Herpes simplex virus type 1 (HSV-1) is a member of the family Herpesviridae and subfamily Alphaherpesvirinae. The virus enters a cell by fusion of the virus envelope with the cell membrane through a poorly understood cascade of molecular interactions involving multiple viral glycoproteins and cellular receptors (Campadelli-Fiume et al., 2000; Clement et al., 2006; Spear & Longnecker, 2003). Initially, cell-surface heparan sulfate (HS) plays an important role in the attachment of viral glycoproteins B (gB) or C (gC) to the target-cell surface (Herold et al., 1991; Shieh et al., 1992; Shukla & Spear, 2001; WuDunn & Spear, 1989). HS, however, is not essential for viral entry into cultured cells, as it can be substituted functionally by low-speed spinoculation (Scanlan et al., 2005) or glycoprotein D (gD) receptor overexpression (Shukla et al., 1999). Viral attachment is followed by fusion between the viral envelope and the target-cell membrane. This process is essential for entry of HSV-1 into cells. Fusion is mediated by binding of viral gD to one of its cell-surface receptors in association with three other essential glycoproteins: gB, gH and gL (Browne et al., 2001; Spear & Longnecker, 2003; Turner et al., 1998; Yoon et al., 2003). The receptors for gD include two members of the immunoglobulin superfamily, nectin-1 and nectin-2 (Cocchi et al., 2000; Geraghty et al., 1998; Warner et al., 1998), herpesvirus entry mediator (HVEM), which is a member of the TNF receptor family (Montgomery et al., 1996), and specific sites in HS or 3-O-sulfated HS (3-OHS) generated by certain members of the 3-O-sulfotransferase (3-OST) family of HS-modifying enzymes (O’Donnell et al., 2006; Shukla et al., 1999; Tiwari et al., 2004; Xia et al., 2002; Xu et al., 2005).

Members of the 3-OST family act to modify HS, which is composed of repeating disaccharide units containing D-glucuronic or iduronic acid and N-acetylglucosamine, late in its biosynthesis (reviewed by Lindahl et al., 1998; Rosenberg et al., 1997). Apparently, each 3-OST isoform recognizes as substrate glucosamine residues in regions of the HS chain having specific, but probably different, prior modifications, including epimerization and sulfation at other positions (Esko & Lindahl, 2001). Thus, different 3-OSTs can generate potentially unique protein-binding sites in HS (Liu & Rosenberg, 2002; Liu et al., 1999; Rosenberg et al., 1997; Shworak et al., 1999). For HSV-1, it is supported by the fact that most 3-OSTs, but not 3-OST-1, can generate HSV-1 receptors (O’Donnell et al., 2006; Shukla et al., 1999; Tiwari et al., 2005a; Xia et al., 2002; Xu et al., 2005). Recently, we showed that 3-OS HS generated

---

Byline and acknowledgments

Correspondence

Deepak Shukla
dshukla@uic.edu

Received 15 August 2006
Accepted 4 December 2006

---

**Soluble 3-O-sulfated heparan sulfate can trigger herpes simplex virus type 1 entry into resistant Chinese hamster ovary (CHO-K1) cells**

Vaibhav Tiwari,1 Christopher O'Donnell,1,2 Ronald J. Copeland,3 Tanya Scarlett,3 Jian Liu3 and Deepak Shukla1,2

1Department of Ophthalmology and Visual Sciences, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA
2Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA
3Division of Medicinal Chemistry and Natural Products, University of North Carolina, Chapel Hill, NC 27599, USA

Herpes simplex virus type 1 (HSV-1) interaction with glycoprotein D (gD) receptors facilitates virus entry into cells. Chinese hamster ovary (CHO-K1) cells lacking cellular receptors allow virus to attach, but not to enter, implying a role for receptors during the post-attachment (entry) phase of HSV-1 infection. Here, it is shown that the presence of soluble heparan sulfate (HS) modified by 3-O-sulfotransferase-3 (3-OST-3), but not by 3-OST-1, triggered HSV-1 entry into resistant CHO-K1 cells. It was further demonstrated that a CHO-K1 mutant deficient in glycosaminoglycan synthesis became susceptible to entry when spinoculated in the presence of 3-OST-3-modified soluble HS, indicating that the role of the gD receptor is to trigger entry rather than cell attachment. In separate experiments, 3-OST-3-modified soluble HS also triggered fusion of HSV-1 glycoprotein-expressing cells with CHO-K1 cells. Taken together, these results show that association of gD with cell surface-bound receptor is not essential for HSV-1 entry and spread.
by the 3-OST-3 isoform is the predominant receptor for HSV-1 entry into cells of the corneal stroma (Tiwari et al., 2006).

