JVI Accepted Manuscript Posted Online 14 October 2015
J. Virol. doi:10.1128/JVI.01828-15

THE EBOLAVIRUS GLYCOPROTEIN DIRECTS FUSION THROUGH NPC1$^+$ ENDOLYSOSOMES

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Word Count: Abstract, 75; Main Text, 1,254; Methods, 456
Running Title: Ebola enters cells through NPC1$^+$/TPC2$^+$ endolysosomes

ABSTRACT

Ebolavirus, a deadly hemorrhagic fever virus, was thought to enter cells through endolysosomes harboring its glycoprotein receptor, Niemann-Pick C1. However, an alternate model was recently proposed in which ebolavirus enters through a later NPC1-negative endosome that contains two pore Ca^{2+} channel 2 (TPC2), a newly identified ebolavirus entry factor. Here, using live cell imaging we provide evidence that in contrast to the new model, ebolavirus enters cells through endolysosomes that contain both NPC1 and TPC2.

MAIN TEXT

As evidenced by the recent crisis in West Africa, ebolavirus (EBOV) can cause widespread disease and death in human populations. Entry of EBOV into cells, which is mediated by its sole glycoprotein (GP), is a target for therapeutic intervention (1, 2). EBOV entry is unusual in that it requires proteolytic-priming of GP followed by engagement of Niemann-Pick C1 (NPC1), a thirteen-pass membrane protein that aids cholesterol transport from endolysosomes (LE/Lys). Since the realization that NPC1 functions as an EBOV receptor (3-6), models have invoked entry through $NPC1^+$ LE/Lys (7-9). However, a recent study proposed a very different model: passage through $NPC1^+$ LE/Lys followed by traffic to and entry in endosomes that lack NPC1 but contain two pore Ca^{2+} channel 2 (TPC2), a recently emerged EBOV entry factor (10, 11). The recent studies for (9) and against (10) entry in $NPC1^+$ LE/Lys were based on static microscopic assessment of colocalization of virus-like particles (VLPs) with

endosomal markers. We found (9) that VLPs bearing EBOV GP enter the cytoplasm shortly after colocalization with NPC1$^+$ LE/Lys, assessed at fixed timed intervals in parallel samples analyzing VLP colocalization with NPC1 and VLP entry, which was based on delivery of VP40-β-lactamase into the cytoplasm. A limitation of the static colocalization analyses is that the observer cannot tell if the colocalized particle goes on to fuse. Here, to circumvent that limitation, we used live cell microscopy to determine whether EBOV entry does or does not occur in NPC1$^+$ LE/Lys.

For these experiments we used Moloney Murine Leukemia Virus (MoMLV) particles (Fig. 1) containing gag-mKO (red) in their core and the far-red probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-4-chlorobenzenesulfonate salt (DiD) incorporated into the pseudovirus membrane during particle production, as described previously (12, 13). We used retroviral pseudoparticles since we previously showed that retroviral particles bearing EBOV GP, either full-length or pre-primed (cleaved *in vitro* to the ~20 kDa form), enter cells with the same kinetics as filamentous EBOV VLPs (9). We prepared particles with a biosynthetically pre-primed (21 kDa) version of EBOV GP since, as seen previously (14), higher entry levels were seen with particles bearing primed vs. full length GP (Fig. 2e). In practice, three types of particles are produced: ones with both gag-mKO and DiD, ones with only gag-mKO, and ones with only DiD (Fig. 1a, b). When a double-labeled (mKO/DiD) particle fuses, the lipid dye (blue) diffuses into the endosome membrane and the core (red) enters

the cytoplasm (Fig. 1c). For the studies presented we only analyzed double-labeled particles.

