Chapter 10

Methods for Studying the Function of Cytomegalovirus GPCRs

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Abstract

All of the cytomegaloviruses discovered to date encode two or more genes with significant homology to G-protein coupled receptors (GPCRs). The functions of these cytomegalovirus GPCRs are just beginning to be elucidated; however, it is clear that they exhibit numerous interesting activities in both in vitro and in vivo systems. In this chapter, we review the various methodologies that can be used to examine biochemical aspects of viral GPCR signaling in vitro as well as examine the biological activity of these viral GPCRs in vitro and in vivo in virus infected cells using recombinant cytomegaloviruses.

Key words G-protein coupled receptors, Human cytomegalovirus, Murine cytomegalovirus, Virus genetics, Signal transduction, Virological methods

1 Introduction

Human cytomegalovirus (HCMV) is a betaherpesvirus that infects a large majority of the world's population. Infection with HCMV in utero is the leading cause of infectious congenital birth defects in developed countries, resulting in developmental disabilities. Although infection with the virus remains, for the most part, asymptomatic in healthy individuals, immunocompromised individuals who undergo viral reactivation or receive a primary infection suffer from severe morbidity and often mortality as a direct consequence of HCMV-associated disease [1].

Sequence analysis of the HCMV genome reveals that this virus encodes at least four G protein-coupled receptors (GPCRs), including UL33, UL78, US27, and US28 [2, 3]. GPCRs are cell surface molecules that contain seven transmembrane domains and function in signal transduction [4]. The binding of an appropriate ligand to a given GPCR activates the receptor while dissociation of the ligand converts the GPCR to an inactive state. Some GPCRs exhibit significant activity in the absence of a bound ligand and in this case the signaling is termed constitutive.

Andrew D. Yurochko and William E. Miller (eds.), *Human Cytomegaloviruses: Methods and Protocols*,
Methods in Molecular Biology, vol. 1119, DOI 10.1007/978-1-62703-788-4_10, © Springer Science+Business Media New York 2014

When in their active state, GPCRs induce a variety of signal transduction pathways that alter the cellular environment by activating molecules involved in adhesion, migration, proliferation, differentiation, cytoskeletal dynamics, contractility, etc. [5]. Both the primate (i.e., human, rhesus) and non-primate (i.e., murine, rat) CMVs encode members of the UL33 and UL78 family, whereas only the primate CMVs additionally encode US27 and US28 [6].

Cytomegalovirus encoded GPCRs have been demonstrated to respond to external ligands and/or signal constitutively (some examples are provided with references—HCMV US28 [7–11], HCMV UL33 [12], Rat CMV (RCMV) R33 [12, 13], and Murine CMV (MCMV) M33 [9, 14]). The murine UL33 orthologue M33 contributes in vivo to pathogenesis, as assessed by a requirement of the GPCR and its constitutive signaling for viral replication within the host's salivary glands [15–18]. Similar results were demonstrated with the RCMV UL33 orthologue, R33 [19]. Although members of the UL33 gene family are required for pathogenesis in vivo; UL33, R33, and M33 are dispensable for replication in fibroblasts [20, 21]. Similar to UL33, HCMV UL78 has orthologues across the betaherpesvirus family. MCMV M78 is a virion constituent, and upon infection of host cells, promotes immediate early (IE) viral mRNA accumulation [22]. Infection of the respective host with a virus harboring a deletion of M78 or R78 decreases viral titers in the spleen, liver, and salivary glands, while increasing the survival rates in these animals [22, 23], suggesting a role for these GPCRs in viral pathogenesis in both the mouse and rat CMV models. HCMV UL78 is assembled into the mature viral particle [24], and although not essential for efficient viral replication in fibroblasts [24, 25] or in a renal artery tissue culture model [25], it is critical for replication in epithelial and endothelial cells [24]. In epithelial cells, UL78 is necessary for appropriate delivery of the viral particle to the nucleus [24]. Recent investigations into the role of US27 during viral infection have revealed that this protein is important for efficient spread of HCMV via the extracellular route in both endothelial cells and fibroblasts [26]. Currently UL78 and US27 signaling (constitutive and/or ligand-induced), chemokine interaction(s), and natural ligand(s) remain unknown.

