RESEARCH NOTE

The herpes simplex virus 1 Us3 kinase is involved in assembly of membranes needed for viral envelopment and in distribution of glycoprotein K [version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Background: Capsids of herpes simplex virus 1 (HSV-1) are assembled in cell nuclei, released into the perinuclear space by budding at the inner nuclear membrane acquiring tegument and envelope. Alternatively, capsids gain access to the cytoplasm via dilated nuclear pores. They are enveloped by Golgi membranes. Us3 is a non-essential viral kinase that is involved in nucleus-to-cytoplasm translocation, preventing apoptosis and regulation of phospholipid-biosynthesis. Us3-deletion mutants (HSV-1ΔUs3) accumulate in the perinuclear space. Nuclear and Golgi membranes proliferate, and homogeneous, proteinaceous structures of unknown identity are deposited in nuclei and cytoplasm. Glycoprotein K (gK), a highly hydrophobic viral protein, is essential for production of infectious progeny virus but, according to the literature, exclusively vital for envelopment of capsids by Golgi membranes. In the absence of Us3, virions remain stuck in the perinuclear space but mature to infectivity without reaching Golgi membranes, suggesting further function of gK than assumed.

Methods: We constructed a HSV-1ΔUs3 mutant designated CK177ΔUs3gK-HA, in which gK was hemagglutinin (HA) epitope-tagged in order to localize gK by immunolabeling using antibodies against HA for light and electron microscopy.

Results: CK177ΔUs3gK-HA-infected Vero cells showed similar alterations as those reported for other HSV-1ΔUs3, including accumulation of virions in the perinuclear space, overproduction of nuclear and Golgi membranes containing electron dense material with staining property of proteins. Immunolabeling using antibodies against HA revealed that gK is overproduced and localized at nuclear membranes, perinuclear virions stuck in the perinuclear space, Golgi membranes and on protein deposits in cytoplasm and nuclei.

Conclusions: Us3 is involved in proper assembly of membranes needed for envelopment and incorporation of gK. Without Us3, virions derived by budding at nuclear membranes remain stuck in the perinuclear space but incorporate gK into their envelope to gain infectivity.
Keywords
HSV-1, envelopment, nuclear membranes, Golgi membranes, gK, immunogoldlabeling, immunofluorescence, TEM

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Author roles: Tobler K: Methodology, Validation; Senn C: Formal Analysis, Investigation, Methodology; Schraner EM: Investigation, Methodology, Visualization; Ackermann M: Resources, Supervision, Validation, Writing – Review & Editing; Fraefel C: Resources, Writing – Review & Editing; Wild P: Conceptualization, Investigation, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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**Introduction**

Capsids of herpes simplex virus 1 (HSV-1) are assembled in host cell nuclei, transported to the nuclear periphery and translocated to the perinuclear space (PNS) or into the cytosol (Roizman et al., 2007). The HSV-1 Us3 kinase phosphorylates numerous viral and cellular substrates (Kato & Kawaguchi, 2018). It is involved in nucleus-to-cytosol capsid translocation (Kato et al., 2009; Mou et al., 2009; Reynolds et al., 2002; Ryckman & Roller, 2004; Wisner et al., 2009), in regulation of phospholipid synthesis induced by HSV-1 (Sutter et al., 2012; Wild et al., 2012), and in blocking apoptosis induced by HSV-1 (Galvan & Roizman, 1998; Goodkin et al., 2004; Leopardi et al., 1997). The Golgi complex plays a significant role in apoptosis (Cheng et al., 2010; Hicks & Machamer, 2005), in membrane biosynthesis together with the endoplasmic reticulum (Smirle et al., 2013), and in HSV-1 envelopment (Roizman et al., 2014). Glycoprotein K (gK) is a hydrophobic transmembrane protein of the viral envelope (Mo & Holland, 1997) encoded by the UL53 gene (Bond & Person, 1984; Hutchinson et al., 1992). gK is essential for efficient envelopment by Golgi membranes (Baines et al., 1991; Melancon et al., 2007) and for production of infectious progeny virus (Choulenko et al., 2012). HSV-1ΔUs3 virions accumulate in the PNS. Nuclear and Golgi membranes become severely altered by insertion of proteins of unknown nature (Wild et al., 2015). The essentiality of gK in envelopment by Golgi membranes prompted us to identify gK in cells infected with HSV-1ΔUs3. Because of the hydrophobicity of gK, we constructed a HSV-1ΔUs3 with a hemagglutinin (HA) tag at gK, CK177ΔUs3gK-HA. Immunogold labeling shows that gK localizes on amorphous protein structures, nuclear and Golgi membranes, and, importantly, on virions in the PNS.