We analyzed pseudovirus particles with pre-primed (21 kDa) EBOV GP entering live cells expressing either NPC1-GFP (Fig. 2) or GFP-Rab5 (Fig. 3) using spinning disk confocal microscopy. In brief, pseudovirus particles were spinfected onto the surface of BSC-1 cells at 4^{o}C after which the dishes were placed on a spinning disk confocal microscope stage maintained at 37^{o}C. After 5 to 15 min, the medium was replaced with 37^{o}C medium and images were captured at intervals of 5 or 10 frames/sec. Only double-labeled pseudoparticles were monitored, as separation of the content (gag-mKO) from the membrane (DiD) gives the clearest measure of a fusion event (Fig. 1c) (12, 13). In experiments represented in Fig. 2, the cells were transfected to express NPC1-GFP. The particle tracked in Fig. 2a (Movie 1) fused 5.5 min after associating with an NPC1^{+} LE/Lys (green). In this case, fusion was evidenced by an increase of the DiD signal (due to dequenching upon diffusion into the endosome membrane) and loss of the gag-mKO signal that had been associated with the DiD (blue) and NPC1-GFP (green) signals. The particle tracked in Fig. 2b (Movie 2) associated with an NPC1^{+} LE/Lys (green) in two steps, and fused 2 min after the second step. In this case the gag-mKO signal was lost, but the DiD signal remained the same, as seen upon fusion of MoMLV pseudovirions bearing HIV Env in endosomes (13). This is because the starting concentration of DiD in this particle was not high enough to observe a dequenching signal (12, 13).

All observed EBOV GP-mediated fusion events (43/43; 100%; traces similar to those in Fig. 2a or Fig. 2b) occurred in endosomes tagged with NPC1-GFP (Fig. 2c). Fusion did not occur if target cells were pretreated with bafilomycin or E64d, chemical inhibitors that nullify, respectively, the low endosomal pH and cathepsin activities needed for EBOV fusion (14, 15) (Fig. 2d). We have previously shown that these treatments do not block trafficking of EBOV GP particles to $NPC1^+$ LE/Lys (9). Moreover, if the particles contained the I544A EBOV GP fusion loop mutant (16), the number of fusion events was strongly reduced (Fig. 2d), in line with reduced infectivity of the same particles (Fig. 2e). For WT (21 kDa) EBOV GP pseudoparticles, the dwell times between stable association with an NPC1-GFP-tagged endosome and fusion ranged from 3-10 min, with shorter dwell times seen for traces exemplified in Fig. 2b.

21 kDa EBOV GP pseudoparticles that fused did so after passage through GFP-Rab5-tagged endosomes (Fig. 3a and Movies 3 and 4; Movie 4 is the continuation of Movie 3). In Movie 3 the pseudovirus associates with a GFP-Rab5 endosome at ~ 3 min. By the end of Movie 3, the endosome has lost most of its GFP-Rab5 signifying its maturation into a later endosome. Continuation of imaging (Movie 4) reveals that the particle fuses much later, at ~ 54 min total timing; based on data presented in Fig. 2, this fusion presumably occurs in a $NPC1^+$ LE/Lys. These findings are consistent with previous observations on the route of EBOV entry (9, 17). And, in contrast to particles bearing EBOV GP,

which only fused in NPC1-GFP-tagged endosomes (Fig. 2 and Movies 1 and 2), similarly prepared particles bearing VSV-G fused primarily in GFP-Rab5-tagged endosomes (Fig. 3b and Movie 5), consistent with prior results (18, 19).

Our results provide strong evidence that EBOV GP-mediated fusion does, indeed, occur in $NPC1^+$ LE/Lys (3, 4, 6-8, 20). After internalization, EBOV passes through a $Rab5^+$ endosome, which then loses Rab5 as it matures into an $NPC1^+$ LE/Lys. In this compartment, primed GP binds to NPC1 (3, 4, 6), and fusion occurs from within an $NPC1^+$ LE/Lys. Our findings are not consistent with the recently proposed model in which EBOV enters from a later putative endosome that lacks NPC1 but contains TPC2 (10, 11). Given the high colocalization of TPC2 with Lamp2 (21) and of Lamp2 with NPC1 (9), we predicted that most $NPC1^+$ LE/Lys contain TPC2 (and vice versa). Indeed, NPC1-GFP and TPC2-mCherry (22) are virtually completely colocalized (Fig. 4a, b) and remain together throughout live cell imaging (Movie 6).

Although TPC2 is required for EBOV entry (10), our results showing (a) that all EBOV GP fusion events occur in $NPC1^+$ LE/Lys (Fig. 2c) and (b) that virtually all $NPC1^+$ LE/Lys contain TPC2 (Figs. 4a,b; Movie 6) argue against the recently proposed model, which requires dissociation of primed GP from NPC1 and subsequent passage of EBOV particles out of the NPC1 compartment followed by transport to a later $NPC1^-/TPC2^+$ endosome (10, 11). Instead, our results argue that EBOV GP directs fusion in a LE/Lys that contains both NPC1 and

TPC2 (Fig. 4c). Hence, rather than playing a direct role in EBOV fusion, a more likely possibility is that TPC2 is required for LE/Lys maturation (23), which is clearly required for EBOV entry (3, 24), and which involves Ca^{2+} dependent endosome fusion (25-27). Proper maturation of $NPC1^{+}$/$TPC2^{+}$ LE/Lys is likely needed to provide the environmental cue(s) that trigger(s), and the physical conditions (e.g., lipid composition) that support, EBOV fusion (9).

