In-vivo DAB cytochemistry and high-pressure freezing to determine the source of the human cytomegalovirus envelope

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Background/aims:
The human cytomegalovirus (HCMV), an enveloped DNA virus, significantly alters host cell morphology during infection. Formation of infectious virus progeny requires two critical envelopment processes: primary and secondary envelopment. Secondary envelopment occurs in a specialized juxtanuclear region known as the cytoplasmic viral assembly complex (cVAC), where capsids bud into the lumen of cellular vesicles and by this acquire the final virion envelope. The identity of these vesicles is debated, with both trans-Golgi and endosomal vesicles proposed as potential sources (1-3, and see abstract by T. Bergner, L. Cortez Rayas, J. von Einem, C. Read).

To address this, we adapted the protocol by Ellinger et al. 2010 (4) for cytochemical labelling of endocytic membranes for visualization by electron microscopy (EM). The protocol uses wheat germ agglutinin (WGA) conjugated to horseradish peroxidase (HRP) for labelling and diaminobenzidine (DAB) cytochemistry that is performed in living cells. WGA specifically binds to N-acetyl-glucosamine and sialic acid, abundant in the plasma membrane, whereby it is endocytosed in large quantities. The peroxidase-catalyzed DAB reaction results in a specific labelling of the endocytic compartment. Performing this reaction in vivo allows cryo-immobilization through high-pressure freezing. The combination with freezing within milliseconds allows capturing snapshot of the dynamic membrane system, making this approach suitable for pseudo-dynamic EM studies. Moreover, high-pressure freezing provides excellent structural preservation, allowing differentiated visualization of the endocytic compartment, HCMV capsids in different maturation stages, and their interaction during the secondary envelopment process in EM. This is a prerequisite for detailed 3D EM analysis.

Methods:
The study was performed with human foreskin fibroblasts infected with HCMV for 5 days, following the adapted protocol of Ellinger et al. 2010 (4). For pulse-chase experiments, WGA-HRP was added to the living cells and incubated for specific pulse times (e.g., 60 or 10 minutes). After the pulse, WGA-HRP was removed, and the samples were incubated for an additional chase period of 30 minutes. In vivo DAB cytochemistry was then performed by applying DAB to induce the formation of an insoluble reaction product at intracellular membranes, visible as a dark precipitate in EM. Samples were immediately cryo-immobilized through high-pressure freezing, freeze-substituted, and embedded in epoxy resin. Transmission electron microscopy (TEM) was used for quantitative analysis of 10 infected cells. Capsids were categorized as budding or enveloped and further categorized as either WGA-labelled or not labelled. 3D visualization of the endocytic compartment was achieved using STEM tomography.

Results:
TEM analysis revealed that the WGA-HRP precipitate is clearly visible and located along the intraluminal face of various intracellular membrane compartments, including vesicles, endosomes, multivesicular bodies, and the trans-side of the Golgi apparatus. Examination of the cVAC showed numerous capsids associated with WGA-labelled membranes. Notably, within 90 minutes of WGA-HRP pulse-chase, about 90% of budding capsids and 50% of enveloped capsids were WGA-labelled. This indicates rapid plasma membrane endocytosis,

translocation to the cVAC and completion of the secondary envelopment process. Reducing the pulse-chase time to 30 minutes still resulted in WGA-labelled membranes that were used for secondary envelopment, suggesting that this process is even faster. STEM tomography further unambiguously identified capsid budding at the trans-side of Golgi cisternae.

Conclusion:
This study demonstrated that combining peroxidase-catalyzed cytochemistry with high-pressure freezing and freeze substitution ensures optimal structural preservation and specific labelling of the endocytic compartment suitable for EM studies. With this approach, we showed that endocytosed membranes are the primary source of the HCMV envelope. Additionally, the findings highlight the rapidity of secondary envelopment.

Keywords:

herpesvirus, cytochemistry, dynamics, TEM, STEM

Reference:

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