Herpes simplex virus type-1 (HSV-1) is a ubiquitous human virus, commonly associated with the outbreaks of facial cold sores. Recurrent infections in the eye can cause corneal blindness (Farooq et al., 2010). Severe complications especially in neonates and immunocompromised patients may result in retinitis and inflammation of the brain tissues that leads to encephalitis (Liesegang et al., 1989; reviewed by Liesegang, 2001; Roizman & Sears, 1996; Whitley et al., 1998). HSV-1 infects host cells through initial attachment to cells via surface heparan sulfate (HS) followed by fusion of the virion envelope with the plasma membrane (Shukla & Spear, 2001; Spear et al., 2000). The current model suggests that entry of virus requires four HSV glycoproteins (gB, gD, gH and gL) (Browne et al., 2001; Davis-Poynter et al., 1994; Forrester et al., 1992; Muggeridge, 2000; Pertel et al., 2001; Turner et al., 1998) and at least one cellular receptor for gD (Cocchi et al., 2000; Terry-Allison et al., 2001; Tiwari et al., 2004). The receptors for HSV-1 gD include a member of the tumour necrosis factor-receptor family named herpesvirus entry mediator (HVEM) (Montgomery et al., 1996; Tiwari et al., 2005a), a member of the immunoglobulin superfamily commonly known as nectin-1 (Cocchi et al., 1998; Shukla et al., 2006) and modifications in HS by multiple D-glucosaminyl 3-O-sulfotransferase (3-OST) isoforms. Among the known 3-OST isoforms, all but one (3-OST-1) isoforms mediate HSV-1 entry (O’Donnell et al., 2006; Shukla et al., 1999; Tiwari et al., 2005b; Xia et al., 2002; Xu et al., 2005) and cell-to-cell fusion (O’Donnell & Shukla, 2009; Tiwari et al., 2004). It has also been proposed that paired immunoglobulin-like receptor alpha can serve as a co-receptor for HSV-1 by interaction with glycoprotein B (gB) (Satoh et al., 2008; Shukla et al., 2009).

HSV can also induce host cell cytoskeletal rearrangements to facilitate infection (Akhtar & Shukla, 2009). In this regard, HSV-1 entry and cell-to-cell spread may particularly be benefited by changes in cytoskeletal rearrangements (Farooq et al., 2010; Tiwari et al., 2008). For instance, cytoskeletal elements such as actin filaments may be reorganized in parallel bundles to form filopodia for viral surfing/transport to reach the cell body (O’Donnell & Shukla, 2008; Oh et al., 2010). Similarly, alterations in cytoskeleton may be needed when membranes fuse during HSV-1 entry or cell-to-cell spread (Spear et al., 2000; O’Donnell et al., 2010). In addition, HSV-1 also utilizes microtubules for transport from the cell periphery towards the nucleus (Marozin et al., 2004).

Recent studies have shown that HSV-1 relies heavily on actin cytoskeleton during phagocytic-uptake by primary cultures of human corneal fibroblasts (CF) and for surfing in retinal pigment epithelial (RPE) cells (Clement et al., 2006; Tiwari et al., 2008). Induction of filopodia formation provides a unique large surface area for virus to surf and...
PI3K signalling is required for HSV-1 entry

find the target cell (Oh et al., 2010). This mode of HSV-1 entry activates Rho-GTPase signalling pathways within a target cell that helps facilitate viral entry (Clement et al., 2006; Oh et al., 2010). It has been previously shown that Rho-GTPases (Rho-A and cdc42) are key modulators that facilitate filopodia formation during viral entry (Clement et al., 2006). One downstream signalling pathway for filopodia-induction is phosphoinositide 3 kinase (PI3K) (Greber, 2002). PI3Ks are a cellular family of heterodimeric enzymes that consist of a regulatory subunit (p85) activated by tyrosine phosphorylation, which recruits inositol phospholipids that are phosphorylated by the catalytic subunit (p110) (Carpenter et al., 1992; Skolnik et al., 1991; Stoyanov et al., 1995). These lipids serve as second messengers that regulate the phosphorylation of other kinases such as Akt/PKB, cyclic AMP-dependent protein kinase A, some protein kinase C isoforms, and the ribosomal S6 kinases p70 and p85 (Coffer et al., 1998). The ability of PI3K to regulate multiple cellular pathways, coupled with the need for HSV to induce an environment favourable for viral entry via changes in actin cytoskeleton in host cells, prompted us to examine the role of PI3K signalling in HSV-1 entry into natural target cells from the human eye (Farooq et al., 2010). Our study demonstrates that PI3K activity is exploited by HSV-1 during filopodia induction and also during cell-to-cell fusion.