SPECIAL METHODS

Production of DiD and mKO-labeled MLV Pseudovirions: Plasmids (0.5 µg 21 kDa GP (full length GP for Fig. 2e); 2 µg MLV-gag-mKO; 1 µg pHIT60; 3 µg pFB-luc (Agilent); 2.5 µg pCAGGS) were diluted to 300 µl in phenol red free DMEM without serum or additives. For 21 kDa EBOV GP (WT or I544A), pFurin was added (1:5 ratio furin:21 kDa GP plasmid). Polyfect (Qiagen) (10 µl per µg DNA) was added and the mixture was incubated for 10 min. HEK293T/17cells (~80% confluent) were then transfected according to the Qiagen protocol. 16-20 h post-transfection, HEK293T media was replaced with OMEM containing 10 µM DiD and incubated at 37°C (5% CO_2) for 4 h. The cells were then washed and covered with HEK293T medium without phenol red. After 24 h (DiD labeling), the media was collected, centrifuged (250 x g; 10 min; 4°C), filtered through a 0.45 µm syringe filter, aliquoted (1 ml aliquots), and stored at -80°C until used. This procedure does not yield uniformly DiD-labeled particles. While some particles contain self-quenching levels of DiD, many do not (12, 13).

Live-cell Imaging of Double Labeled MLV Pseudovirions and Data Analysis: MLV pseudovirions (100-500 µl) were diluted to a total volume of 2 ml in imaging medium (10% FBS-FluoroBrite-DMEM, 10 mM HEPES, 1% sodium pyruvate, 1% antibiotic/antimycotic, and 1% L-glutamine) and kept on ice. BSC-1 cells which had been transiently transfected with the indicated plasmid (7-8 hr) and then replated (50,000 to 150,000 cells) on 35 mm glass bottom tissue culture dishes (MatTek) for ~16-40 h, were cooled to 4^{o}C for 15-30 min and then covered with the diluted MLV pseudovirion solution. The dishes were centrifuged for 1 h at 250 x g (4°C) and then immediately transferred to a Nikon Eclipse TE2000-E microscope equipped with a Yokogawa CSU 10 spinning-disk confocal unit, a 512x512 Hamamatsu 9100c-13 EM-BT camera, a motorized stage maintained at 37^{o}C, and a Nikon Perfect Focus System. Samples (in a single focal plane) were imaged using a 60X/1.45 NA Nikon Plan Apo TIRF oil immersion objective. GFP fluorescing cells were found within 5-15 min, after which the imaging medium was replaced with fresh 37^{o}C imaging media, and image acquisition was started 0-15 min later for a total of 60-90 min at 5 or 10 second frame intervals. Where indicated cells were pre-treated with bafilomycin (200 nM) or E64d (10 µM) for 1 h, and then processed as above. Still images and movies of the tracked, double-labeled particles were generated using Nikon Elements (Nikon). Data analysis was carried out using Micro-Manager (28), the Speckle TrackerJ plugin (29) in ImageJ (30), and Microsoft Excel. Individual particles were tracked separately in each live cell experiment, which entailed image acquisition from 1 or 2 cells per experiment.

ACKNOWLEDGMENTS

The work was supported by R21 AI103601 (to JMW). JS was supported in part by T32AI007046. AG is a Senior Investigator of the Wellcome Trust. GR was funded by an MRC (UK) program grant to AG. We thank the following for plasmids: 21kDa EBOV GP (P. Bates, U. Penn); pFurin (G. Thomas, U. Pitt.); MLV-gag-mKO (G. Melikyan, Emory U.); MLV-gag-pol (pHIT60) (A. Kingsman, U. Oxford); GFP-Rab5 (M. Zerial, (Max Planck Inst., Dresden); NPC1-GFP (M. Scott, Stanford U).

