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Optimized method for isolation of immature intracytoplasmic retroviral particles from mammalian cells

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ABSTRACT

To biochemically and structurally characterize viral intracytoplasmic particles (ICAPs), a sample of high purity and homogeneity is usually required. Production of ICAPs in the system closely related to their natural host cells is crucial for the analysis of host-cell binding proteins involved in ICAPs assembly, transport and budding. However, this approach is often hampered by problems with low yield of the ICAPs due to either low expression or fast release from the host cell. Another obstacle may be a low stability or fragility of the intracellular particles. The published methods for ICAPs isolation often involved several time-consuming centrifugation steps yielding damaged particles. Other papers describe the ICAPs production in non-natural host cells. Here, we optimized the method for purification of unstable Mason-Pfizer monkey virus (M-PMV) ICAPs from non-human primate derived cells, commonly used to study MPMV replication i.e. African green monkey kidney fibroblast cell line (COS-1). Our simple and rapid procedure involved separation of the intracytoplasmic particles from the cell debris and organelles by differential, low-speed centrifugation, their purification using sucrose velocity gradient and final concentrating by low-speed centrifugation. Importantly, the method was established for unstable and fragile M-PMV intracytoplasmic particles. Therefore, it may be suitable for isolation of ICAPs of other viruses.

1. Introduction

The assembly of immature retroviral particles is driven by mutual interactions of specific domains of Gag polyprotein precursor and by binding of viral genomic RNA. Retroviruses assemble by two, morphologically distinct pathways. In the first one, the assembly is facilitated by the plasma membrane. This so-called C-type assembly is utilized by the majority of retroviral genera: alpharetroviruses (e.g. Rous sarcoma virus), gammaretroviruses (e.g. murine leukemia virus) and lentiviruses (e.g. human immunodeficiency virus, type 1). Betaretroviruses that utilize the second pathway, assemble at the pericentriolar region of the infected cells (Sfakianos et al., 2003). The preassembled particles are then transported to the plasma membrane, where the budding occurs. This type of immature particles assembly is named a D-type and its typical representatives are Mason-Pfizer monkey virus (M-PMV) and mouse mammary tumor virus (Sommerfelt et al., 1993). Following the assembly, maturation takes place during or shortly after budding. These processes i.e. maturation and budding, are similar for both morphological types. During the maturation, the Gag polyprotein precursor is cleaved by activated retroviral protease into several structural proteins: matrix (MA), capsid (CA) and nucleocapsid that subsequently reassemble to form mature, fully infectious virus.

The intracytoplasmic D-type of assembly provides the opportunity for the isolation of immature particles from the cytoplasm of the host cells. This contrasts with the C-type retroviruses that do not form any distinct intracytoplasmic immature particles because the assembly is spatially and temporally connected with the budding. The intracytoplasmic immature particles provide an excellent tool to examine the biochemical and structural aspects of the retroviral assembly (Bohmova et al., 2010; Rumlova-Klikova et al., 2000; Strohalmova-Bohmova et al., 2014) as well as to identify the cellular proteins participating on their assembly and transport (Vlach et al., 2008). The in vitro interactions of purified viral intracytoplasmic particles (ICAPs) with liposomes or isolated cellular membranes may mimic the cellular environment required for budding and maturation, and thus may be a plausible approach to such studies.

The separation of viruses from the subcellular components of tissue homogenates by centrifugation through either velocity or isopycnic sucrose gradients was first shown by Anderson (Anderson et al., 1966). Later, Kuff et al. (1968) described the isolation of intracisternal A-

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particles from BALB/c mice plasma-cell tumors by two rounds of centrifugation comprising the centrifugation through 48% sucrose cushion followed by the centrifugation through 33–68% (w/vol) sucrose density gradient. Smith and Wivel applied this protocol for the isolation of intracytoplasmic A particles from mammary tumors (Smith and Wivel, 1972). However, as the vast majority of the ICAPs was lost in discarded nuclear pellet fraction, Smith and Wivel optimized the Kuffs method by introducing different cell lysis procedure and also by applying additional purification steps. Their optimized purification procedure comprises a series of sucrose gradient centrifugations: discontinuous centrifugation to the interface of two sucrose layers (50% (w/w) and 63% (w/w) sucrose) and continuous isopycnic 20–66% (w/w) sucrose gradient centrifugation (Smith and Wivel, 1973). The first attempt of ICAPs isolation from HeLa, HEp-2 and AO cells infected with M-PMV was described by Bukrinskaya et al. (Bukrinskaya et al., 1974). However, the particles obtained after a long-lasting (16 h) centrifugation were not related to M-PMV as they documented immunochemically. Therefore, the authors concluded that the particles represented an independent intracellular oncornavirus. They also reported that the isolated particles were extremely fragile. The fact that they failed to isolate M-PMV ICAPs, suggests that their stability is even lower. The more simple protocol for M-PMV ICAPs purification was described for production in insect *Spodoptera frugiperda* (Sf9) cells (Parker and Hunter, 2001). However, the use of the insect host cells considerably limits the studies aimed at the factors controlling the particles assembly and transport in M-PMV natural host cells.

