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Short communication

Calcium-signal facilitates herpes simplex virus type 1 nuclear transport through slingshot 1 and calpain-1 activation

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) can establish its latency in neurons and is associated with virus-induced pathological neurodegeneration in the nervous system. Here we show that viral penetration-induced calcium release facilitated HSV-1 intracellular trafficking through activating slingshot 1 (SSH), a phosphatase regulating actin filament dynamics. More detailed studies revealed that phospholipase C gamma 1, and the inositol 1,4,5-trisphosphate receptor isoform 1 were required for SSH activation. Besides, calpain-1, a calcium-dependent cysteine protease, was involved in viral intracellular migration. These results may lead to new targets for antiviral therapy.

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1. Introduction

Herpes simplex virus type 1 (HSV-1), a member of the *Herpesviridae*, can spread from epithelial cells to neurons and is typically responsible for complications ranging from mucosal lesions to deadly brain infections (e.g., herpes simplex encephalitis, HSE) (Weller, 2011). After primary infection, HSV-1 can establish its latency in the trigeminal or cervical ganglia and reactivate to induce neurite damage and neuronal death under appropriate circumstances. Although rapid progress has been achieved based on the deep understanding of HSV-1 life cycle including viral replication, assembly and release, there are still some unsolved processes such as intracellular translocation of viral particles to nucleus (Coen and Schaffer, 2003). Intracellular migration of pathogen relies on the cytoskeleton-mediated transportation system (Lyman and Enquist, 2009; Sodeik, 2000). Cortical actin (a thin, cross-linked actin network lying immediately beneath the plasma membrane) is the first obstacle encountered by the virus upon infection. The cortex has the potential to prevent or delay the transit of large molecular assemblies. After membrane fusion and penetration, those assembled F-actin at cell periphery could prevent viral cell cortex traversing and transport of viral particle to nucleus requires F-actin depolymerization to release incoming virions from actin-mediated transport. Besides, interruption of F-actin dynamics impaired HSV-1 entry and trafficking (Zheng et al., 2014). However, litter is known regarding the mechanism used by viruses to overcome such restrictions.

Calcium is involved in many signaling cascades that have diverse outcomes depending on the spatiotemporal aspects of the calcium release. The most common signaling pathway that increases cytoplasmic calcium concentration is the phospholipase C (PLC)–inositol triphosphate (IP_3)–IP_3 receptor (IP_3R) pathway. Many cell surface receptors activate the PLC enzyme, which then hydrolyses the membrane phospholipid PIP_2 to form IP_3. IP_3 diffuses to the endoplasmic reticulum, binds to its receptor (IP_3R), which is a Ca^{2+} channel, and thus releases Ca^{2+} from the endoplasmic reticulum. Increase in the intracellular Ca^{2+} concentration has been associated with membrane fusion for a variety of biological membranes, including some enveloped viruses. Several viruses have been reported to utilize Ca^{2+}-signaling pathway for entry, replication and releasing (Chami et al., 2006; Scherbik and Brinton,

Abbreviations: HSV-1, herpes simplex virus type 1; IP_3, inositol triphosphate; IP_3R, inositol triphosphate receptor; PLC, phospholipase C; PLCγ1/PLCG1, phospholipase C gamma 1; SSH, slingshot 1.

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2010). For instance, binding of HIV-1 gp120 to CD4 and engagement of chemokine coreceptors resulted in activation of Ca^{2+}-signaling pathways (Holden et al., 1999). Similarly, HSV-1 infection also triggered elevation of the intracellular Ca^{2+} concentration (Cheshenko et al., 2003; 2007), which was still evident 12 h post-infection (Piacentini et al., 2011). Fusion of the viral envelope and cell membrane and/or an immediate post-entry event (penetration) initiated Ca^{2+}-signaling, which promoted the nuclear transport of HSV-1 particle (Cheshenko et al., 2005). However, the specific molecular effectors mediating Ca^{2+}-signaling have not been illuminated. Herein, we investigated the mechanism of incoming virions nuclear transport facilitated by Ca^{2+}-signaling.

