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Assays to Assess Arenaviral Glycoprotein Function

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Abstract

Arenaviruses, such as Lassa virus (LASV) and Pichinde virus (PICV), are enveloped viruses with a bi-segmented ambisense RNA genome. The large (L) genomic segment encodes the Z matrix protein and the L RNA-dependent RNA polymerase, whereas the small (S) genomic segment encodes the nucleoprotein (NP) and the glycoprotein precursor complex (GPC). GPC is processed by signal peptidase in the endoplasmic reticulum into the stable signal peptide (SSP) and GP1/GP2, which is further cleaved by the Golgi-resident subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P) into the cellular receptor-recognition subunit GP1 and the transmembrane subunit GP2, which helps promote the membrane fusion reaction to allow virus entry into the cell. This article describes assays to assess PICV GPC expression, proteolytic processing, fusion function, and GPC-mediated virus-like particle (VLP) entry into cells under tissue-culture conditions.

Keywords

Arenavirus; Lassa virus (LASV); Pichinde virus (PICV); glycoprotein precursor complex; GPC; cell entry; receptor recognition; cell membrane fusion

1. Introduction

The first step of arenavirus infection is mediated by cellular attachment and internalization. Arenavirus utilizes its envelop glycoprotein as its receptor-binding protein (1). During infection of the host cells, arenaviral glycoprotein is expressed as a single polypeptide called glycoprotein precursor complex (GPC), which undergoes cellular proteolytic cleavages, myristoylation and glycosylation to produce the individual GP1 (cellular receptor-binding subunit), GP2 (membrane fusion subunit) and SSP (stable signal peptide), which together assemble into the mature trimeric complex that is inserted into the membrane of the budding virions (2–8). The envelope glycoprotein (GPC) mediates the entry of the virus into its host cell via its specific recognition by the different cellular protein receptors. Pathogenic New

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⁴Notes

⁵GP-mediated VLP entry was monitored by microscopy of eGFP expression in infected cells (Fig 4A).

World arenaviruses (e.g., Junin virus), bind the host cell transferrin receptor 1 (TfR1) through the GP1 receptor-binding subunit of GPC (9, 10), whereas Old World arenaviruses [e.g., LASV and lymphocytic choriomeningitis virus (LCMV)], as well as several nonpathogenic New World viruses, use the cellular α -dystroglycan protein for uptake into the endosome (11, 12). Fusion reaction of the viral and cellular membranes is activated by low pH in the endosome and is promoted by the GP2 subunit of GPC (13, 14). Here we described several methods to analyze GPC functions, including GPC expression and proteolytic cleavage, pH-dependent membrane fusion, and virus-like particle (VLP) entry into cells under tissue-culture conditions. A comprehensive level of understanding of arenaviral glycoprotein functions will aid the development of the much needed vaccine and antivirals against deadly human arenaviruses, such as LASV that is responsible for hundreds of thousands of annual human infections and thousands of deaths in several endemic countries in West Africa (15)

2. Materials

1. Plasmids: pCAGGS-GPC plasmid, which contains the PICV glycoprotein gene; pT7-Fluc has the Firefly luciferase reporter under a T7 promoter (obtained from Jack H. Nunberg, University of Montana); pE-GFP, a lentivirus transfer plasmid containing e-GFP (pHR-CMV-eGFP), lentivirus packaging plasmid (pCMV-dR8.2), lentivirus VSV-G plasmid (pCMV-VSV-G). pCMV-EGFP, a plasmid expressing eGFP under CMV promoter. p β -gal, a plasmid expressing β -gal under CMV promoter.
2. BSRT7–5 cells, obtained from K. Conzelmann at Ludwig-Maximilians-Universität, Germany. These cells were originally derived from BHK-21 cells that were transformed to constitutively express the T7 RNA polymerase (16). BSRT7–5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mg geneticin per ml, and 50 mg penicillin and streptomycin per ml.
3. 293T cells, a highly transfectable cell line originally derived from human embryonic kidney, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 mg penicillin and streptomycin per ml.
4. DMEM (Sigma), MEM (Invitrogen), FBS (Sigma), Penicillin and Streptomycin (Invitrogen), Geneticin (Invitrogen), trypsin-EDTA (Invitrogen), PBS (Sigma).
5. 2x Laemmli Sample Buffer (Bio-Rad).
6. Polyacrylamide gel solutions: Resolving gel: 3.3 ml Acrylamide/Bis-acrylamide (30% solution, Sigma), 2.5 ml Resolving buffer (Bio-Rad), 4.1 ml water, 100 μ l 10% SDS, 50 μ l 10% APS (Sigma), 5 μ l TEMED (Sigma). Stacking gel: 1.3 ml Acrylamide/Bis-acrylamide (30% solution, Sigma), 2.5 ml Stacking buffer (Bio-Rad), 6.1 ml water, 100 μ l 10% SDS, 100 μ l 10% APS (Sigma), 10 μ l TEMED (Sigma).
7. Mini-PROTEAN® Tetra Cell Systems.

