Supplemental Methods for:

Comparative Proteomics of Human Monkeypox and Vaccinia Intracellular Mature and Extracellular Enveloped Virions

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Virion Preparation

HeLa cells (ATCC, Manassas, VA) were grown in Dulbecco's modification of Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Near confluent monolayers of HeLa cells were infected with MPV or VV at a multiplicity of infection of 0.1 plaque-forming units per cell in a Biosafety Level 3 facility. After 48 h the cells were lifted into the culture medium by scraping and were centrifuged at 2,000 rpm (avg. RCF = $650 \times g$) for 10 min in a GS-6KR centrifuge with a GH-3.8 rotor (Beckman Coulter, Inc., Fullerton, CA). The cell pellets and clarified culture medium were retained for IMV and EEV purification, respectively.

The cell pellets were resuspended in 4 mL of ice-cold 10 mM Tris-HCl pH 8.0, and then placed on ice for 15 min (with vortexing every 3 min) to cause cell swelling. The samples were sonicated twice for 30 sec and centrifuged to pellet cell nuclei at 2,000 rpm (avg. $RCF = 650 \times g$) for 10 min in a GS-6KR centrifuge with a GH-3.8 rotor. The supernatants were then layered onto 6 mL 36% (w/v) sucrose cushions. The nuclei pellets were resuspended in 2 mL of ice-cold 10 mM Tris-HCl pH 8.0, sonicated twice for 30 sec, centrifuged as above, and the supernatants were layered onto the same sucrose cushions.

In parallel, EEV particles from the clarified culture medium were pelleted by centrifugation at 24,000 rpm (avg. $RCF = 76,000 \times g$) for 30 min at 4°C in an L8-M ultracentrifuge with a SW28 rotor (Beckman Coulter, Inc., Fullerton, CA). The supernatants were discarded and the viral pellets were resuspended in 1 mL of ice-cold 10 mM Tris-HCl pH 8.0 by sonication twice for 30 sec. The samples were then layered onto 6 mL 36% (w/v) sucrose cushions.

The IMV and EEV samples were then centrifuged to pellet the virus particles at 18,000 rpm (avg. RCF = 40,000 × g) for 80 min at 4°C in an L8-M ultracentrifuge with a SW 41 Ti rotor (Beckman Coulter, Inc., Fullerton, CA). The supernatants were discarded and the viral pellets were resuspended in 1 mL of ice-cold 10 mM Tris-HCl pH 8.0 by sonication twice for 30 sec. The samples were layered onto 11 mL 25-40% (w/v) continuous sucrose gradients in ultraclear ultracentrifuge tubes (Beckman Coulter, Inc., Fullerton, CA) and centrifuged at 13,500 rpm (avg. RCF = 22,500 × g) for 40 min at 4°C in an L8-M ultracentrifuge with a SW 41 Ti rotor. When we compared the IMV and EEV sucrose gradient ultracentrifuge tubes side by side, we observed that the EEV particles formed a band at a lower sucrose density than the IMV particles, likely

because of its extra membrane. The viral bands were collected from the top by pipetting, transferred to fresh ultracentrifuge tubes, diluted with 10 mL of ice-cold 10 mM Tris-HCl pH 8.0, and then centrifuged to pellet the virus particles at 13,500 rpm (avg. RCF = $22,500 \times g$) for 40 min at 4°C in an L8-M ultracentrifuge with a SW 41 Ti rotor. The supernatants were discarded, and a final centrifugation step through a 10% sucrose cushion was performed at 18,000 rpm (avg. RCF = $40,000 \times g$) for 80 min at 4°C in an L8-M ultracentrifuge with a SW 41 Ti rotor (the initial MPV purification did not include this step). The supernatants were discarded, and the viral pellets were resuspended in 0.5 mL of ice-cold 10 mM Tris-HCl pH 8.0 and stored at -80° C.

Transmission Electron Microscopy

Unsectioned electron microscopy samples were prepared by placing a drop of concentrated virus onto 200-Cu copper mesh grids (Electron Microscopy Sciences, Hatfield, PA) and covering the sample with Karnovsky's fixative (5% (v/v) glutaraldehyde, 4% (v/v) formaldehyde, 80 mM sodium phosphate pH 7.2, Electron Microscopy Sciences)¹. The samples were incubated for 30 min at room temperature, and then rinsed twice with H₂O. The samples were stained for 2 min with 2% phosphotungstic acid, viewed on a FEI/Philips Morgagni 268 transmission electron microscope (FEI, Hillsboro, OR), and photographed with a Redlake MegaPlus 2K camera (Roper Industries Inc., Duluth, GA).

