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Preparation of the Human Cytomegalovirus Nuclear Egress Complex and Associated Proteins

Mayuri Sharma¹, Jeremy P. Kamil^{1,2}, and Donald M. Coen¹

¹Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston MA 02115, USA

²Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

Abstract

Herpesviruses, like most DNA viruses, replicate their genomes in the host cell nucleus. Their DNA is then packaged and assembled into viral nucleocapsids, which, in most cases, are too large to pass through the nuclear pore complex (NPC). Instead, herpesviruses use a complex multi-step pathway, termed nuclear egress, to exit the nucleus. Key players in this process include two conserved viral proteins that form the nuclear egress complex (NEC). In human cytomegalovirus (HCMV), these NEC proteins are UL50, embedded in the inner nuclear membrane, and its nucleoplasmic partner UL53. Both are essential for viral nuclear egress. However other viral components as well as host nuclear envelope proteins may also participate in nuclear egress. Identifying these viral and cellular factors may provide important insight into the herpesvirus lifecycle and its relationship to the underlying, yet still-mysterious, host nuclear egress pathway. We developed an immunoprecipitation-based protocol, described herein, to identify protein-protein interactions involving the NEC from cells that express an epitope-tagged version of NEC subunit UL53 from the nuclear fraction of infected cells.

Keywords

Nuclear egress complex; proteomics; herpesvirus; human cytomegalovirus; protein kinase; nuclear envelope; nuclear lamina

1. Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus that can cause life-threatening complications in immunocompromised individuals, and is the leading infectious cause of congenital defects in newborns (Britt, 2008; Mercorelli et al., 2011; Pultoo et al., 2000). Current therapies to control HCMV infection are limited in terms of their efficacy, potency, selectivity, and pharmacokinetic properties (Coen & Schaffer, 2003; Hakki & Chou, 2011). Knowledge of the viral life cycle can provide insight into pathways that might be targeted for development of new therapeutics. The process of nuclear egress during herpesvirus replication offers such a target. Nuclear egress is a multi-step pathway during which herpesvirus nucleocapsids translocate from the nucleoplasm to the cytoplasm, without going through nuclear pore complexes (Hennig & O'Hare, 2015; Johnson & Baines, 2011;

Mettenleiter, 2002). Instead, following virus-promoted disruption of nuclear lamina structure, nucleocapsids access the inner nuclear membrane (INM), and are then enveloped by the INM as they bud into the nuclear envelope lumen, a step known as ‘primary envelopment’. These enveloped nucleocapsids then fuse with the outer nuclear membrane (ONM) and are released into the cytoplasm. Nuclear egress is orchestrated by two viral proteins that form the nuclear egress complex (NEC). In HCMV, these viral subunits consist of UL50 (an integral INM protein) and its nucleoplasmic partner UL53. Both are required for nuclear egress and viral replication; interestingly they also recruit other proteins to facilitate specific steps during nuclear egress (Sharma *et al.*, 2014).

Herpesvirus nuclear egress may be related to a recently discovered host pathway by which large ribonucleoproteins (RNPs) exit the nucleus (Razafsky & Hodzic, 2015; Speese *et al.*, 2012). However, there appear to be key differences in the players involved in the two pathways, suggesting their molecular details may differ. Notably, a virus-encoded kinase is critical for HCMV nuclear egress, whereas a cellular kinase is important for the host pathway (Sharma & Coen, 2014; Sharma *et al.*, 2014; Speese *et al.*, 2012). To understand these pathways, and identify differences that might be exploited as antiviral drug targets, we first need to identify key viral and cellular proteins involved in nuclear egress. The protocols below were designed to facilitate the identification of NEC-associated proteins in HCMV-infected cells.

2. Sample preparation for isolation of the nuclear egress complex

2.1 Cells

Human cells infected with HCMV serve as the source for NECs. HCMV is propagated in primary human foreskin fibroblasts (HFF) (e.g., strain Hs27, ATCC CRL-1684; American Type Culture Collection; Manassas, VA). The cells are passaged in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC 30-2002), supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, at 37°C and 5% CO₂ in ~95% humidity. After reaching confluence, cells are maintained in DMEM containing 5% FBS (maintenance medium).

