confirmed the cell-surface expression of NMHC-IIA in normal Vero cells and upregulation of the cell-surface expression of NMHC-IIA at 15 min after the temperature shift to 37°C (Fig. 3b). These results indicate that the cell-surface expression of NMHC-IIA is upregulated after viral adsorption at 4°C followed by a
temperature shift to 37 °C, a finding that has not been previously reported for other viruses.

Subcellular localization of NM-IIA is regulated in part by phosphorylation of regulatory light chains at the threonine residue at position 18 (Thr 18) and the serine residue at position 19 (Ser 19). One of the protein kinases responsible for the NM-IIA regulatory light chain phosphorylation at these sites is Ca2+-calmodulin-dependent myosin light chain kinase25. In fact, diphosphorylation of regulatory light chains at Thr 18 and Ser 19 was enhanced greatly after viral adsorption at 4 °C followed by a temperature shift to 37 °C (Fig. 3d). Therefore, we examined the effects of ML-7—a specific inhibitor of myosin light chain kinase35—on the phosphorylation of regulatory light chains at Thr 18 and Ser 19 (Fig. 3d) as well as enrichment of NMHC-IIA at the plasma membrane and upregulation of cell-surface expression of NMHC-IIA after viral adsorption at 4 °C followed by a temperature shift to 37 °C (Fig. 3c, e). Vero-cell susceptibility to HSV-1(GFP) infection was inhibited by ML-7 in a dose-dependent manner (Fig. 4a), whereas susceptibility to influenza virus infection was not affected by ML-7 (Fig. 4b). ML-7 seemed to inhibit HSV-1 infection at the level of virus entry, based on the observation that PEG treatment significantly enhanced susceptibility to HSV-1(GFP) in the presence of ML-7 (Supplementary Fig. 9). Furthermore, effects on HSV-1 infection similar to those seen with ML-7 were also observed when cells were treated with BAPTA-AM, a cell-permeable cytosolic Ca2+ chelator14 (Supplementary Fig. 10) or transfected with a plasmid expressing a dominant-negative mutant of myosin light chain kinase (Supplementary Fig. 11), indicating that ML-7 in fact inhibited HSV-1 infection by blocking the myosin light chain kinase. In the murine model for herpes stromal keratitis, treatment of mouse eyes with ML-7 before HSV-1 inoculation significantly reduced viral replication in the eyes and herpes stromal keratitis severity, and the survival curve of mice treated with ML-7 was significantly greater than that for mock-treated mice (Fig. 4c–e). These results indicate that ML-7 efficiently inhibits HSV-1 infection in both cultured cells and the murine model, and suggest that regulation of NM-IIA, including NMHC-IIA redistribution to the cell surface during initiation of HSV-1 penetration, is required for efficient HSV-1 infection in vitro and in vivo (Supplementary Discussion 2 and 3).

NMHC-IIA is a subunit of NM-IIA, and NM-IIA is central to the control of cell adhesion, cell migration and tissue architecture7. As expected, because NM-IIA has essential roles in basic cellular functions,
NMHC-IIA is expressed in a wide variety of cultured cell lines and in various tissues and cell types in vivo.\(^1\) Furthermore, NMHC-IIA is expressed on the cell surface\(^2\) (Fig. 3b) and is enriched in lipid rafts\(^3,4\), with which HSV-1 gB associates during viral entry.\(^5\) These reports together with the data here presented implicate NMHC-IIA as a functional gB receptor enabling HSV-1 to infect various cell lines and important in vivo cellular targets. Future clarification of the precise roles of NMHC-IIA and its regulators in HSV-1 infection will define the molecular basis of HSV-1 entry. These results may indicate new prophylactic and therapeutic approaches for the development of antitherpes drugs that target NM-IIA and NM-IIA regulators.