Arguably the most-studied HCMV GPCR is encoded by US28. US28 exhibits both constitutive and agonist-dependent signaling and has been demonstrated to bind numerous C–C chemokines (RANTES, MIP-1α, MIP-1β, and MCP-1) and the CX3C-chemokine fractalkine [7, 8, 10, 27–29]. US28 constitutive signaling is exemplified by its ability to activate phospholipase C–β in the absence of ligand, while its agonist-dependent signaling is exemplified by its ability to modulate Ca^{2+} flux and direct vascular smooth muscle cell migration [7, 10, 11, 30–32]. HCMV US28 appears to be "promiscuous" in its G-protein coupling as it is able to activate

either $G_{q/11}$or $G_{12/13}$ G proteins, or both [7, 32–34]. As a consequence of this G-protein signaling, US28 is able to influence the activity of a variety of downstream effectors such as NFκB, NFAT, CREB, MAPK, Rho, and STAT/IL6 [9, 33, 35–38]. In addition to its involvement in cell migration, HCMV US28 has been shown to have oncogenic potential. Expression of US28 enhances cell growth and cell cycle progression, and induces a pro-angiogenic and transformed phenotype in vitro [39, 40]. In vivo, injection of NIH3T3 fibroblasts expressing US28 into nude mice does indeed promote tumorigenesis, possibly via COX-2 up-regulation [40]. More recently, investigators have shown that the HCMV US28 RNA is found in both glioblastomas [36] and medulloblastomas [41], and in the former, promotes an invasive and angiogenic phenotype [36]. Taken together, these results argue for a role of US28 as a viral oncogene.

This chapter focuses on recent advancements in methodologies used for studying the function of CMV GPCRs, emphasizing protocols that can be performed in the context of infection. In particular, we will address methods for examining viral GPCR signaling by transient assays and focus on the recent breakthroughs in generating viral mutants making possible the investigation of GPCR function in the context of infection. Finally, we will discuss the importance of animal models to our understanding of the CMV GPCRs in viral pathogenesis.

2 Materials

2.1 Cell Culture

1. Primary human fibroblasts, such as HS68 cells (ATCC, CRL-1635) used between passages 10 and 20 (*see* **Note 1**).
2. Human embryonic kidney cell line HEK-293 (ATCC, CRL-1573).
3. Primary human retinal pigment epithelial cells (ARPE19 [ATCC, CRL-2302]) used between passages 22 and 35.
4. Human umbilical vascular endothelial cells (HUVECs) isolated from umbilical cords by collagenase digestion (or purchased from Lonza) maintained on either Primaria tissue culture plates (BD Falcon) or plates pre-coated with 0.1 % pig gelatin (Sigma) in 1× PBS, and used between passages 2 and 8.
5. Dulbecco's modified eagle medium (DMEM) containing 10 % Fetal Clone III serum (Hyclone), and supplemented with 100 U/ml each of penicillin and streptomycin is used to culture primary human fibroblasts.
6. Minimal essential medium MEM containing 10 % fetal bovine serum (Hyclone), and supplemented with 100 U/ml each of penicillin and streptomycin is used to culture HEK-293 cells.

7. DMEM-HAM's F12 containing 10 % FBS, 2.5 mM L-GLUTAMINE, 0.5 mM sodium pyruvate, 15 mM HEPES, 1.2 g/l $NaHCO_3$, and 100 U/ml each of penicillin and streptomycin is used to culture ARPE19 cells.
8. EGM-2 medium supplemented with the EGM-2 additives (Lonza) is used to culture HUVECs.
9. Trypsin–EDTA: 0.05 % Trypsin, 0.53 mM EDTA.
10. Transfection reagent (e.g., Mirus TransIT® LT1 or Invitrogen Lipofectamine 2000).