**Methods**

**Cells and viruses**

Vero cells (European Collection of Cell Cultures, ECACC, 84113001) were grown in Dulbecco’s modified essential medium (DMEM; 31885-023; Gibco, Bethesda, MD, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (Anti-Anti, 15240-062, Gibco) and 10% fetal bovine serum (FBS; 2-01F10-I, Bio Concept, Allschwil, Switzerland). The Us3-deletion mutant R7041ΔUs3 was kindly provided by Bernard Roizman (The Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Illinois, USA). Wild-type (wtHSV-1) strain F (Ejercito et al., J. Gen. Virol. 2:357–364, 1968), R7041ΔUs3 were propagated in Vero cells.

**Generation of recombinants CK177ΔUs3gK-HA and CK177gK-HA**

Recombineering of pYEbac102 (Tanaka et al., 2003) was done in two steps in Escherichia coli strain SW102 (Warming et al., 2005). First, a galK expression cassette was amplified by PCR (Phusion R, High-Fidelity DNA Polymerase; M0530L, BioLabs, Ipswich, MA, USA, according to manufacturer’s recommendations) with homology arms flanking UL53 using the following primers: primer forward, 5'-tct tcg tgt cca gtc cgc tgc acc gat gta ttt acg egg tac gcc cca ccc CCT GTT GAC AAT TAA TCA TCG GCA -3'; primer reverse, 5'-gtt tcc aat tgt cat atg ccc tta...
plates for TEM, or in 75 cm² cell culture flasks for immunogold-labeling, for 2 days prior to inoculation with CK177ΔUs3gK-HA, R7041ΔUs3, CK177gK-HA or wtHSV-1 at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu)/ml.

**Cryo-fixation for transmission electron microscopy**

Cells on sapphire disks were frozen 16 to 24 hpi in a high-pressure freezer (HPM010; BAL-TEC Inc., Balzers, Liechtenstein) and prepared for sectioning as described in detail previously (Wild et al., 2018; Wild et al., 2002). Cells were analyzed in a transmission electron microscope (CM12; FEI, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000; Gatan, Pleasanton, CA, USA).

**Immunofluorescence**

Cells inoculated with CK177ΔUs3gK-HA or CK177gK-HA at MOI 1 were incubated for 20 h, briefly washed with PBS, fixed with 2% formaldehyde for 25 min at room temperature, washed with cold PBS, permeabilized with 0.1% Triton-X-100 at room temperature for 7 min, and blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). Cells were labeled with mouse monoclonal HA-probe antibodies (1:500) (SC-7392, Santa Cruz Biotechnology, Dallas, TX, USA), followed by anti-mouse Alexa 488-conjugated secondary antibodies (1:500) (A1101; Thermo Fisher) as well as with polyclonal antibodies (1:500) raised in rabbits against Us3 (kindly provided by Bernard Roizman) followed by anti-rabbit Alexa 594-conjugated secondary antibodies (1:500) (A11037; Thermo Fisher). After staining nuclei with 4',6-diamidino-2-phenylindol (DAPI; Roche, Mannheim, Germany), cells were embedded in glycergel mounting media (C0563; Dako North America, Carpinteria, CA, USA) and 25 mg/ml DABCO (1,4-diazabicyclo [2.2.2] octane; 33480, Fluka, Buchs, Switzerland) and analyzed using a confocal laser scanning microscope (SP2, Leica, Wetzlar, Germany).