First we tested whether HSV-1 infection induced intracellular calcium release. Human neuroblastoma SK-N-SH cells were loaded with 5 μM Furo-3 AM, a specific calcium fluorescent probe, at 37 °C for 1 h and then incubated with HSV-1 (MOI 20) at 4 °C for 1 h. After washing, the cells were transferred to 37 °C for indicated times and images were captured with a Zeiss LSM510 Meta confocal system (Carl Zeiss). We observed that intracellular Ca^{2+} level was increased upon HSV-1 infection at 30 min post-infection (mpi) and 60 mpi (Fig. 1A), suggesting a Ca^{2+}-signaling activated by HSV-1 (Cheshenko et al., 2003). Because the most common signaling pathway that increases cytoplasmic calcium concentration is the PLC–IP_3–IP_3R pathway, we then determined whether PLC was involved in HSV-1-induced Ca^{2+}-signaling. A specific PLC inhibitor U73122 was used to test its effect on HSV-1 infection. The cytotoxicity of U73122 was tested and cell viability was not affected by 50 μM U73122 (Fig. S1A). Cells in 12-well plate incubated with virus (MOI 20) at 4 °C for 1 h were washed, transferred to 37 °C for another 1 h. The cells were then washed with cold-PBS (pH3.0) to remove bound but not penetrated virions and the internalized viral DNA was extracted, quantified by detecting viral UL46/UL47 gene using real-time PCR as described previously (Schmidt et al., 2011; Xiang et al., 2012; Zheng et al., 2014). As shown in Fig. 1B, pretreatment with U73122 at 37 °C for 1 h decreased the level of internalized HSV-1 DNA following infection. In contrast, exposure of cells to U73122 at a post-entry time point (30 mpi) had no effect. Besides, the effect of U73122 on viral nuclear transport was also be examined by real-time PCR detecting the immediate early gene (UL54) expression at 3 hours post-infection (hpi), a measurement for viral nuclear translocation. After the cells were incubated with HSV-1 at 4 °C and transferred to 37 °C, inhibitors were added for 3 h and total RNA was extracted. We found that U73122 inhibited HSV-1 nuclear transport in a concentration-dependent manner (Fig. 1C). Treatment cells with U73122 also reduced the level of intracellular Ca^{2+} increased by HSV-1 infection (Fig. 1D). Because U73122 targeted a wide range of PLC isoforms, we used siRNA to investigate whether phospholipase C gamma 1 (PLCγ1/PLCG1), a known mediator of intracellular calcium release, was specifically activated (Bozym et al., 2010). For a full list of siRNAs and primer sequences used, please refer to supplemental tables. All the cytotoxicities of siRNAs and inhibitors used in this work have been tested by MTT assay and all the siRNAs and inhibitors were used in a non-cytotoxic concentration (data not shown). The cells were transfected with 2 μg siRNA as described previously (Xiang et al., 2012). As a result, depletion of PLCγ1 inhibited viral entry (Figs. 1E and S1B). Together, these results suggest a role for PLCγ1 in the induction of intracellular calcium release in response to HSV-1 penetration.