2.2 Assessing PLC-β Activity by Measuring IP_3 Accumulation

1. Wash dowex in formate phase (AG1-X8, Bio-Rad 140-1444) with 20 l dH_2O. Store as 50 % slurry in dH_2O at 4 °C. Add 1 ml of slurry to column prior to use. A variety of reusable columns can be used. Bio-Rad Poly-Prep® chromatography columns (part no. 731-1550) work well.
2. [2-^{3}H(N)]-myo-inositol (PerkinElmer, NET-114A).

2.3 Cell Lysis

1. Standard lysis buffer: 50 mM Hepes, pH 7.4, 0.5 % NP-40, 250 mM NaCl, 10 % Glycerol, 2 mM EDTA, 1 mM PMSF, 2.5 μg/ml aprotinin, 5.0 μg/ml leupeptin, 200 μM activated sodium orthovanadate, 1 mM Sodium Fluoride (*see* **Note 2**).
2. RIPA lysis buffer: 10 mM Tris pH 7.5, 0.1 % SDS, 1.0 % Triton X-100, 1.0 % deoxycholate, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2.5 μg/ml Aprotinin, 5.0 μg/ml Leupeptin, 200 μM activated sodium orthovanadate, 1 mM sodium fluoride.
3. Laemmli Sample Buffer: 14.0 ml 4× Tris Stacking buffer pH 6.8, 14.4 ml 50 % Glycerol, 2.0 g SDS, 240 μl beta-mercaptoethanol, 9.4 ml dH_2O.
4. 10× Red Blood Cell (RBC) Lysis Buffer: 40.15 g NH_4Cl, 5.0 g $NaHCO_3$, 0.186 g EDTA in 200 ml dH_2O. Dilute RBC Lysis buffer to 1× prior to use.

2.4 Western Blotting

1. Tris buffered saline containing Tween-20 (TBST): 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20.
2. Blocking Buffer: TBST containing 5 % nonfat dried milk.
3. Phospho-p38 MAPK (Thr180/Tyr182) Antibody, Cell Signaling Technology, Cat#9211.
4. Supported nitrocellulose (e.g., Schleicher and Schuell).
5. Chemiluminescence Detection Kit (e.g., Amersham ECL or Pierce SuperSignal western blotting kits).
6. Infrared Detection Kit (e.g., Li-Cor Odyssey western blotting kits).