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Figure 1: Experimental Setup. Cartoon (a) and representative image (b) of double (#1) and single (#s 2, 3) labeled MLV pseudovirions expressing EBOV 21 kDa GP. The image (b) is from an early frame of a movie in which MLV pseudovirions expressing EBOV 21 kDa GP were added to a BSC-1 cell expressing NPC1-GFP. Only the red and blue channels are shown, for clarity. Scale bar, 2 μm. (c) Expected behavior of gag-mKO and DiD (from a double labeled MLV pseudovirus) upon delivery, hemifusion and fusion in a GFP labeled endosome.

Figure 2: Pseudovirions bearing 21 kDa EBOV GP fuse in NPC1-GFP-tagged endosomes. (a, b) Two examples of live cell imaging experiments (of BSC-1 cells transfected to express NPC1-GFP). Left: Still images of movie frames taken at the indicated times from Movies 1 and 2, respectively. Asterisks indicate the pseudovirus being tracked (yellow before fusion; white after fusion). Right: Corresponding fluorescence intensity traces of DiD, gag-mKO and NPC1-GFP. Double-headed arrows indicate times of corresponding still frames shown in the Left panels. 'A' and 'F' indicate when the indicated pseudovirion associates ('A') and fuses ('F') with the NPC1-GFP-marked endosome. In (b) two stages of association ('A1' and 'A2') are seen. Scale bar, 1 μm; all panels, same magnification. (c) Fraction of observed fusion events that occurred ('colocalized') in endosomes tagged ('yes') with NPC1-GFP (43/43 fusion events) or not tagged ('no') with NPC1-GFP (0/43 fusion events): 43/482 double-labeled particles were observed to fuse and all 43 fusion events occurred in $NPC1^+$ LE/Lys. (d) Fusion

Journal of Virology

(separation of red and blue fluorescence) is significantly depressed in cells treated with 200 nM bafilomycin (baf) (fusion was seen for 0/96 double-labeled particles observed) or 10 μM E64d (fusion was seen for 0/165 double-labeled particles observed), or if the pseudovirions contained the I544A fusion loop mutation (fusion was seen for 4/253 double-labeled particles observed). Data in (d) for WT 21 kDa GP without drug treatment are for the 43 fusion events (43/482 double-labeled particles observed) depicted in Fig. 2c. (e) Relative infectivity of equivalent volumes of MLV pseudovirions bearing full length WT GP or WT or I544A 21 kDa GP. Data are from a standard infection assay based on the luciferase reporter in the pseudovirions. Western blot analysis confirmed that all forms of GP were incorporated and that comparable amounts of pseudovirions (based on MLV gag) were employed (data not shown).

Figure 3: Pseudovirions bearing 21 kDa EBOV GP pass through Rab5 endosomes prior to fusion; VSV-G pseudovirions fuse in Rab5 endosomes. (a) Single particle tracking of double-labeled 21 kDa EBOV GP pseudovirion entering a BSC-1 cell expressing GFP-Rab5. Left: Still images of movie frames taken at the indicated times from Movies 3 and 4. (Note that Movie 4 is the continuation of Movie 3.) Asterisks indicate the pseudovirus being tracked (yellow before fusion; white after fusion). Right: Corresponding fluorescence intensity traces of DiD, gag-mKO and GFP-Rab5. Scale bar, arrows and labeling are as in Fig. 2 with the addition that 'D' denotes dissociation of Rab5 from the pseudovirion containing compartment. Note the break in the time scale, which

corresponds to the transition between Movie 3 and Movie 4. (b) As in (a) but for a pseudovirion expressing VSV-G (data from Movie 5). Five out of six observed VSV-G fusion events occurred in $Rab5^+$ endosomes.

Figure 4: EBOV enters cells through LE/Lys that contain both NPC1 and TPC2. (a) Still images (from Movie 6) and (b) Manders coefficients (based on 1,959 endosomes) demonstrating high colocalization between NPC1-GFP and TPC2- mCherry. (c) Model for EBOV entry pathway: EBOV-GP coated particles are internalized into the cell and pass through $Rab5^+$ endosomes, which mature (including early endosome (EE) docking and fusion) to generate LE/Lys containing both NPC1 and TPC2 (designated LE, in the figure). The virus particles then fuse with the limiting membrane of these LE/Lys. En route GP is proteolytically processed (e.g., by endosomal cathepsins) and exposed to low pH and other environmental factors (in $NPC1^+/TPC2^+$ LE/Lys) that trigger and support fusion.