2.2. Viruses

We use a virus expressing a version of UL53 bearing a FLAG-epitope at the C-terminus, designated UL53-FLAG AD169rv (referred to as ‘53-F’) (Sharma *et al.*, 2014). 53-F was derived from a bacterial artificial chromosome (BAC) clone of HCMV strain AD169, named AD169rv, with the BAC-derived wild type virus as the control. High titer stocks for each virus are generated by infecting HFFs at low multiplicity of infection (MOI) (typically 0.001), with a previously titrated stock, and harvesting the supernatant after 80–90% of the cells show cytopathic effects. The supernatant containing the virus is centrifuged at 1000×*g* for 10 min at room temperature to pellet cell debris. The supernatant is dispensed in 1 ml aliquots and frozen in liquid nitrogen until further use. One aliquot of each virus is used for titration by serial dilution as described (Kamil & Coen, 2007).

2.3 Infection

Seed HFFs ($\sim 3 \times 10^6$ cells per 100-mm dish) and incubate until they reach $\sim 90\%$ confluence (typically ~ 3 days). Count cells from a representative dish, and then infect two 100-mm dishes of HFF ($\sim 1 \times 10^7$ cells per dish) at an MOI of 1 with either UL53-FLAG AD169rv, or, as a specificity control, with wild-type AD169rv virus. To infect cells, replace -medium with 7 ml maintenance medium containing diluted virus; incubate 2 h and then replace with fresh maintenance medium lacking virus. To determine the optimal time post-infection at which to isolate NECs, we measured UL50 and UL53 expression levels by Western blotting (Fig. 1A). In cells infected at an MOI of 1, we found NEC expression peaks at 72 h post-infection (hpi).

2.4 Prepare nuclear fractions from infected cells

Pre-chill all equipment and glassware on ice, and follow the steps outlined in Figure 2 (Sharma *et al.*, 2014).

1. At 72 hpi, remove medium from infected cells and wash cells once with 5 ml ice-cold 1X Dulbecco's Phosphate buffered saline (DPBS).
2. Resuspend cells by scraping into 3 ml Hypotonic Buffer (10 mM HEPES, pH 7.4, 1 mM dithiothreitol [DTT], 1 mM MgCl_2 , 10 mM KCl and protease inhibitor cocktail [Roche Applied Sciences, Indianapolis, IN]).
3. Incubate resuspended cells on ice for 15 min. This incubation in hypotonic buffer allows cells to swell.
4. Transfer cells into a 5-ml Dounce homogenizer (Wheaton, USA) and homogenize cells using 40 strokes, on ice.
5. Centrifuge the homogenate (1000g, 10 min) to pellet the nuclei. Remove the supernatant (cytoplasmic fraction) and retain a small aliquot (mix with Laemmli sample buffer) for subsequent Western blotting analyses, and store remainder at -80°C if needed. Resuspend the nuclear pellet in 3 ml Lysis Buffer (50 mM HEPES, pH 7.4, 1 mM DTT, 200 mM NaCl, 5 mM EDTA, 50 mM arginine, 0.5% Triton X-100, EDTA-free protease inhibitor cocktail [Roche Applied Sciences, Indianapolis, IN]). Arginine reduces nonspecific protein binding to resins.
6. Rotate the nuclear fraction end-over-end at 4°C for 15 min.
7. Homogenize the nuclear suspension with 40 strokes, on ice, to disrupt nuclear membranes.
8. Centrifuge 25 min at $18,000g$ (Eppendorf centrifuge), and recover the supernatant (nuclear lysate) for further steps.
9. Monitor enrichment in each fraction by SDS-PAGE and immunoblotting with antibodies specific for cytoplasmic and nuclear markers. We use glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the cytoplasmic marker (Abcam ab8245; dilution 1:5000) and lamin A/C as the nuclear marker (Santa Cruz,

N-18; dilution 1:1000). This protocol greatly enriches for nuclear proteins as shown in Figure 1B.

2.5 Immunoprecipitation

Pre-clear lysate—To reduce non-specifically associated proteins, “pre-clear” the nuclear lysate from Step 8 by incubating with resin bearing an isotype-matched control of the antibody to be used for immunoprecipitation. We prepare IgG agarose (Sigma) resin as suggested by the manufacturer. Briefly, centrifuge 600 μ l of the resin slurry (handled with wide-bore tips) for 30 sec (6,000g, 4°C), and remove the supernatant. Wash the settled resin three times (3 ml DPBS each), centrifuging 30 sec between washes. Mix the nuclear lysate from Step 8 with 300 μ l of washed/settled anti-IgG agarose resin. Rotate 3 h end-over-end at 4°C. Centrifuge (30 sec, 6000 $\times$ g) and recover the cleared nuclear supernatant for use in Step 10, below.