**METHODS SUMMARY**

**Identification of proteins that interact with gB during viral entry.** Mouse embryonic fibroblasts immortalized by simian virus 40 large T antigen\(^5\) and IC\(2\) cells were infected with HSV-1 expressing MEF–gB at 4 °C for 2 h, then transferred to 37 °C for 2 min, harvested, treated with PBS containing 2 mM 3,3’-dithiobis(sulphosuccinimidyl propionate) (DTSSP; Piers) at 4 °C for 2 h, and lysed in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA). After centrifugation, the supernatants were immunoprecipitated with an anti-Myc monoclonal antibody (LP14; MBL) and the immunoprecipitates were reacted with AcTEV protease (Invitrogen). After another centrifugation, the supernatants were immunoprecipitated with an anti-Flag monoclonal antibody (M2; Sigma), and these immunoprecipitates were electrophoretically separated in a denaturing gel and visualized by silver staining. Bands detected only in immunoprecipitates from mouse embryonic fibroblasts were excised and digested in the gel with trypsin as described previously.\(^5\) The purified peptides were then loaded on an automated nanoflow liquid chromatograph (Dina) and tandem mass spectrometry (LC-MS/MS; Q-Star Elite, Applied Biosystems). The peptide masses obtained by LC-MS/MS analysis were processed against mouse protein sequences in the National Center for Biotechnology Information (NCBI) RefSeq database (35,662 sequences as of 14 July, 2009) using the Mascot algorithm (Matrix Science).

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Author Contributions** Y. Kawaguchi and J.A. designed the experiments; J.A., H.G., T.S., Y. Kawaguchi and J.A. wrote the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to Y. Kawaguchi (ykawaguchi@ims.u-tokyo.ac.jp).

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METHODS
Identification of proteins that interact with gB during viral entry.
Mouse embryonic fibroblasts immortalized by simian virus 40 large T antigen25 and IC21 cells were infected with HSV-1 expressing MEF-gB at 4 °C for 2 h, then transferred to 37 °C for 2 min, harvested, treated with PBS containing 2 mM 3,3′-dithiobis(sulphosuccinimidyl propionate) (DTSSP; Piers) at 4 °C for 2 h, and lysed in radioimmunoprecip assay (RIPA) buffer (1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA). After centrifugation, the supernatants were immunoprecipitated with an anti-Myc monoclonal antibody (LP14; MLB) and the immunoprecipitates were reacted with AcTVE protease (Invitrogen). After another centrifugation, the supernatants were immunoprecipitated with an anti-Flag monoclonal antibody (M2; Sigma), and these immunoprecipitates were electrophoretically separated in the gel with trypsin as described previously26. The purified peptides were then loaded on an automated nanoflow liquid chromatograph (Dina) and tandem mass spectrometry (LC-MS/MS; Q-Star Elite, Applied Biosystems). The peptide masses obtained by LC-MS/MS analysis were processed against mouse protein sequences in the National Center for Biotechnology Information (NCBI) RefSeq database (35,662 sequences as of 14 July, 2009) using the Mascot algorithm (Matrix Science).

Cells and viral infection. CHO-PLRL2 and CHO-nectin-1 cells are CHO-K1 cells stably expressing human PILRα and human nectin 1, respectively27. Medium 199, Ham’s F-12 medium, and RPMI1640 medium supplemented with 1% FCS were used for virus infection of Vero, CHO and HL60 cells, respectively.