We began our study using HeLa, RPE and primary cultures of CF isolated from the stroma of human cornea (obtained from the Illinois Eye Bank, Chicago, IL, USA; processed by using the institution approved protocol in accordance with the Declaration of Helsinki). Human CF is a natural target cell type that has been shown to exploit 3-O-sulfated heparan sulfate as a receptor (Tiwari et al., 2006, 2007). RPE cells also get infected during a natural infection and virus entry is mediated by nectin-1 receptor (Tiwari et al., 2008). Cultures of CF were grown in L-glutamine containing minimum essential medium (MEM; Invitrogen) supplemented with 10% FBS (Sigma) and 5% calf serum (CS) as described previously (Yue & Baum, 1981). The transformed HeLa and RPE cells were grown in Dulbecco’s modified Eagle medium (Invitrogen) containing 10% FBS as described previously (Tiwari et al., 2008). The cells were trypsinized and passaged after reaching confluency. CFs from third passages were used for this study.

To determine the effect of the PI3K inhibitor (LY294002; Cell Signaling Technology) on HSV-1 entry, we first tested the ability of HSV-1 to infect cells in the presence and absence of LY294002. The inhibitor is stable at 37 °C and an extremely potent and specific inhibitor of PI3K activity (King et al., 1997; Wennstrom & Downward, 1999). HSV-1 entry into the cell was determined by using β-galactosidase-expressing HSV-1 reporter virus (KOS gL86). As shown in Fig. 1, the PI3K inhibitor significantly blocked viral entry in a dose-dependent manner in RPE, HeLa and CF cells. The blocking activity of the PI3K inhibitor was seen at concentrations as low as 0.05 mM as well. Interestingly, either pre-treatment of HSV-1 with the PI3K inhibitor or pre-treatment of cells showed similar results. This is probably not due to any virucidal effects of the inhibitor. Its well sustained cellular kinase inhibition activity is probably responsible for the inhibition of entry. The net concentration of the inhibitor does not change in either case as it has a relatively long half-life (3.5–10.0 h) (Gervais et al., 2006; Jones et al., 1999), which may cause the inhibitor to remain effective on cells regardless of whether the virus or the cells were treated first. The negative effect on entry was not associated with any particular gD receptor since the effect was repeatedly observed with each gD receptor expressed alone in Chinese hamster ovary (CHO-K1) cells (Fig. 1d), which do not normally express them (Shukla et al., 1999).

We also evaluated whether the effect is limited to HSV-1 (KOS) strain or if other HSV-1 strains would also be negatively affected by the inhibitor. Nectin-1 expressing CHO Ig8 cells that express β-galactosidase upon viral entry (Montgomery et al., 1996) were used to examine additional virulent strains of HSV-1 (F, MP and 17) (Dean et al., 1994). The cells were pre-incubated with the PI3K inhibitor and then infected with various HSV-1 strains. The results from this experiment again showed that the inhibitor blocks entry of all HSV-1 strains in a dose-dependent manner (Fig. 1e). Next, to demonstrate that the inhibitory effect of LY294002 on HSV-1 entry was specific, we used a highly related compound LY303511 (Calbiochem Inc.) that does not affect PI3K activity. As shown in Fig. 1(f) the inactive compound had no effect on HSV-1 entry, while LY294002 significantly affected HSV-1 entry. A more potent PI3K signalling inhibitor wortmannin has also been shown to inhibit HSV-1 transport (Nicola & Straus, 2004). At this point we did not rule out that the effects seen on entry could have also been due to the inhibition of the capsid transport. The significance of PI3K signalling during HSV-1 entry was further ascertained by overexpressing a dominant-negative PI3K mutant lacking the p110-catalytic subunit-binding domain (ΔSH2) (Ueki et al., 2000). This mutant significantly reduced viral entry into the cells (Fig. 1g), suggesting once again an important role for PI3K in HSV-1 entry.

We next investigated other herpesviruses and their dependence on PI3K signalling during entry. As shown in Fig. 2(a)–(c), pre-treatment of natural target cells with the PI3K inhibitor significantly reduced the entry of HSV-1 (z-herpesvirus), cytomegalovirus (CMV, Towne strain; β-herpesvirus) and human herpes virus-8 (HHV-8; γ-herpesvirus), suggesting that the effect of PI3K signalling may be universal among herpesviruses.