To identify such host-cell proteins binding to the M-PMV particles, we intended to produce the ICAPs in the African green monkey kidney fibroblast cell line (COS-1), rather than in the previously used insect Sf9 cells. COS-1 cells are non-human primate derived cells, commonly used to study MPMV replication. To do so, we combined and optimized the available methods for purification of the M-PMV ICAPs from both mammalian (Bukrinskaya et al., 1974; Kohoutova et al., 2009) and insect cells. Initially, we used a transport and budding defective M-PMV mutant that accumulates high amounts of ICAPs due to the A18V mutation in the MA domain (A18V MA) (Parker et al., 2001; Rhee and Hunter, 1991). These A18V MA ICAPs are not released from the cell in contrast to those of the wild-type (wt) and thus do not undergo maturation. Therefore we used this mutant for optimization of the ICAPs preparation. Initially, we optimized the cell disintegration conditions and next the conditions for M-PMV ICAPs isolation. Finally, we verified the suitability of the optimized procedure for the isolation of the wild type M-PMV intracytoplasmic particles.

2. Materials and methods

2.1. Expression plasmids of M-PMV

The proviral vectors pSARM4, encoding M-PMV genome, and A18V MA pSARM4, carrying the A18V mutation in the MA domain (A18V MA), were kindly provided by Dr. Eric Hunter. The construct with double point mutations A18V in MA and D26A in protease (D26A PR) was created using standard subcloning techniques; plasmids were propagated in *E. coli* DH5α and verified by DNA sequencing.

2.2. Cell line and transfection

COS-1 (ATCC, USA) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich, USA) supplemented with 10% fetal bovine serum (Sigma–Aldrich, USA) and 1% L-glutamine (Sigma–Aldrich, USA) at 37 °C in 5% CO_2 atmosphere. Cells were transfected with the appropriate DNA construct using polyethylenimine (PEI) (Polysciences, Inc., USA) as a transfection reagent. The medium was changed 5 h post transfection and the cells were maintained at 37 °C in 5% CO_2 atmosphere.

2.3. Purification of immature intracytoplasmic virus particles from cell lysate

72 h post the transfection, the cells were washed by phosphate-buffered saline (PBS) and lysed for 30 min on ice in lysis buffer consisting of 20 mM Tris (pH 7.6) (Trizma® base, Sigma-Aldrich, USA), 1 mM EDTA (Sigma-Aldrich, USA), 500 mM NaCl (Penta, Czech Republic) and 1% Triton™ X-100 (Sigma-Aldrich, USA), supplemented with Halt™ Protease Inhibitor Cocktail (100X) (Thermo Fisher Scientific, USA). The cell lysate was successively centrifuged for 5 min 3,000 *g*, 10,000 *g* and 16,000 *g* to remove bulky, pelletable cell debris (Kohoutova et al., 2009; Parker et al., 2001; Rumlova et al., 2014). The final supernatant was optionally filtered and loaded onto the top of a velocity 5% to 20% (w/vol) sucrose gradient in the lysis buffer. The centrifugation proceeded at 25,000 rpm (Beckmann SW41 rotor) for 25 min at 4 °C. Eight fractions (1 ml each) were collected and the presence of ICAPs in the gradient fractions was analyzed by western blot (WB). The fractions containing the highest amounts of M-PMV Gag polyprotein were pooled and dialyzed overnight against the storage buffer (20 mM Tris pH 7.6, 0.5 M NaCl). Following day, the sample was centrifuged at 16,000*g* for 10 min at 4 °C and the pellet was resuspended in the storage buffer. The final sample was immediately analyzed using western blot and transmission electron microscopy (TEM).

2.4. Western blot

The samples were solubilized by boiling in protein loading buffer and separated by the polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a nitrocellulose membrane (SpinChem®, Sweden) and detected with polyclonal antibody against M-PMV CA (in-house production) and HRP-conjugated anti-rabbit IgG antibody (Sigma-Aldrich, USA) using a chemiluminescent substrate SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA) and LAS-3000 imager.

2.5. Transmission electron microscopy

Immature intracytoplasmic particles were negatively stained with 2% sodium phosphotungstate (pH 7.3) on carbon-coated grids as described earlier (Hadravova et al., 2012). The samples were visualized by TEM using a JEOL transmission electron microscope JEM-1010 at 80 kV. Images were recorded with an AnalySIS MegaView III Digital Camera.

3. Results

To isolate authentic M-PMV intracytoplasmic A particles, we combined and optimized the procedures described earlier (Bukrinskaya et al., 1974; Kohoutova et al., 2009; Parker et al., 2001). To enhance the amount of the ICAPs within the cells for the initial optimization of isolation procedure, we used the transport and budding defective M-PMV mutant A18V MA (Rhee and Hunter, 1991). This mutant accumulates a high amount of ICAPs under the plasma membrane. These ICAPs neither bud nor undergo maturation, representing thus an ideal material for the method set-up. Initially, as the procedures of M-PMV ICAPs isolation from insect cells seemed to be the most straightforward, we repeated the protocol published by Parker et al. (Parker et al., 2001). In this protocol, the producing cells were lysed in hypotonic lysis buffer containing 1% Triton X-100. The ICAPs were then separated from cleared cell lysate by centrifugation and then concentrated at the 35% sucrose–75% sucrose interface. Following this protocol; however, we obtained a very low yield of the intracellular particles most probably due to insufficient cell lysis. To overcome this problem, we optimized the process of the cell lysis and separation of ICAPs from the bulky cellular material. To do so, we used three low-speed centrifugation