8. Molecular weight markers (Bio-Rad).
9. PVDF or Nitrocellulose membranes (Millipore).
10. Tris-buffered saline (TBS) (pH=7.4, Fisher Scientific).
11. 5% non-fat milk (in TBS).
12. Guinea pig anti-PICV serum.
13. 4% paraformaldehyde (PFA) (Fisher Scientific).
14. IFA blocking buffer: 200mM glycine, 2% BSA, 2% goat serum in PBS.
15. IRDye-labeled anti-guinea pig antibody (Licor).
16. Firefly luciferase assay kit (Promega) and cell-culture lysis reagent (Promega).
17. Ultracentrifuge (Beckman Coulter).
18. 2.5 M calcium chloride (CaCl_2) (Fisher Scientific).
19. 2X HBS: 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4 , pH 7.10.
20. Acidic DMEM (pH 5.0 adjusted by adding HCl) (*see* Note 1).
21. Polybrene, a cationic polymer used to enhance transfections (Sigma).
22. Power supply: PowerEase 500 (Thermo Fisher).
23. Trans-blot Turbo Blotting system (Bio-Rad).
24. Odyssey imaging system (Licor).
25. Fluorescence-activated cell sorter (FACS) (Becton Dickinson).
26. Synergy 2 plate reader (BioTeK).
27. Fluorescence microscope (Nikon).
28. Beta-Galactosidase Assay 2X Buffer: 200 mM sodium phosphate buffer (pH 7.3), 2 mM MgCl_2 , 100 mM beta-mercaptoethanol, 1.33 mg/ml ONPG

3. Methods

3.1 Intracellular GPC expression and processing

1. Seed 293T cells into 6-well plates at 6×10^5 cells/well with complete DMEM.
2. In the morning of the day of transfection, replace culture medium with an equal aliquot of fresh complete DMEM.
3. Prepare DNA and calcium chloride mix fresh as follows:
 - 2 μl pCAGGS-GPC (1 $\mu\text{g}/\mu\text{l}$)
 - 10 μl 2.5 M CaCl_2

¹.Arenavirus GPC-mediated membrane fusion is pH-dependent. It is critical to prepare acidic DMEM media with accurate pH by using a pH meter.

88 µl water

1–2 h post-medium replacement, add 100 µl of 2X HBS dropwise into each well of 293T cells. Incubate the final DNA mix at ambient temperature for 4 mins and then add it dropwise into wells. 6 h post-transfection, replace the medium with fresh complete DMEM and continue to culture in CO₂ incubator at 37°C.

4. 48 h post-transfection, aspirate the medium, and lyse cells by adding 200 µl of lysis buffer for 10 min on ice. Concentrate the cell lysates by centrifugation at 15,000g for 3 min, collect the supernatants, and mix them with 2x Laemmli Sample Buffer.
5. 20 µl samples were loaded into SDS-PAGE gel with molecular weight marker. The samples were separated at a constant 100V for 1–1.5 h.
6. Transfer the proteins from gel to either nitrocellulose or PVDF membrane by Trans-blot Turbo Blotting system (Bio-Rad) following the manufacturer's instructions.
7. Block the membrane with 5% non-fat milk-TBS for 1 h at ambient temperature and then incubate it with guinea pig anti-PICV serum over night at 4°C. The next day, wash the membrane in TBS 3 times and incubate with IRDye-labeled anti-guinea pig antibody for 1 h at ambient temperature. Then wash the membrane with TBS three times at ambient temperature and then scan and analyze it with an Odyssey imaging system (*see Note 2*).