**Immunogold labeling**

Inoculated cells were harvested at 20 hpi and prepared according to (Tokuyasu, 1973; Tokuyasu, 1980). Cells fixed with 4% formaldehyde in 0.1 M Na-phosphate, pH 7.4, for 2 h at room temperature were scraped from the flasks, washed three times with 0.1 M Na-phosphate by centrifugation at 13,000g for 30 s, and pelleted in 12% gelatine by centrifugation at 13,000g for 3 min at 37°C. After gelation at 4°C, 1 mm³ blocks were immersed overnight in 2.3 M sucrose constantly rotating at 4°C. The infiltrated blocks were mounted on specimen holders, frozen by plunging into liquid nitrogen, and placed into the cryo-microtome (UC6, Leica, Vienna, Austria) at -120°C. Ultra-thin sections of 80–100 nm were collected on carbon-coated formvar films mounted on hexagonal 100 mesh/inch copper grids. Sections were washed by floating on several drops of buffer and blocking solutions prior to routine labeling procedure (Slot et al., 1991) with primary antibodies (1:30) against HA (SC-805, Santa Cruz Biotechnology), and secondary anti-rabbit antibodies (1:30) coupled to 12 nm colloidal gold (111-205-144, Jackson ImmunoResearch, West Grove, PA, USA). After labeling, sections were washed on distilled water droplets, stained by immersing in a mixture of 1.8% methyl cellulose and 0.4% uranyl acetate (Griffiths et al., 1983) for 10 min, and dried for imaging by TEM (Philips, CM12). For controls, primary antibodies were omitted, and labeling was also performed in wtHSV-1 infected cells.

**Results**

**CK177ΔUs3gK-HA induces alteration of nuclear and Golgi membranes**

Infection with HSV-1ΔUs3 induces formation of folds and invagination of nuclear membranes associated with accumulation of virions (Poon et al., 2006; Reynolds et al., 2002; Wisner et al., 2009). Infection with CK177ΔUs3gK-HA, a similar mutant lacking Us3 but equipped with an epitope-tagged gK, also induced multiple folds and invaginations of nuclear membranes containing virions. Moreover, homogenous, proteinaceous structures occurred within nuclei and cytoplasm of CK177ΔUs3gK-HA (Figure 1) and R7041ΔUs3 (Figure 2A) infected cells. Golgi complexes consisted of multiple stacks comprising thick electron dense membranes especially at the trans face (Figure 3) similarly as described in R7041ΔUs3 (Figure 2B) infections (Wild et al., 2015). We conclude that the absence of Us3 is responsible for the alterations in the Golgi architecture, while the epitope-tagged gK-HA has no obvious impact on Golgi disorganization. Unprocessed images of this, and all other figures, are available as Underlying data (Tobler et al., 2019).
gK localizes on Golgi membranes, nuclear membranes, virions, and proteinaceous deposits

Budding of capsids at nuclear and Golgi membranes starts by insertion of budding proteins that appears in electron micrographs as a dense layer (Leuzinger et al., 2005). The major budding proteins at nuclear membranes are UL31/UL34 (Bigalke et al., 2014; Hagen et al., 2015), while gK and UL20 protein are responsible for budding at Golgi membranes (Melancon et al., 2007). Infection with CK177gK-HA revealed that gK localizes in cytoplasm, nuclear rim and even in nuclei (Figure 4A) by immunofluorescence using antibodies against HA. Following infection with CK177ΔUs3gK-HA, gK-HA signals are strongly enhanced in the cytoplasm (forming large clusters), at the nuclear rim and in nuclei (Figure 4B). At the ultrastructural level, Golgi membranes (Figure 5), nuclear membranes and viral envelopes (Figure 6) and the homogeneous structures in nuclei and cytoplasm (Figure 7) were distinctly labeled in CK177ΔUs3gK-HA infected cells. Immunogold labeling agrees with the distribution of gK-HA visualized by immunofluorescence implying that gK was, in addition to Golgi localization, translocated into nuclei, incorporated into nuclear membranes, and became part of the viral envelope during budding.