Despite our growing knowledge on the identification of gD receptors, the exact mechanism by which a gD receptor works remains poorly understood. It is not known whether the receptors, which belong to three diverse families, are needed for anchoring of the virions to certain specific locations on the cell surface (such as lipid rafts or cell junctions), or whether their more important function is to activate the viral glycoproteins for the membrane-fusion process. A recent finding that the addition of soluble forms of nectin-1 and nectin-2 facilitates entry into HSV-resistant wild-type Chinese hamster ovary (CHO-K1) cells (Kwon et al., 2006) supports the latter possibility. The purpose of this study was to determine whether soluble 3-OS HS, a polysaccharide receptor, also functions in a manner similar to that of the nectins in triggering HSV-1 entry. We also found that not only entry, but also cell-to-cell fusion, can be triggered specifically by soluble 3-OS HS.

The first experiment was to see whether the presence of soluble 3-OS HS would allow viral entry into HSV-resistant wild-type CHO-K1 cells. A standard entry assay was used as described previously (Kwon et al., 2006; Shukla et al., 1999). CHO-K1 cells (3 x 10^5 cells) suspended in cold PBS were preincubated with a β-galactosidase-expressing recombinant HSV-1 (KOS) glB6 virus (provided by P.G. Spear, Northwestern University, Chicago, IL, USA) for 90–120 min at 4 °C on a rocking device in a volume of 300 µl, before the cells were incubated with 1.5 µg soluble 3-OS HS generated in vitro by the action of purified 3-OST-1 or 3-OST-3 (as described previously; Tiwari et al., 2004) or 4.3 µg soluble nectin-1 (HveC-346t, kindly provided by G. Cohen, R. Eisenberg and C. Krummenacher, University of Pennsylvania, Philadelphia, PA, USA) (Krummenacher et al., 1998). The mixtures were incubated for 2 h at 37 °C under constant rocking. The cells were collected by low-speed centrifugation, washed once with PBS, resuspended in 50 µl F-12 medium containing 10 % fetal bovine serum and seeded in a single well of a 96-well plate. After incubation for 15–17 h in a 5 % CO2 incubator at 37 °C, the cultures were processed for an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) assay. Viral entry was also measured quantitatively by using an ONPG (O-nitrophenyl-β-D-galactopyranoside) assay at 410 nm in a microplate reader (Spectra Max 190; Molecular Devices). As evident from Fig. 1(a), compared with the untreated CHO-K1 cells (middle panel), treatment with soluble 3-OS HS resulted in HSV-1 entry into CHO-K1 cells (right panel). Cells transfected with the 3-OST-3 expression construct (pDS43) were used as a positive control (left panel). The ONPG assay provided similar results (Fig. 1b). Cells incubated with soluble 3-OS HS generated by 3-OST-3, but not by 3-OST-1, were rendered susceptible to HSV-1 entry. 3-OS HS generated by 3-OST-1 is non-gD-binding and does not allow viral entry (Shukla et al., 1999). The observation that entry...
into non-permissive CHO-K1 cells was seen only when a soluble gD-binding form of 3-OSt HS was present reflects the specificity of interaction between gD and soluble 3-OSt HS generated by 3-OST-3. Quite remarkably, compared with the corresponding untreated cells, both 3-OST-1- and 3-OST-3-generated 3-OSt HS samples had somewhat negative effects on entry into two HSV-permissive cell lines, HeLa and Vero. Although the mechanism for this phenomenon needs further investigation, it is likely that soluble 3-OSt HS would not enhance entry into naturally permissive cells. Our data with resistant cells imply that soluble 3-OSt HS generated by 3-OST-3 is able to bind to HSV-1 gD and initiate entry independently of gD–cell-surface association. Soluble receptor-mediated infection has been reported previously for other viruses, including subgroup A avian sarcoma and leukemia viruses (ASLV-A), ASLV-B (Damico & Bates, 2000; Knauss & Young, 2002), Human immunodeficiency virus 2, a simian immunodeficiency virus strain and Murine hepatitis virus (Allan et al., 1990; Werner et al., 1990).

Spinoculation is a low-speed centrifugation-based virus inoculation method (Scanlan et al., 2005). This technique has previously been shown to enhance viral entry through increased virus interaction at the cell surface (Scanlan et al., 2005). In this study, spinoculation was used to determine whether viral entry could be enhanced by soluble 3-OSt HS in cells naturally lacking HS. For this experiment, we used CHO-pgsA cells, which are deficient in glycosaminoglycan synthesis (CHO-745) (Shieh et al., 1992). Cells were either mock-infected (uninfected) or infected with HSV-1 (KOS gL86) and entry was determined as described above. Regardless of treatment with 3-OSt HS and infection with HSV-1, no viral entry was detected in CHO-745 cells that were kept on the benchtop (Fig. 2). Similar results were obtained with spinoculated cells with one exception: spinoculated cells that were treated with 3-OST-3-modified 3-OSt HS and infected with HSV-1 (KOS gL86) allowed entry (Fig. 2). This further reinforces the claim that the HSV-1 entry seen is due specifically to the presence of soluble 3-OSt HS.