Plasmids. For generating a fusion protein of GST and the rod domain of NMHC-IIA (GST–NMHC-IIA), a plasmid (pGEX-NMHC-IIA) was constructed by amplifying the sequence containing NMHC-IIA codons 1665–1690 by PCR from pEGFP-ARF296 and cloning the DNA fragment into pGEX-4T3 (GE Healthcare) in-frame with GST. For generating a fusion protein of Fc and the rod domain of NMHC-IIA (NMHC-IIA–Fc), which was used for detection of cell surface gB, a plasmid (pME-Fc-NMHCIIA) was constructed by the procedure used to generate pGEX-NMHC-IIA. However, instead of pGEX-4T-3, we used a modified pMIE18S expression vector containing a mouse CD150 leader sequence and the amino terminus and the Fc fragment of human IgG1 at the carboxy terminus, in which the leucines at the Fc codons 266 and 267 were mutated to alanine and glutamine, respectively, to reduce the binding affinity to cellular Fc receptors, and the histidine at codon 467 was mutated to arginine to reduce the binding affinity to HSV-1 gE, an Fc ligand25. The NMHC-IIA open reading frame was amplified from Addgene plasmid 11347 (Addgene) was cloned into pMxs-puro23 and designated pMxs-NMHC-IIA-puro. Plasmids pPEP98-gB, pPEP99-gD, pPEP101-gL and pPEP100-gH were used for purification of gB, gD, gL and gH, respectively, as described previously28. Plasmids pCAGT7, encoding T7 RNA polymerase, was transfected into IC21 cells was infected with HSV-1 expressing MEF–gB or MEF–gH at a multiplicity of infection (MOI) of 50 at 4 °C for 2 h. The cells were then transferred to 37 °C for 2 min, harvested, washed with PBS, and lysed in TNE buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA) containing a proteinase inhibitor cocktail. After centrifugation, the supernatants were pre-cleared by incubation with protein-A-sepharose beads for 30 min at 4 °C. After a brief centrifugation, the supernatants were reacted with an anti-Flag or anti-gB antibody for 2 h at 4 °C. Protein–A-sepharose beads were then added and allowed to react, with rotation, for a further 1 h at 4 °C. The immunoprecipitates were collected by a brief centrifugation, washed extensively with TNE buffer, and analysed by immunoblotting with an anti-NMHC-IIA antibody.

Fc-fusion protein. Plasmid pMIE-Fc-NMHC-IIA, encoding the rod domain of NMHC-IIA fused to a mutant Fc fragment of human IgG1 with low binding affinity for cellular Fc receptors and for the HSV-1 gE Fc ligand, was constructed as described previously. Soluble NMHC-IIA–Fc was produced and used to detect gB on the cell surface of 293T cells infected with HSV-1 at an MOI of 1 or transfected with a gB or gD expression plasmid as described previously28. As a control, purified human CD200–Fc protein was used. For transfection of 293T cells with the gB or gD expression plasmids, nearly confluent cells in 6-well plates were transfected with 4 μg of each of the plasmids by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 18 h after transfection, cells were harvested, stained with NMHC-IIA–Fc or control–Fc (human CD200–Fc) and analysed by FACSCalibur with Cell Quest software (Becton Dickinson).

Affinity precipitation with NMHC-IIA fused to GST. GST or GST–NMHC-IIA was expressed, purified on glutathione-sepharose beads and quantified as described previously28. Affinity precipitation with GST or GST–NMHC-IIA fusion protein was performed as described previously28.

Biotinylation of cell surface proteins of HSV-1-infected cells. Vero cells were exposed to wild-type HSV-1 or HSV-1 expressing MEF–gB or MEF–gH at an MOI of 50 at 4 °C for 2 h. At 2 min and 15 min after transfer of the cells to 37 °C, cell samples were washed four times with ice-cold PBS, and biotinylated twice with cleavable sulfo-NHS-SS-Biotin (Pierce) for 15 min each. After two washes with DMEM supplemented with 0.2% bovine serum albumin (BSA), free thiol groups were quenched with 5 mg iodoacetamide (in PBS supplemented with 1% BSA) per ml and cells were harvested, solubilized in RIPA or TNE buffer containing a proteinase inhibitor cocktail, precipitated with streptavidin beads or an anti-Flag (mAb) antibody, and analysed by immunoblotting with an anti-NMHC-IIA antibody or streptavidin horseradish peroxidase.