Sectioned electron microscopy samples were prepared by resuspending $\sim 10^7$ plaque forming units of virus in 0.5 mL of Karnovsky's fixative, incubating the samples 2 h at room temperature, and then incubating the samples at 4°C for 12 h. The samples were centrifuged at 20,000 rpm (avg. RCF = $18,000 \times g$) for 20 min at 4°C in an OptimaTM TL ultracentrifuge with a TLA 55 rotor (Beckman Coulter, Inc., Fullerton, CA). The pellets were resuspended in 0.5 mL of buffer (100 mM sodium cacodylate, 100 mM sodium phosphate, pH 7.4) and centrifuged a second time to pellet the virus at 20,000 rpm (avg. $RCF = 18,000 \times g$) for 20 min at 4°C in an Optima[™] TL ultracentrifuge with a TLA 55 rotor. The samples were washed in a post-fix solution (100 mM sodium cacodylate, 1% (w/v) osmium tetroxide, 0.8% (w/v) K₃Fe(CN)₆) for 1 h, and then the samples were microcentrifuged and the pellet was rinsed in H₂O for 10 min. The samples were dehydrated with a graded series of acetone and infiltrated with a 1:1 (v/v) mixture of acetone and Poly/Bed[®] 812 (Polysciences Inc., Warrington, PA) for 12 h on a rotator. The infiltration solution was removed, and the samples were incubated in fresh 100% Poly/Bed[®] 812 for 2 h and then polymerized in fresh Poly/Bed[®] 812 for 24 h at 60°C. The samples were removed and sectioned at 60 - 80 nm on a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on 300-Cu copper mesh grids (Electron Microscopy Sciences), stained with 2 mL 8% (w/v) uranyl acetate (Electron Microscopy Sciences) for 30 min, and rinsed in H₂O. The sections were post-stained with lead citrate for 10 min, viewed on a FEI/Philips Morgagni 268 transmission electron microscope, and photographed with a Redlake MegaPlus 2K camera.

LC-LTQ-OrbitrapTM-MS(/MS)

The HPLC system consisted of two 100 mL Isco Model 100DM syringe pumps with a Series D controller (Isco, Inc., Lincoln, NE), an in-house manufactured stir-bar style mobile phase mixer (2.5 mL), three 4-port, 2-position valves (Valco Instruments Co., Houston, TX) for mobile phase, purge/split, and capillary column selection, and a 6-port, 2-position injection valve (Valco Instruments Co.) equipped with a 10 μ L sample loop. The mixer and valves were mounted on an in-house manufactured rack assembly that was custom fit to a PAL autosampler (Leap Technologies, Carrboro, NC) for unattended routine analysis. Reversed-phase capillary HPLC columns were manufactured in-house by slurry packing 3 μ m Jupiter C₁₈ stationary phase (Phenomenex, Torrance, CA) into 60 cm lengths of 50 μ m i.d., 360 μ m o.d. fused silica capillary tubing (Polymicro Technologies Inc., Phoenix, AZ) that incorporated a 0.5 μ m retaining screen in a 1/16 inch custom-bored (50 μ m i.d.) union (Valco Instruments Co.).

The mobile phase consisted of 0.2% (v/v) acetic acid, 0.05% (v/v) trifluoroacetic acid in H_2O (mobile phase A) and 0.1% (v/v) trifluoroacetic acid, 89.9% (v/v) acetonitrile in H_2O (mobile phase B). The mobile phase was degassed using an in-line vacuum degasser (Alltech Associates, Inc., Deerfield, IL). The HPLC system was equilibrated at 10,000 psi with 100% mobile phase A, and then the mobile phase selection valve was switched 50 min after injection from position A to B, which created an exponential gradient as mobile phase B displaced A in the mixer. A 33 cm length of 15 μ m i.d., 360 μ m o.d. fused silica tubing was used to split ~20 μ L/min of flow before it reached the injection valve. The split flow controlled the gradient speed under conditions of constant pressure operation. Flow through the capillary HPLC column when equilibrated to 100% mobile phase A was ~200 nL/min.

MS analysis was performed using an LTQ-OrbitrapTM mass spectrometer (Thermo Electron Corp.) with nanoelectrospray ionization. The HPLC column was coupled to the mass spectrometer by an in-house manufactured interface. The heated capillary temperature and spray voltage were 200°C and 2.0 kV, respectively. Data acquisition began 80 min after sample injection and continued for 120 min at AGC settings of 3×10^4 and 1×10^6 for the LTQTM and OrbitrapTM, respectively. OrbitrapTM spectra were collected from 400-2000 *m/z* at a resolution of 100k followed by data-dependant LTQTM MS/MS spectra of the three most abundant ions using a collision energy setting of 35%. A dynamic exclusion time of 60 sec was used to discriminate against previously analyzed ions.

References

1. Ito, S.; Karnovsky, M. J., Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. *J Cell Biol* **1968**, 39, 168a–169a (abstract number 418).