Further, to gain an understanding of the specific effects of the PI3K inhibitor, we asked whether the inhibitor can affect HSV-1-induced filopodia formation (Oh et al., 2010). To answer this question, immunofluorescence was used to stain wild-type HSV-1 (KOS)-infected HeLa and RPE cells in the presence and the absence of the inhibitor. As shown in Fig. 2(d) and (e), HSV-1 failed to induce filopodia in both types of cells pre-incubated with the PI3K inhibitor. This probably affects the entry, since the inability
Fig. 1. PI3K inhibitor negatively affects HSV-1 entry. (a–c) Dose-dependent effect of LY294002 on HSV-1 entry into natural target cells. Experiments were performed in two different ways. As indicated either virus or target cells (RPE, HeLa or CF) were first pre-incubated with the PI3K inhibitor (LY294002) at stated concentrations for 90 min in 96-well plate dishes at room temperature followed by the addition of recombinant HSV-1 (gL86 virus at 40 p.f.u. per cell). After 2 h, cells were washed with 1× PBS and incubated in the culture medium for an additional 4 h. Finally, the cells were permeabilized and incubated with O-nitrophenyl-β-D-galactopyranoside (ONPG) substrate for the quantification of β-galactosidase activity expressed from the input viral genome. The enzymic activity was measured (Spectra Max 190; Molecular Devices) at an optical density of 410 nm. (d) PI3K signalling during HSV-1 entry is not gD receptor specific. CHO-K1 cells transiently expressing gD receptors: nectin-1, HVEM and 3-OST-3 were pre-treated with LY294002 for 45 min followed by infection with the recombinant HSV-1 (KOS) gL86 virus (40 p.f.u. per cell). After 6 h, entry was measured as described above (a–c). In this and other figures each value shown is the mean of three or more determinations (± SD). (e) PI3K inhibitor also inhibits entry of additional wild-type strains of HSV-1. CHO Ig8 cells that express β-galactosidase upon viral entry were pre-incubated with 1× PBS (control) or with the PI3K inhibitor (LY294002) at indicated concentrations for 45 min at room temperature. After 45 min, HSV-1 (F, MP or 17 at 50 p.f.u. per cell) were incubated with the cells. Viral entry blocking was measured as described above (a–c). (f) The inactive compound LY303511 has no effect on HSV-1 entry. The target CF cells were first pre-incubated with LY303511 or with LY294002 at 0.05 mM concentrations for 90 min in 96-well plate dishes at room temperature followed by the addition of recombinant HSV-1 (gL86 virus at 40 p.f.u. per cell). After 6 h, entry was measured as described above (a–c). (g) Over expression of PI3K dominant-negative in CF cells significantly affects HSV-1 entry. CFs were transfected with PI3K dominant-negative expression plasmid (ΔSH2) (Ueki et al., 2000) or empty vector at 2.5 µg DNA. About 18 h post-transfection, cells were infected with HSV-1 KOS (gL86) and 6 h later entry was measured as described above (a–c). Each value shown is the mean of three or more determinations (± SD).
of cells to form filopodia has been shown to result in significant reduction of virus infectivity (Oh et al., 2010).

Finally, we tested the role of PI3K signalling during HSV-1 glycoproteins-mediated cell-to-cell fusion. Cell fusion has been used to demonstrate viral and cellular requirements during entry and spread (Pertel et al., 2001). Quite evidently, target cells expressing individual HSV-1 gD receptor nectin-1, HVEM and 3-OST-3 treated with the PI3K inhibitor demonstrated impaired cell-to-cell fusion with effector cells expressing four HSV-1 (KOS) glycoproteins, gB (pPEP98), gD (pPEP99), gH (pPEP100) and gL.