Immunofluorescence and detection of diphosphorylated regulatory light chain. Vero cells were mock-incubated or exposed to wild-type HSV-1 at an MOI of 50 for 2 h at 4 °C. At various times after exposure of the cells to HSV-1, cell samples were fixed, permeabilized, and then stained with anti-NMHC-IIA as described previously28. To examine the effects of ML-7, a specific inhibitor of myosin light chain kinase, or BAPTA-AM, a cell-permeable cytosolic Ca++ chelator, on localization of NMHC-IIA during the initiation of HSV-1 entry, Vero cells pretreated with the indicated concentrations of ML-7 or BAPTA-AM for 30 min and then mock-incubated for 1 h were exposed to wild-type HSV-1 at an MOI of 50 for 1 h (BAPTA-AM) or 2 h (ML-7) at 4 °C in the same concentrations of ML-7 or BAPTA-AM. After removal of the inoculum, the cells were re-fed with pre-warmed medium and 15 min after being transferred to 37 °C, they were fixed, permeabilized, and then stained with anti-NMHC-IIA as described earlier, or analysed by immunoblotting with an anti-regulatory-light-chain antibody or an antibody to phosphorylated regulatory light chain at Thr 18 and Ser 19.

Establishment of HL60 cells stably overexpressing NMHC-IIA. Plat-GP cells, a 293T-derived murine-leukaemia-virus-based packaging cell line29, were transduced with pMxs-NMHC-IIA-puro or pMxs-puro in combination with pMDG encoding envelop glycoproteins of the murine-leukaemia virus. Two days after transfection, supernatants were harvested. HL60 cells were transduced by infection with retrovirus-containing supernatants of transfected Plat-GP cells and selected with 0.5 μg puromycin per ml in maintenance medium. Resistant cells transduced by recombinant retrovirus derived from pMxs-NMHC-IIA or
pMxs-puro were designated HL60/NMHC-IIA or HL60/puro cells, respectively. HL60/puro and HL60/NMHC-IIA cells in 24-well plates were inoculated with HSV-1(GFP) or pseudorabies virus expressing GFP at an MOI of 1 or 0.5, followed by centrifugation at 32 °C at 1100g for 1 h. The inoculum was then removed, and the cells were washed and re-fed with the appropriate medium. At 12 h after infection, cell samples were analysed by fluorescence microscopy (Olympus IX71) or a FACSCalibur with Cell Quest software.

Infection inhibition assays. For inhibition with an anti-NMHC-IIA antibody, Vero and CHO-nectin-1 cells in 24-well plates were pretreated with various concentrations of anti-NMHC-IIA or control serum for 30 min and then inoculated with HSV-1(GFP), pseudorabies virus expressing GFP, or influenza virus at an MOI of 1 or 0.5 in the same serum concentrations. After viral adsorption for 30 min, the inoculum was removed and the cells were washed twice and re-fed with the appropriate medium. CHO-PILRx and HL60/NMHC-IIA cells in 24-well plates were pretreated with various concentrations of anti-NMHC-IIA or control serum for 30 min and then inoculated with HSV-1(GFP), pseudorabies viruses expressing GFP, or influenza virus at an MOI of 1 in the same serum concentrations, followed by centrifugation at 32 °C at 1100g for 1 h. The inoculum was removed, and the cells were washed twice and re-fed with the appropriate medium. At 6 h, 7 h (Vero, CHO-PILRx and CHO-nectin-1 cells), or 12 h after infection (HL60/NMHC-IIA cells), cell samples were analysed by use of FACSCalibur with Cell Quest software. For inhibition with ML-7 or BAPTA-AM, Vero cells pretreated with various concentrations of ML-7 or BAPTA-AM for 30 min were inoculated with wild-type HSV-1, HSV-1(GFP) or influenza virus at an MOI of 1 in the same concentrations of ML-7 or BAPTA-AM. After removal of the inoculum, the cells were re-fed with medium containing the same concentrations of ML-7 or BAPTA-AM. At 5 h after infection, cell samples were analysed by use of FACSCalibur with Cell Quest software.