Immunoprecipitate with anti-FLAG antibody—We use the EZ-View anti-FLAG M2 monoclonal antibody-conjugated agarose (Sigma) for immunoprecipitation. Prepare the resin for use as described above.

10. Add the entire pre-cleared nuclear supernatant from Step 9, to 300 μ l of settled, EZ-View anti-FLAG M2 affinity resin. Rotate 4 hours at 4°C.
11. Centrifuge (6,000g for 30 sec) to pellet the beads. Remove the supernatant (flowthrough, FT; unbound proteins); mix 100 μ l of this supernatant with 100 μ l Laemmli sample buffer, boil and retain for subsequent Western blotting analyses. Store the rest at –80°C.
12. Wash beads (bearing the immunoprecipitated proteins) four times by rotating 15 min in 3 ml Lysis Buffer at 4°C, and re-pelleting (6,000g, 30 sec).
13. Elute bound proteins by low pH: add 270 μ l of 0.1 M glycine, pH 2.5, and incubate 5 min at room temperature. Centrifuge at 6,000g for 10 sec, and collect the supernatant. Neutralize the supernatant by adding 30 μ l Neutralization Buffer (0.5 M Tris pH 7.4, 1.5 M NaCl) and glycerol (final concentration 10%) to a final volume of 300 μ l. Split this eluate into three 100- μ l aliquots. Two of the 100- μ l aliquots are stored at –80°C until further processing for mass spectrometry analysis (Section 2.6, below). The last 100- μ l aliquot is reserved for Western blot validation of candidates identified by mass spectrometry; add 100 μ l Laemmli sample buffer, boil and freeze until needed.
14. Add 300 μ l Laemmli sample buffer to the post-elution beads, heat denature at 85–90°C for 5 min, then centrifuge (6,000g, 30 sec) and recover the supernatant (‘resin’; proteins that resisted low-pH elution). This ‘resin’ sample has a protein composition similar to the eluate (unpublished results), and is used for qualitative assessment by silver staining and Western blotting in Step 15 (shown in Figure 3), prior to mass spectrometry.
15. To monitor results, resolve aliquots of boiled ‘resin’ samples by SDS-PAGE and silver staining (Figure 3A). As expected for conventional antibodies,

immunoglobulin heavy and light chains are abundant contaminants (Fig. 3A, IgHC and IgLC, respectively). Bands corresponding to UL50 and UL53 should be highly enriched in the 53-F lane, compared to WT-infected samples (Fig. 3A), as confirmed by MS analysis of this region of the gel (bracket, Figure 3A), and immunoblotting (Sharma *et al.*, 2014). Importantly, at least 14 additional bands representing candidate NEC-associated proteins also specifically co-precipitated from 53-F-infected samples (Fig. 3A).

2.6 Sample processing for mass spectrometry (MS)

Please consult your MS experts before proceeding. They may suggest useful alternatives. Based on the advice of our MS facility, we concentrate the samples, resolve proteins by SDS-PAGE and excise proteins from specific regions of each lane for MS identification. Concentrate each low-pH eluate (100 µl) to a final volume of 30 µl using the Wessel Flügge protocol (Wessel & Flugge, 1984), as follows.

1. Add 400 µl methanol and vortex, add 100 µl chloroform and vortex, add 300 µl water and vortex, then centrifuge (14,000g) for 1 min.
2. Remove the top (aqueous) layer carefully and discard. Add 400 µl methanol to the organic phase, vortex, and centrifuge (14,000g, 2 min).
3. Remove the supernatant without disturbing the pellet.
4. Air-dry the pellet. Resuspend the pellet in 30 µl Laemmli buffer, and resolve proteins by gradient (4–20%) SDS-PAGE.
5. Visualize proteins using Colloidal blue (Life Technologies), according to manufacturer's instructions.
6. The samples and gels are handled with gloves. Excise gel slices containing protein bands in the molecular weight range of 10–25, 25–37, 37–50, 50–75, 75–100, 100–150, and 150–250kD using separate razor blades for each gel slice. Avoid the immunoglobulin heavy and light chain bands that can be observed at 50 and 25kD, respectively. Place each gel slice in its own microcentrifuge tube, containing water to cover the gel slice, and submit for MS analysis.