After the role of soluble 3-OSt HS in viral entry had been established, we continued our investigation by determining the effects of soluble 3-OSt HS on cell-to-cell fusion, an indicator of HSV-1 spread. We examined whether the soluble receptor generated by 3-OST-3 could facilitate cell-to-cell fusion by using HSV-resistant CHO-K1 cells, because these cells are also resistant to cell fusion due to the absence of a gD receptor (Pertel et al., 2001). A standard luciferase reporter gene assay was performed to quantify the cell fusion induced between cells treated with 3-OST-3-modified soluble 3-OSt HS and HSV-1 glycoproteins (Pertel et al., 2001; Tiwari et al., 2004). The ‘effector’ CHO-K1 cells were transfected transiently with each of four glycoprotein plasmids: pPEP98 (gB), pPEP99 (gD), pPEP100 (gH) and pPEP101 (gL), as well as the plasmid pT7EMC Luc, which expresses a luciferase reporter gene. The control ‘target’ cells were co-transfected with a plasmid expressing 3-OST-3 (or nectin-1) and the plasmid pCAGT7, or the pCAGT7 plasmid by itself for use with soluble 3-OSt HS generated by 3-OST-3 (or nectin-1) as test. The pCAGT7 plasmid expresses T7 RNA polymerase to induce expression of the luciferase gene. The test ‘target’ cells were preincubated for 2 h with 1.5 μg soluble 3-OSt HS modified by either 3-OST-1 or 3-OST-3 (or 4.3 μg soluble nectin-1, HveC-346t) before mixing with effector cells. As shown in Fig. 3, soluble 3-OSt HS generated by 3-OST-3, but not by 3-OST-1, was able to induce cell-to-cell fusion in CHO-K1 cells. In similar experiments, the effect of soluble 3-OSt HS was also examined on HeLa and Vero cells. In either case, soluble 3-OSt HS did not have any detectable effect on cell-to-cell fusion (data not shown). These results imply that soluble 3-OSt HS (and nectin-1) can mediate cell-to-cell fusion in non-permissive cells, but perhaps not in naturally susceptible cells.

In summary, we have shown that, similar to soluble nectin-1, soluble 3-OSt HS has the ability to make HSV-1-resistant CHO-K1 cells permissive to HSV-1 infection. Soluble 3-OSt HS showed entry and cell fusion comparable with those of membrane-bound 3-OSt HS and these phenomena were specific to HS modification by 3-OST-3 and not 3-OST-1. Our virus entry result agrees with that of Kwon et al. (2006) in that soluble gD receptors are able to trigger entry in HSV-resistant cells when the virus is first allowed to associate with the cell surface. In addition, our results provide novel evidence that soluble receptors such as 3-OSt HS and nectin-1 can also mediate cell-to-cell fusion. Our results also reinforce the claim that HSV-1 must be
used, it is likely that the virus enters into each of the abovementioned cell types by using a common gD-mediated mechanism, and targeting that mechanism itself would probably prevent infection of these and, perhaps, all cell types.

**Acknowledgements**

We thank Gary Cohen, Roselyn Eisenberg and Claude Krummenacher (University of Pennsylvania, Philadelphia, PA, USA) and Patricia Spear (Northwestern University, Chicago, IL, USA) for reagents. This work was supported by grants from the National Institute of Allergy and Infectious Diseases (AI057860 to D. S. and AI50050 to J. L.) and a postdoctoral fellowship from the American Heart Association (AHA0525768Z to V. T.). R. J. C. is the recipient of a predoctoral fellowship from the David and Lucile Packard Foundation.

**References**


---

**Fig. 3.** Soluble 3-OS HS triggers cell fusion in CHO-K1 cells devoid of the gD receptor. A standard luciferase reporter assay was performed to test for cell fusion between two populations of cells: the ‘effector’ cells expressing HSV-1 gB, gD, gH and gL and the ‘target’ cells expressing either cell-associated 3-OST-3, nectin-1 or no gD receptor. In the latter case, soluble 3-OS HS modified by either 3-OST-1 or 3-OST-3, or soluble nectin-1 was added. The untreated target cells expressing gD receptors (3-OST-3 and nectin-1) were used as positive controls. The treated and untreated cells were incubated for 2 h at 37 °C under constant rocking. The cells were then plated before luciferase activity was measured 24 h after co-cultivating effector and target cells. Relative luciferase units (RLU) were determined by using a Sirius luminometer (Berthold Detection Systems) and are from experiments performed in triplicate; error bars represent SD.
Soluble 3-OS HS-mediated HSV-1 entry