PEG treatment. Vero cells pretreated with anti-NMHC-IIA or control serum at a 1:5 dilution at 37 °C for 30 min were inoculated with HSV-1(GFP) at an MOI of 1 in the same serum concentration. After viral adsorption for 30 min at 37 °C, the inoculum was removed, and the cells were washed twice and exposed to 40% PEG in the same serum concentration. After viral adsorption for 30 min at 37 °C, the inoculum was removed, and the cells were washed twice and re-fed with the appropriate medium. At 6 h, 7 h (Vero, CHO-PILRx and CHO-nectin-1 cells), or 12 h after infection (HL60/NMHC-IIA cells), cell samples were analysed by use of FACSCalibur with Cell Quest software. Vero cells pretreated with 20 μl of Medium 199 supplemented with 1% FCS and then frozen at –80 °C. Frozen samples were later thawed and thoroughly mixed, and infectious virus was quantified by using standard plaque assays on Vero cells. The total p.f.u. per cotton tip was determined and divided by two to calculate the approximate viral titre per eye. Mice were monitored daily for mortality, and the clinical severity of keratitis of individually scored mice was recorded as described previously14,21. The clinical scoring system was as follows: 0, normal cornea; 1, mild haze; 2, moderate haze, iris visible; 3, severe haze, iris not visible; 4, severe haze, corneal ulcer; and 5, corneal rupture. All of the animal studies were carried out with the approval of the Ethical Committee for Animal Experimentation at the University of Tokyo.

Supplementary Discussion-1

We showed that infection of HL60/NMHC-IIA cells with HSV-1 GFP resulted in a remarkable increase in the percentage of virus-infected cells compared to control HL60/puro cells (Fig. 1e and Supplementary Fig. 4a). These results are in agreement with previous observations, made independently, that expression of NMHC-IIA increases markedly during differentiation of HL60 cells and that the susceptibility of these cells to HSV-1 infection also increases upon their differentiation.

Supplementary Discussion-2

We showed that in the murine model for herpes stromal keratitis, treatment of mouse eyes with ML-7 prior to HSV-1 inoculation significantly reduced viral replication in the eyes and herpes stromal keratitis severity. Moreover, the survival curve of mice treated with ML-7 was significantly greater than that for mock-treated mice (Fig. 4c to e). Although the mechanism by which ML-7 functions in the murine model remains unclear, the inhibitory effect of ML-7, when administered prior to viral inoculation, on viral replication and pathogenicity implies that this drug blocks the initial step of HSV-1 infection, as observed in vitro.

Supplementary Discussion-3

We demonstrated up-regulation of NMHC-IIA cell surface expression after viral adsorption at 4°C followed by a temperature shift to 37°C. This observation is consistent with, and may explain, previous reports of a 10-15 min time lag between viral adsorption at 4°C and initiation of viral penetration after a temperature shift to 37°C, observed with HSV-1 but not other viruses. HSV-1 entry triggers a quick release of Ca²⁺ (within 16 sec after a shift to 37°C) and this Ca²⁺ signaling plays a critical role in HSV-1 entry. Ca²⁺ is an essential cofactor for activation of myosin light chain kinase, which controls functions and localization of NM-IIA by phosphorylation. The data presented here that ML-7, a specific inhibitor of myosin light chain kinase, as well as BAPTA-AM, a Ca²⁺ chelator (Supplementary Fig. 10), prevented translocation of NMHC-IIA upon initiation of viral penetration as well as HSV-1 infection support the model that translocation of NMHC-IIA during initiation of viral penetration, which is required for efficient HSV-1 infection, is regulated by a signaling event(s) that occur(s) during the initial step of viral penetration.
Supporting Bibliography