3.2 Cell-surface GPC expression

1. Follow steps 1–3 of Methods 3.1.
2. 48 h post transfection, the cell medium was aspirated off, and cells were washed once with PBS. Then, the cells were trypsinized with trypsin-EDTA and concentrated by centrifugation at 120g for 5 min. The cells were washed once with PBS
3. Fix the cells with 4% PFA for 5 min. Wash the cells once with PBS and block them with IFA-blocking buffer for one h at 4°C. Then incubate the cells with guinea pig anti-PICV serum over night at 4°C.
4. The following day, wash cells with PBS three times and incubate with Alexa Fluor® 488 dye-labeled goat anti-guinea pig antibody for 1 h at ambient temperature. Wash cells with PBS three times and analyze by FACS (*see Note 3*).

²Western blots were used to monitor PICV GPC expression and processing into GP1 and GP2 (Fig 1). The full-length GPC precursor is approximately 70 kDa, whereas the processed gene products are 37 kDa and 35 kDa, respectively. The higher than expected molecular weight of the proteins based on their amino acid compositions and the fuzziness of the bands are due to different amounts of N-linked glycosylation.

³Flow cytometry was used to detect PICV GP expression on the cell surface (Fig. 2). A typical transfection resulted in 44% of the cells expressing GP on their surfaces.

3.3 GPC fusion assays

3.3.1 eGFP-based assay

1. 293T cells were seeded for 18–24 h before transfection in a 12-well plate at density of 3×10^5 cells/well. 1–2 h before transfection, the medium was replaced by fresh complete DMEM.
2. Prepare DNA and calcium chloride mix as follows:
2 μ l pCAGGS-GPC (1 μ g/ μ l)
1 μ l pCMV-EGFP (1 μ g/ μ l)
5 μ l 2.5 M CaCl_2
42 μ l nuclease-free water
1–2 h after medium replacement, 100 μ l of 2X HBS was dropwise added into each well. The final mix was incubated at ambient temperature for 4 min and then dropwise added into wells. 6 h post transfection, the medium was replaced by a fresh aliquot of complete DMEM and continued to culture in CO_2 incubator at 37°C.
3. 36 h post transfection, replace the medium with acidic DMEM (pH=5.0) (*see* Note 1), incubate cells for 5 min at 37°C, in a CO_2 incubator, and replace with an aliquot of complete DMEM for an additional 12 h of incubation at 37°C.
4. 48 h post transfection, the supernatants were aspirated off, and cells were washed with PBS for one time. 4% PFA was used to fix the cells for 5 mins. Cells were washed with PBS for one time.
5. Cells were analyzed by fluorescence microscopy.

3.3.2 Luciferase based assay

1. 293T cells were seeded into 12-well plates at 3×10^5 cells/well with complete DMEM.
2. In the morning of the day of transfection, culture medium was replaced by an equal aliquot of fresh complete DMEM.
3. Prepare DNA and calcium chloride mix as follows:
2 μ l pCAGGS-GPC (1 μ g/ μ l)
1 μ l pT7-Fluc (100 ng/ μ l)
1 μ l p β -gal (50 ng/ μ l)
5 μ l 2.5 M CaCl_2
41 μ l nuclease-free water
1–2 h after medium replacement, 100 μ l of 2X HBS was dropwise added into each well. The final mix was incubated at ambient temperature for 4 min and then dropwise added into wells. 6 h post transfection, the medium was replaced

by a fresh aliquot of complete DMEM and continued to culture in CO₂ incubator at 37°C for 18 h.

4. 24 h post transfection, 293T cells were mixed with BSR-T7 cells (at 1:1 ratio) in 12 wells and cultured for 12 h. 36 h post transfection, the medium of the mixed cells was replaced with acidic DMEM (pH=5.0) (*see* Note 5.1) and incubated for 5 min and then replaced by fresh complete DMEM for additional 12 h of culturing.
5. 48 h post transfection, the supernatants were aspirated off, and cells were washed once with PBS. Cells were lysed with cell-culture lysis reagent.
6. The firefly luciferase was analyzed by firefly luciferase assay kit according to the manufacturer's instructions. Briefly, 20 µl of cell lysis were mixed with 100 µl of firefly luciferase assay reagent in 96-well plates. The light production was measured by a plate reader (*see* Note 4). To measure the transfection efficiency, 50 µl of cell lysis were mixed with 50 µl of Beta-Galactosidase Assay 2X Buffer for 10 min and the reaction was stopped by adding 150 µl of 1M Tris-HCl (pH=8.0). Use the plate reader to analyze the samples at the absorbance of 420 nm. The fusion activity was calculated by taking the ratio of firefly luciferase over beta-gal activities.