Figure 2. Transmission electron microscopy images of Vero cells 16 hpi with R7041ΔUs3. (A) homogenous, proteinaceous deposits (p) adjacent to altered Golgi membranes (Go), and (B) Golgi fields containing thick, electron dense membranes with the staining properties of proteins. Note the virion within double-coated structures (arrow), and the one in the endoplasmic reticulum (beside the arrowhead). Bars, 200 nm.

Figure 3. Transmission electron microscopy of Vero cells 24 hpi (A) and 20 hpi (B) with CK177ΔUs3gK-HA. The nucleus in (A) is tangentially sectioned so that invaginations of the inner nuclear membrane (arrow) appear as hibernations. The Golgi complex (Go) comprises multiple stacks consisting of electron dense membranes similar as in R7041ΔUs3 infections. One capsid (c) is in the cytoplasm, and two virions (one indicated by “v”) are within small concentric vacuoles. Note the well-preserved mitochondria (m). (B) shows details of Golgi membranes, which continue into rough endoplasmic reticulum membranes (thick arrow), and a tangentially sectioned Golgi field. Bars, 200 nm.

Figure 4. Immunofluorescence microscopy of Vero cell at 20 hpi with CK177gK-HA (A) or CK177ΔUs3gK-HA (B). CK177gK-HA infected cells show fine but dense distribution of gK-HA over the entire cytoplasm, at the nuclear rim and, at a less extent, in the nucleus. Distribution of Us3 is similar though more intense at the nuclear rim. In the absence of Us3, gK-HA signals are markedly enhanced in all three compartments. gK-HA partly localizes with wheat germ agglutinin (WGA) as a marker for Golgi membranes. The fate of the Golgi complex is under investigation using specific markers.

Figure 5. Golgi membranes 20 hpi with CK177ΔUs3gK-HA after immunogoldlabeling using antibodies against HA. Gold particles are irregularly distributed on electron dense Golgi membranes (Go) as well as on proteinaceous deposits (p). Bars, 200 nm.
Discussion

The precise functions of Us3 kinase has been reviewed by Kato & Kawaguchi (2018). However, the mechanism of regulation phospholipid-biosynthesis is unknown (Wild et al., 2012). Membrane enlargement in the absence of Us3 is associated with structural alterations including folding of nuclear membranes and malformation of Golgi stacks, respectively, and insertion of proteins into nuclear and Golgi membranes. Immunolabeling using antibodies against the HA tag recognized gK to be one component of altered membranes suggesting that deposition/insertion of gK is related to membrane overproduction in the absence of Us3.

HSV-1ΔUs3 virions are infective even though they are not released from the PNS, and do not pass the Golgi complex. gK has been reported to be involved in viral envelopment by Golgi membranes, not by nuclear membranes (Melancon et al., 2005). gK is essential for infectivity playing a significant role in viral entry (Foster et al., 2001; Musarrat et al., 2018). Therefore, gK must be provided during budding of HSV-1ΔUs3 at nuclear membranes. The intense labeling of gK on nuclear membranes and viral envelopes clearly demonstrates that gK becomes part of the viral envelope during budding of HSV-1ΔUs3. gK was also found in nuclei in both HSV-1ΔUs3 and CK177gK-HA infected cells indicating that gK is transported into the nucleus in the presence or absence of Us3. Indeed, others have also observed gK incorporated into nuclear membranes in the context of wtHSV-1 infection (Rajcani & Kudelova, 1998) suggesting that gK may also play a significant role in envelopment of wtHSV-1 at nuclear membranes.