Supplementary Figure 1. Schematic diagrams and characterization of recombinant viruses generated in this study.  a, Schematic diagrams of the genome structures of wild-type HSV-1(YK304) and the relevant domains of recombinant viruses.  Diagram 1: linear representation of the HSV-1(YK304) genome.  Diagram 2: diagrams of the gB and gH gene products.  Diagram 3: schematic diagrams of the gB gene product and the gH gene product in recombinant HSV-1 expressing Myc-TEV-Flag tagged gB (MEF-gB) and HSV-1 expressing Myc-TEV-Flag tagged gH (MEF-gH), respectively.  b and c, Immunoblots of electrophoretically separated virions of wild-type HSV-1(YK304), HSV-1 expressing MEF-gB or HSV-1 expressing MEF-gH purified by sucrose gradient centrifugation (b), and of lysates of Vero cells infected with wild-type HSV-1 (YK304), HSV-1 expressing MEF-gB or HSV-1 expressing MEF-gH (c) and reacted with an antibody to gB (upper) or FLAG (lower).  MEF-gB, HSV-1 expressing MEF-gB; MEF-gH, HSV-1 expressing MEF-gH.  These results indicate that recombinant viruses were constructed appropriately.
Supplementary Figure 2. Identification of a gB receptor by immunoprecipitation and mass spectrometry analysis. Preliminary experiments demonstrated that in a recombinant HSV-1 in which alanine substituted for two gB threonines (at positions 53 and 480), gB binding to PILRα was completely blocked. This recombinant virus was unable to infect IC21 cells, a PILRα-positive murine macrophage line, but efficiently infected PILRα-negative cell lines, such as mouse embryonic fibroblasts (data not shown). These observations suggested that IC21 cells express only a single gB receptor, PILRα, whereas mouse embryonic fibroblasts express gB receptor(s) other than PILRα. Therefore, we attempted to identify cellular protein(s) that co-precipitate with gB from mouse embryonic fibroblasts but not from IC21 cells. a, Mouse embryonic fibroblasts and IC21 cells were exposed to HSV-1 expressing MEF-gB at 4°C for 2h, followed by a temperature shift to 37°C for 2 min, treated with the membrane-impermeable cross-linker DTSSP, and sequentially immunoprecipitated with anti-Myc and anti-Flag antibodies. Immunoprecipitates were separated in a denaturing gel and silver stained. Bands I and II were analyzed by mass spectrometry and identified as NMHC-IIA and actin, respectively. Mouse fibroblasts, mouse embryonic fibroblasts. b, Amino acid sequence of mouse embryonic fibroblast NMHC-IIA determined by mass spectrometry analysis. The 42 peptide sequences identical to NMHC-IIA are shown in red. Actin protein was also identified (data not shown). NM-IIA is an actin-binding protein, which explains why actin co-precipitated with gB.
Supplementary Figure 3. HSV-1 gB specifically associates with NMHC-IIA. a, Vero cells exposed to wild-type HSV-1 at an MOI of 50 at 4°C for 2 h, followed by a temperature shift to 37°C for 2 min, were immunoprecipitated with an anti-gB or anti-Flag antibody and analyzed by immunoblotting with an anti-NMHC-IIA antibody. b, Lysates of Vero cells infected with wild-type HSV-1 at an MOI of 5 for 24 h were reacted with GST or GST-NMHCIIA immobilized on glutathione-Sepharose beads. The beads were then washed extensively and analyzed by immunoblotting with an anti-gB antibody. c, 293T cells mock-infected (red line) or infected with wild-type HSV-1, gB-deficient HSV-1, or revertant gB-deficient HSV-1 in which gB deficiency was restored (green line) at an MOI of 1 were stained with NMHC-IIA-Fc or control-Fc at 18 h post-infection and analyzed by flow cytometry.
Supplementary Figure 4. NMHC-IIA mediates HSV-1 infection. a, HL60/puro and HL60/NMHC-IIA cells were infected with HSV-1 GFP at the indicated MOIs, analyzed by flow cytometry, and the percentage of infected cells determined at 12 h post-infection. Data are shown as means and s.e.m. (n=3). b, HL60/NMHC-IIA cells were infected with HSV-1 GFP at an MOI of 1 in the presence of various concentrations of anti-NMHC-IIA or control antiserum, analyzed by flow cytometry, and the percent infected cells determined at 12 h post-infection. Relative infection represents the percentage of infected cells in antiserum-treated cultures compared with untreated controls. Data are shown as means and s.e.m. (n=3).