3.4 GPC-mediated virus-like particle (VLP) entry assay

3.4.1 GPC VLP production

1. 293T cells were seeded into 10 cm dish at 3×10^6 cells/well with complete DMEM.
2. In the morning of the day of transfection, culture medium was replaced by an equal aliquot of fresh complete DMEM.
3. Prepare DNA and calcium chloride mix as follow:
 100 µl of DNA mix (for one 6-well plate transfection) contains:
 5 µl pCAGGS-GPC (1 µg/µl)
 10 µl lentivirus packaging plasmid (1 µg/µl)
 15 µl pHR'-eGFP (1 µg/µl)
 50 µl 2.5 M CaCl₂
 420 µl nuclease-free water
 1–2 h after medium replacement, 500 µl of 2X HBS was dropwise added into each well. The final mix were incubated at ambient temperature for 4 min and then dropwise added into wells. 6 h post-transfection, the medium was replaced by fresh complete DMEM and cultured in CO₂ incubator at 37°C for 48 h.

⁴. GPC fusion function was assessed by the observation of syncytia in cell culture. Fig 3A shows eGFP-expressing cellular syncytia formation in GPC transfected cells (right panel) but not in mock transfected cells (left panel).

4. 48 and 72 h post-transfection, supernatants were collected and filtered through a 0.45 µm filter and then concentrated by ultracentrifuge at 140,000g for 2 h. The concentrated VLPs were resuspended in 300 µl PBS and stored at −80°C.

3.4.2 GPC-mediated VLP entry assay

1. 293T cells were seeded in a 12-well plate at 2×10^5 cells/well with complete DMEM.
2. 16–20 h after seeding, the cell medium was replaced with a fresh aliquot of complete DMEM. Concentrated VLP (100 µl) (*see* 3.4.1 above) was mixed with 8 µg polybrene and dropwise added into wells.
3. 6–8 h post transduction, the cell medium was replaced with a fresh aliquot of the complete DMEM.
4. 48 h post transduction, the cell medium was aspirated off, and cells were washed once with PBS. Then, the cells were trypsinized with trypsin-EDTA and concentrated by centrifugation at 120g for 5 min. The cells were washed with PBS for one time and fixed with 4% PFA for 5 min. The cells were washed once with PBS and then analyzed by microscopy (*see* Note 5) or by FACS (*see* Note 6).

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⁵ GPC fusion function was assessed by the detection of luciferase in cells. Fig 3B shows T7 promoter-driven Fluc expression, which indicates the GP-mediated cell-cell fusion between 293T cells and the BSR-T7 cells.

⁶ VLP entry could also be monitored by flow cytometry showing the percentage of eGFP-positive cells (Fig 4B).

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Figure 1. Western blot showing PICV GPC expression and proteolytic processing. Left lane contains cell lysate of the mock transfection with an empty plasmid. Right lane shows the unprocessed GP1/2 (top band) and processed GP1/GP2 (bottom bands).

Figure 2. GPC cellular surface expression analysis.

FACS analysis shows virtually no GPC expression on the surface of the mock-transfected 293T cells as a control (left panel) and 44.1% of GPC expression on the surface of 293T cells transfected with a plasmid expressing the GPC (right panel).

Figure 3A. GPC fusion function.

Right panel shows syncytia of cells expressing eGFP under the condition of GPC gene expression. Left panel is a negative control that shows no evidence of cell-to-cell membrane fusion.

Figure 3B. GPC fusion function.

Luciferase assays show no activity in the empty-plasmid transfected (negative) control cells, whereas high luciferase activity in cells that have been fused as a result of GPC expression.

Figure 4A. GPC-mediated VLP entry.

Fluorescence microscopy shows no eGFP expression in control 293T cells that have been mock-transduced (left), and high eGFP expression in 293T cells that have been infected with VLPs carrying the GPC (right).

Figure 4B. GPC-mediated VLP entry.

FACS analysis shows no eGFP expression in control 293T cells that have been mock-transduced, and high eGFP expression in 293T cells that have been infected with VLPs carrying the GPC.