Conclusion

Without Us3, Golgi and nuclear membranes proliferate in association with incorporation of gK that also localizes on virions remaining stuck in the PNS but mature to infectivity suggesting that i) Us3 kinase is involved in regulation in nuclear and Golgi membranes assembly possibly in association with gK synthesis and/or distribution, ii) gK may be the target of Us3 phosphorylation, which is needed for virions to proceed out of the PNS, and iii) gK may also be involved in nucleus-to-cytoplasm translocation in wtHS-1 because gK localizes on nuclei and nuclear rim in the presence of Us3. We hypothesize that Us3 interplays with mechanisms regulating synthesis and arrangement of Golgi membranes, nuclear membranes and gK, which might be the most significant role of Us3 remaining to be investigated.

Data availability

Figshare: The herpes simplex virus 1 Us3 kinase is involved in assembly of membranes needed for viral envelopment and in distribution of glycoprotein K. https://doi.org/10.6084/m9.figshare.8131241.v2 (Tobler et al., 2019).

This project contains the raw images captured for each Figure, with the number of the Figure indicated on each file name.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Benedikt Kaufer

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In this manuscript, the authors generated two herpes simplex virus 1 (HSV-1) mutants harboring a deletion of US3 and insertion of a fluorescent tag on gK. They used these viruses to assess the effect of the US3 deletion on the gK expression levels and localization. Intriguingly, they observed an increased expression of gK and localization of the protein to the nuclear lamina. The manuscript is exciting and well written. However, as the other reviewers pointed out, it would be worth sequencing the recombinant BACs (or the resulting viruses) to ensure that the effect is not due to secondary mutations in the virus genome. Nowadays this would be quick and relatively cheap to sequence the virus genome by miSeq and would eliminate the need for a revertant virus.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly
**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Herpesvirus replication and pathogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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HSV-1 accumulates in the perinuclear space when US3 is deleted. The authors investigate the distribution of gK in the context of US3 deletion. gK is a glycoprotein proposed to be involved in the envelopment of capsids by Golgi membranes, but not by nuclear membranes. The incorporation of gK into the virus envelope in spite of virus morphogenesis being arrested in the perinuclear space in the absence of US3 suggests new functions of gK during budding of HSV-1 at the nuclear membranes. This is a novel observation in relation to the function of gK in viral morphogenesis.

The authors generated two new recombinant viruses, CK177deltaUS3gK-HA and CK177gK-HA, using the BAC system. A major concern of the experimental work presented is the limited characterization of the genomic structure of these recombinant viruses. The possibility that undesired mutations in other loci of the viral genome may have been selected during the construction of the recombinant viruses should be controlled by either sequencing of the viral genome or the construction of a revertant virus in which the modification (introduction of gK-HA with or without the deletion of US3) is restored, and demonstration that the revertant viruses behave as WT viruses. Also, there is no evidence that US3 protein expression is absent in CK177deltaUS3gK-HA. A detailed characterization of the recombinant viruses is necessary to fully support the conclusions of the study, and to rule out the introduction of mutations in other genes.

The presentation of more quantitative data to support the distribution of gK by immunoelectron microscopy would strengthen the conclusions.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  
No

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** molecular virology and viral pathogenesis, herpesviruses and poxviruses.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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In this manuscript results are presented indicating that deletion of the HSV-1 US3 causes internal membrane proliferation and rearrangements and is accompanied by over expression of glycoprotein K (gK). The results point into a potential functional relationship between US3 and gK that has not been previously reported. Authors should note that a previous publication showed that overproduction of gK causes accumulation of enveloped capsids inside the nuclear membranes. This reference should be included and discussed. Authors should also sequence the gK region to ensure that the overproduction of gK is not independent of US3 due to secondary mutations in the genomic area.

References

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular virology and immunopathogenesis of herpes viruses

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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