Supplementary Figure 5. NMHC-IIA enhances pseudorabies virus infection. a, HL60/puro and HL60/NMHC-IIA cells were infected with pseudorabies virus expressing GFP at an MOI of 0.5. At 12 h post-infection, GFP expression in infected cells was analyzed by flow cytometry and the percentage of infected cells was determined. Data are shown as means and s.e.m. (n=3). b, HL60/NMHC-IIA cells were infected with pseudorabies virus expressing GFP at an MOI of 0.5 in the presence of anti-NMHC-IIA or control serum at a 1:5 dilution. The percentage of infected cells was determined by flow cytometry. Relative infection represents the percentage of infected cells in antisera-treated cultures compared with untreated controls. Data are shown as means and s.e.m. (n=3).
Supplementary Figure 6. Anti-NMHC-IIA serum specifically blocks HSV-1 infection of Vero cells and PEG treatment enhances viral infection in the presence of anti-NMHC-IIA serum. a, Vero cells infected with HSV GFP at an MOI of 1 in the presence of anti-NMHC-IIA or pre-immune serum at a 1:5 dilution. The percentage of infected cells was determined by flow cytometry at 6 h post-infection. Relative infection represents the percentage of infected cells in antisera-treated cultures compared with untreated controls. Data are shown as means (n=2). b, Vero cells were inoculated with HSV-1 GFP at an MOI of 1 in the presence of anti-NMHC-IIA or control serum at a 1:5 dilution for 30 min and subsequently treated with 40% PEG. The percentage of infected cells was determined by flow cytometry at 6 h post-infection. Relative infection represents the percentage of infected cells in antiserum and/or PEG-treated cultures compared with untreated controls. Data are shown as means and s.e.m. (n=3; two-tailed Student’s t-test).
Supplementary Figure 7. Anti-NMHC-IIA serum inhibits HSV-1 infection in human epithelial cells. Human keratinocytes (HaCaT cells) (a), human lung carcinomas (NCI-H292 cells) (b) or human corneal epithelial cells immortalized by the T antigen of simian virus 40 (HCE-T cells) (c) were infected with HSV-1 GFP at an MOI of 1 in the presence of anti-NMHC-IIA or control serum at a 1:5 dilution. At 6 h post-infection, GFP expression in infected cells was analyzed by flow cytometry and the percentage of infected cells was determined. Relative infection represents the percentage of infected cells in antisera-treated cultures compared with untreated controls. Data are shown as means and s.e.m. (n=3).
Supplementary Figure 8. Anti-NMHC-IIA serum inhibits HSV-1 infection in CHO cells expressing gD receptor nectin-1 but not in those expressing gB receptor PILRα. CHO-hNectin-1 (a) and CHO-hPILRα (b) cells were infected with HSV-1 GFP at an MOI of 1 in the presence of various concentrations of anti-NMHC-IIA or control serum, and the percentage of infected cells was determined by flow cytometry at 6 h post-infection. Relative infection represents the percentage of infected cells in antisera-treated cultures compared with untreated controls. Data are shown as means and s.e.m. (n=3). CHO-K1 cells, which are resistant to HSV-1 infection and express NMHC-IIA endogenously, become susceptible to HSV-1 infection after transduction with gD receptor nectin-1 to produce CHO-hNectin-1 cells, yet anti-NMHC-IIA serum blocked HSV-1 infection of these CHO-hNectin-1 cells (a). Anti-NMHC-IIA serum did not inhibit HSV-1 infection of CHO-K1 cells that acquired susceptibility to HSV-1 infection by overexpression of another gB receptor, PILRα (CHO-hPILRα) (b). This competitive effect, observed in CHO-hPILRα cells, further supports our conclusion that NMHC-IIA is a functional gB receptor for HSV-1.
Supplementary Figure 9. Enhancement of viral infection by treatment with PEG in the presence of ML-7. Vero cells were infected with HSV-1 GFP at an MOI of 1 in the absence or presence of 20 μM ML-7 for 1 h and subsequently treated with 40% PEG. Mean fluorescence intensities (MFIs) were determined by flow cytometry at 5 h post-infection. Relative MFI represents the MFI of infected cells in ML-7- and/or PEG-treated cultures compared with untreated controls. Data are shown as means and s.e.m. (n=3; two-tailed Student’s t-test).
Supplementary Figure 10. BAPTA-AM inhibits enrichment of NMHC-IIA at the plasma membrane, di-phosphorylation of RLC during the initiation of HSV-1 entry, and HSV-1 infection.  