3. Concluding Remarks

MS provides a starting point by identifying candidates that might interact with the UL53 subunit of NEC egress complexes. The untagged WT AD169rv (WT) control sample helps identify proteins that bind the FLAG-antibody resin non-specifically. Indeed, we found no viral proteins in eluates from this sample. Proteins present at similar levels in WT versus 53-F eluates, such as antibody contaminants, are excluded from further analysis. Proteins unique to 53-F eluates, with three or more unique peptide matches, are considered confidently identified, and strong candidates for further investigation. One such candidate is the nuclear lamina-associated protein, emerin (Fig. 3B). Emerin was also identified in a recent proteomics study that immunoprecipitated HCMV NECs from whole cell lysates (Milbradt *et al.*, 2014). Another strong candidate is the host protein, p32 (Fig. 3B; (Milbradt *et al.*, 2014), previously reported to colocalize with NEC subunit UL50, or with the viral

kinase UL97, in co-transfection experiments (Marschall et al., 2005; Milbradt et al., 2009). However, as p32 associates with a variety of proteins (Muta *et al.*, 1997; van Leeuwen & O'Hare, 2001), the functional relevance of its association with the HCMV NEC remains unclear.

Proteins with one or two peptide matches should also be considered. This group can include rare (low-abundance) proteins, weakly interacting proteins, small proteins, and proteins for which peptide sequence coverage is low. For example, we recovered two peptides corresponding to the HCMV protein kinase, UL97, which is important for disruption of the nuclear lamina during nuclear egress (Krosky *et al.*, 2003; Reim *et al.*, 2013; Sharma & Coen, 2014). Further studies showed that UL97 indeed associates with the NEC, and this association is dependent on each NEC subunit (Sharma *et al.*, 2014).

These protocols can be extended to other epitope-tagged components of the HCMV NEC, and likely other epitope-tagged host and viral proteins involved in nuclear events during infection. Key features of this protocol include the control wild type virus expressing a native (untagged) NEC (to exclude non-specifically associating proteins), and the use of nuclear fractions rather than whole cell lysates. Our experience is that nuclear lysates permit identification of associated proteins that are not observed when using whole cell extracts, and help exclude proteins that are likely to be irrelevant to NEC functions during nuclear egress. We emphasize that the protocols described here are merely first steps, and identified candidates must be tested rigorously to determine their biological validity for viral replication and potential roles in nuclear egress.

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Figure 1.

Western blots of UL53 expression time course, and nuclear fractionation. (A) Human foreskin fibroblasts (HFF; 1×10^5 cells per well of a 24-well plate) were infected at an MOI of 1 with UL53-FLAG AD169rv. At each indicated time, cells were washed with 1XDPBS, harvested into 100 μ l Laemmli sample buffer, heated, resolved by SDS-PAGE and immunoblotted with antibodies specific for UL53 or actin as the loading control. (B) At 72 hpi cells infected with either WT or UL53-FLAG ('53-F') AD16rv were fractionated to separate nuclei (nuclear fraction, 'NF') from the cytoplasmic fraction ('CF'), and immunoblotted with antibodies against GAPDH or A-type lamins (lamin A/C) as cytoplasmic and nuclear markers, respectively.

Figure 2.

Overview of NEC immuno-purification protocol from HCMV-infected cells. Fractions destined for silver staining or immunoblot analysis are in bold. The sample destined for gel-separation and mass spectrometry (MS) is indicated in bold and with an asterisk.

Figure 3.

Qualitative assessment of 'resin' and 'FT' samples prior to MS. (A) Silver stained visualization of proteins immunoprecipitated from cells infected with WT or FLAG-tagged UL53 ('53-F') virus. These samples comprise the 'resin' fractions from Figure 2, which have a protein composition very similar to (or in some cases more concentrated than) the eluate. The bracketed region contains the UL50 and UL53 bands. Immunoglobulin heavy and light chains are indicated by IgHC and IgLC, respectively. (B) Immunoblot analysis of the post-immunoprecipitation flow-through (FT; unbound proteins), and the same 'resin'

fraction shown in panel A, using antibodies specific for each indicated protein. Images are from the same samples, resolved and blotted separately.

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