Within cells, activation of PLC led to the hydrolysis of PIP_2 into DAG and IP_3 and the increase in intracellular calcium might be due primarily to the activation of the IP_3 receptor (IP_3R). To determine the role of IP_3R in viral trafficking, we tested the effect of 2-APB (inhibitor of IP_3R). 2-APB clearly reduced the efficacy of HSV-1 entry in non-cytotoxic concentration (Fig. 1F) and viral nuclear transport (Fig. 1G). 2-APB also reduced the elevation of the intracellular Ca^{2+} level (Fig. 1D). Because there are three IP_3R isoforms and due to their different affinities toward IP_3 (Foskett et al., 2007), we explored the use of siRNA to specifically knock down each subtype of IP_3R (Fig. S1C). The knockdown of IP_3R-2 and IP_3R-3 had little effect on HSV-1 entry whereas knockdown of IP_3R-1 slightly reduced the efficiency of virus entry (Fig. 1H). Besides, an immunofluorescence-based assay for viral trafficking was performed (Greene and Gao, 2009; Xiang et al., 2012). By using microscopy, we tested the specific role for IP_3R in HSV-1 nuclear transport. After viral binding at 4 °C for 1 h, the cells were transferred to 37 °C for 1 h and then fixed, immunostained with anti-HSV-1 ICP5 (Abcam, 1:3000) antibody (green). The rate of nuclear viral particles/total viral particles was determined to evaluate the viral trafficking efficiency. As shown in Fig. 1I, most viral particles docked at cytoplasm in IP_3R-1 knockdown group whereas most of the viral particles were in nucleus in control cells. The rate of nuclear viral particles/total viral particles decreased from $80 \pm 10\%$ for the control to $37 \pm 12\%$ for siIP_3R-1 (Fig. 1J). The role for PLCγ1 and IP_3R-1 in virus-induced Ca^{2+}-signaling was also confirmed in Vero cells (data not shown). These results indicate that IP_3R-1 is likely the critical IP_3R isoform involved in the HSV-1-induced intracellular calcium release and a signal as PLCγ1-IP_3-IP_3R-1- Ca^{2+} facilitates HSV-1 nuclear transport.

Next, we intend to determine the downstream effector of the virus-induced Ca^{2+}-signaling that promoted viral transport. Previous studies reported that elevation of the intracellular Ca^{2+} concentration stimulated focal adhesion kinase (FAK) and the non-receptor tyrosine kinase PyK2 to promote viral nuclear transport (Cheshenko et al., 2005). Protein kinase C was also recruited to the nuclear rim (Park and Baines, 2006). In this work we focused on actin regulator cofilin because viral trafficking was dependent on cytoskeletal rearrangement (Lyman and Enquist, 2009) and cofilin is a key regulator for actin filament dynamics and reorganization and it has been reported to affect HSV-1 infection (Zheng et al., 2014). Cofilin activity is negatively regulated by phosphorylation at Ser-3 and reactivated by dephosphorylation through Ca^{2+} dependent activation of slingshot homolog 1 (SSH) (Mizuno, 2013; Wang et al., 2005). Therefore we evaluated whether SSH, a phosphatase that directly dephosphorylated and activated cofilin (Bernstein and Bamburg, 2010; Kurita et al., 2007), was involved in calcium signal and promoted viral intracellular migration. First we tested the role for calcineurin—the direct upstream phosphatase of SSH—in Ca^{2+} signaling by using its specific inhibitor cypermethrin. Pretreatment cells with cypermethrin led to a significant reduction in HSV-1 infection and there was no effect was observed when cypermethrin was added at a post-entry time point (30 mpi) (Fig. 2A). Cypermethrin (100 nM) also prevented the activation of cofilin (dephosphorylated cofilin) (Fig. 2B). Cofilin is activated at 60 mpi (phosphorylated level decreased, lane 3) when compared to 30 mpi (lane 2), indicating that viral trafficking requires F-actin depolymerization to release viral particle from cell periphery where viral penetration occurs (Zheng et al., 2014). Besides, viral immediate early gene (UL54) expression, a measurement for viral nuclear translocation, and viral particle transport to nucleus were inhibited by cypermethrin treatment (Fig. 2C and D). Only few viral particles docked at the nucleus in cells pretreated with inhibitor when compared with the control cells. The ratio about nuclear viral particles/total viral particles was largely reduced. Those results suggest that intracellular Ca^{2+} activates calcineurin, which in turn activates SSH and cofilin to facilitate viral nuclear transport. Next by western blot assay we found that SSH was activated during HSV-1 entry (increased from 30 min) (Fig. 2E), and knockdown of SSH using siRNA prevented its direct substrate—cofilin dephosphorylation (Figs. 2E and S2A). Besides, knockdown of SSH reduced immediate early gene (UL54) expression, a measurement for viral nuclear translocation (Fig. 2F). Knockdown of SSH also reduced HSV-1 intracellular migration, with a reduced nuclear viral particles/total viral

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