Fig. 2. PI3K inhibitor blocks filopodia formation and entry of additional herpesviruses into target cells. (a–c) Inhibition of PI3K signalling pathway also affects other members of herpesvirus family. α [HSV-1; (a)], β [CMV; (b)] and γ [HHV-8; (c)] were examined. CF, RPE and human conjunctival epithelium (HCE) were pre-incubated with the PI3K inhibitor LY294002 at 0.5 mM for 90 min in 96-well plate dishes at room temperature followed by challenge with β-galactosidase expressing recombinant HSV-1 (HeLa cells) and CMV (RPE cells) and GFP-expressing HHV-8 (HCE cells). HCE cells were grown and cultured as previously described by us (Akhtar et al., 2008). β-Galactosidase activity was determined as described in Fig. 1. For HHV-8-mediated entry, fluorescence intensity was measured 2 days post-infection (Clement et al., 2006). Data represent the mean ± SD of results in triplicate wells in a representative experiment. The experiment was repeated three times with similar results. (d, e) PI3K inhibitor significantly affects filopodia formation. In this experiment, F-actin staining was performed on HeLa (d) and RPE (e) cells in various combinations as indicated. Either cells or the virus (KOS) were pre-treated with LY294002. After 90 min of infection, cells were fixed and stained for F-actin using 10 nM rhodamine-conjugated phalloidin (Molecular Probes) dissolved in 1× PBS for 45 min at room temperature. After three washes, cells were mounted on the slides using Vectashield. All the images were captured on confocal microscopy (Leica SP2) with ×63 objective.
Fig. 3. PI3K signalling is critical during HSV-1 glycoprotein-mediated cell-to-cell fusion and filopodia formation. (a, b) LY294002 blocks cell-to-cell fusion. Target cells expressing HSV-1 gD receptor (indicated) were either treated with the PI3K inhibitor (0.5 mM) or not treated and then incubated with effector cells expressing HSV-1 (KOS) glycoproteins, gB, gD, gH and gL. A luciferase-based reporter system was used to measure fusion (all plasmids and the assay are described by Pertel et al., 2001). Relative luciferase activity was measured in relative luciferase units (RLU) (y-axis). (b) Cell fusion was confirmed using a fluorescent-labelled cell fusion assay in which nectin-1 expressing target CHO-K1 cells co-transfected with pDSRed N1 fluorescent plasmid and either untreated (i) or highlighted (ii) or treated with PI3K inhibitor (iii) or highlighted (iv) were co-incubated with effector CHO-K1 cells co-transfected with HSV-1 glycoprotein (gB, gD and gH–gL) and a GFP-expressing plasmid. (c) Inhibition of PI3K signalling prevents RhoA activation by HSV-1 in CF. Western blot analysis shows the inhibition of RhoA in the presence of PI3K inhibitor. Primary cultures of human CF were treated with the PI3K inhibitor or mock treated for 30 min followed by HSV-1 (50 p.f.u. per cell) infection for 15 min. RhoA activation was determined by using Rhotekin-RBD-GST (RhoA) kit using manufacturer’s protocol (Cytoskeleton Inc.). (d) A structural homologue, LY303511, shows no effect on HSV-1 glycoprotein-induced cell fusion. The target CF cells expressing luciferase reporter gene were treated with or without LY303511 (0.05 mM) before co-culture with the effector cells expressing HSV-1 glycoproteins gB, gD, gH, gL and T7 RNA polymerase. A luciferase reporter assay was performed 18 h after the two cell populations were mixed together. Cell fusion was measured in relative luciferase units (RLU) using a Sirius luminometer (Berthold Detection System). The data shown are the means of triplicate measures and are representative of three independent experiments. (e) Over expression of dominant-negative PI3K significantly inhibits HSV-1 glycoprotein-induced cell fusion. The target CF cells were transfected either with PI3K dominant-negative expression plasmid (ΔSH2) or with control plasmid, and luciferase reporter gene. The effector cells were transfected with HSV-1 glycoproteins gB, gD, gH, gL and T7 RNA polymerase. A luciferase reporter assay was performed 18 h after the two cell populations were mixed together. Cell fusion was measured in relative luciferase units (RLU) as described above. The data shown are the means of triplicate measures and are representative of three independent experiments.
(pPEP101) (all plasmids described by Pertel et al., 2001) (Fig. 3a). This response was further confirmed by using a fluorescent-labelled cell fusion assay (Fig. 3b). Nectin-1-expressing target CHO-K1 cells co-transfected with pDSRed N1 fluorescent plasmid incubated with the PI3K inhibitor for 60 min failed to fuse with the effector CHO-pDSRed N1 fluorescent plasmid incubated with the PI3K expressing target CHO-K1 cells co-transfected with fluorescent-labelled cell fusion assay (Fig. 3b). Nectin-1-(Fig. 3a). This response was further confirmed by using a

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