a, Vero cells mock-incubated or exposed to wild-type HSV-1 at an MOI of 50 at 4°C for 2 h, followed by a temperature shift to 37°C for 15 min in the presence or absence of 50 µM BAPTA-AM were analyzed by immunofluorescence with an anti-NMHC-IIA antibody.  
b, Vero cells were mock-incubated or exposed to wild-type HSV-1 at an MOI of 50 at 4°C for 2 h, followed by a temperature shift to 37°C for 15 min in the presence or absence of 50 µM BAPTA-AM, and expression of total and di-phosphorylated RLC was determined by immunoblotting.  
c, Vero cells infected with HSV-1 GFP at an MOI of 1 in the absence or presence of the indicated concentrations of BAPTA-AM were analyzed by flow cytometry at 5 h post-infection and mean fluorescence intensities (MFIs) were determined.  
d, Vero cells were infected with influenza virus at an MOI of 1 in the absence or presence of 50 µM BAPTA-AM, analyzed by flow cytometry at 7h post-infection, and MFIs were determined.  

Data are shown as means and s.e.m. (n=3).  

Data were normalized to the value in the absence of BAPTA-AM.  

The mean value in the absence of BAPTA-AM was normalized to 100% relative MFI.
Supplementary Figure 11. A dominant-negative mutant of myosin light chain kinase inhibits di-phosphorylation of RLC during the initiation of HSV-1 entry and HSV-1 infection. 
a, Vero cells transfected with the empty expression plasmid (Vector) or the expression plasmid for the dominant-negative mutant of myosin light chain kinase (Dn-MLCK) were mock-incubated or exposed to wild-type HSV-1 at an MOI of 50 at 4°C for 2 h, followed by a temperature shift to 37°C for 15 min, and expression of total and di-phosphorylated of RLC was determined by immunoblotting. The data are representative of three independent experiments. 
b, Amount of di-phosphorylated RLC protein normalized to that of total RLC protein in a. Relative amount represents the amount of di-phosphorylated RLC in cells transfected with Vector or Dn-MLCK and subsequently infected with HSV-1 compared with that in cells transfected with Vector and subsequently mock-infected. Data are shown as means and s.e.m. (n=3; two-tailed Student’s t-test). Consistent with the observations in Fig. 3d and Supplementary Figure 10b, di-phosphorylation of RLC was enhanced after viral adsorption at 4°C followed by a temperature shift to 37°C. Dn-MLCK significantly inhibited di-phosphorylation of RLC induced after viral adsorption at 4°C followed by a temperature shift to 37°C. 
c and d, Vero cells transfected with Vector or Dn-MLCK were infected with HSV-1 GFP (c) or influenza virus (d) at an MOI of 1 and analyzed by flow cytometry at 5 or 7 h post-infection, respectively, and mean fluorescence intensities (MFIs) were determined. Data are shown as means and s.e.m. (n=3; two-tailed Student’s t-test). The mean value in cells transfected with Vector was normalized to 100% relative MFI.
Supporting Bibliography