2.5 Luciferase Reporter Assays

1. Dual-Luciferase Reporter Assay System, Promega, Cat#E1910.

2.6 BAC Recombineering

1. 0.2 mg/ml D-biotin: sterile filtered, made fresh.
2. 20 % galactose: autoclaved, stored at 4 °C.
3. 20 % 2-deoxy-galactose: autoclaved, made fresh.
4. 20 % glycerol: autoclaved, stored at room temperature.
5. 10 mg/ml L-leucine: Heat to get into solution but do not let boil. Sterile filtered, stored at 4 °C.
6. 12.5 mg/ml chloramphenicol in EtOH, stored at −20 °C.
7. 1 M $MgSO_4 \cdot 7H_2O$ stored at room temperature.
8. 1× M9 medium (1 l, autoclaved, stored at room temperature): 42.3 mM Na_2HPO_4 (6 g/l), 22 mM KH_2PO_4 (3 g/l), 18.7 mM NH_4Cl (1 g/l), 8.6 mM NaCl (500 mg/l).
9. 5× M63 (1 l, autoclaved, stored at room temperature): 75.5 mM $(NH_4)_2SO_4$ (10 g/l), 0.5 M KH_2PO_4 (68 g/l), 9.0 μM $FeSO_4 \cdot 7H_2O$ (2.5 mg/l). Adjust to pH 7 with KOH.
10. M63 minimal plates (500 ml makes 20–25 plates): 7.5 g agar in 400 ml ddH_2O in a 500 ml bottle with a stir bar and autoclaved, cooled to a “touchable” temperature of ~50–55 °C, 100 ml 5× M63 medium, 500 μl 1 M $MgSO_4 \cdot 7H_2O$ (1 μM), 500 μl chloramphenicol (12.5 μg/ml), 2.5 ml biotin (0.5 mg) (*see* **Note 3**), 5 ml galactose (0.2 %), 2.25 ml leucine (45 mg).
11. 2-DOG plates (500 ml makes 20–25 plates)
    7.5 g agar in 400 ml H_2O in a 500 ml bottle with a stir bar and autoclaved, cooled to a “touchable” temperature of 50–55 °C, 100 ml 5× M63 medium, 500 μl 1 M $MgSO_4 \cdot 7H_2O$ (1 μM), 500 μl chloramphenicol (12.5 μg/ml), 2.5 ml biotin (0.5 mg) (*see* **Note 3**), 5 ml 2-deoxy-D-galactose (0.2 %), 2.25 ml leucine (45 mg), 5 ml glycerol (0.2 %).
12. MacConkey indicator plates: Prepare MacConkey agar plus galactose according to manufacturer’s instructions (e.g., BD, Cat#281810), 12.5 μg/ml chloramphenicol.
13. PCR cleanup columns (e.g., GE Healthcare GFX columns).

2.7 Purification of BAC DNA

1. CMPS1 [Similar to Qiagen P1 buffer, +RNAse]: 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, pH 8.0, 200 μg/ml RNAse A added *just prior to use* (20λ of 10 mg/ml stock, per 1.0 ml CMPS1).
2. Alkaline SDS Solution [Similar to Qiagen P2 buffer]: Final concentrations: 0.2 N NaOH, 1 % SDS. Make stock solutions at 2× concentrations, so mix equal parts *just prior to use*.
3. TEN Solution: 10 mM Tris–HCl, pH 7.4 or pH 8.0, 1 mM EDTA, pH 8.0, 150 mM NaCl.

4. 10.1 TE Solution: 10 mM Tris–HCl, pH 7.4 or pH 8.0, 0.1 mM EDTA, pH 8.0.
5. Endotoxin Removal Kit: Sigma, Cat#E4274.
6. Column BAC purification kit (e.g., Machery-Nagel Nucleobond BAC purification kit).

2.8 Plasmids

1. pcDNA3 (or similar) vector for viral GPCR of choice (HCMV-US27, HCMV-US28, MCMV-M33, etc.).
2. pcDNA3 (or similar) vector for pp71.
3. pGL3 3× MHC-Luc (or similar) to assess NFκB activity.
4. pGL3 9× NFAT-LUC (or similar) to assess NF-AT activity.
5. pFRLUC and pFA2CREB to assess CREB activity (Stratagene).
6. pHRG-TK to control for transfection variation and generalized effects of viral GPCRs on basal transcriptional activity (Promega).

2.9 Miscellaneous

1. Anti-fade mounting medium (e.g., Vector Labs VECTASHIELD or Molecular Probes SlowFade).
2. Biotinylated anti-FLAG antibody (e.g., Sigma M2 biotinylated anti-FLAG).
3. Cell Surface Protein Isolation kit (Thermo Scientific).

3 Methods

While it is not possible to generalize the signaling activities of the cytomegalovirus GPCRs into a single pathway, it is clear that at least several of these receptors (US28/M33/R33) signal via G-proteins such as $G_{q/11}$ and drive a number of downstream signals including accumulation of the second messenger IP_3, activation of protein kinases, and stimulation of transcription factor activity. In this section, we will describe basic methodology that can be used to assess these particular signaling activities.

3.1 Measuring Viral GPCR Stimulated Inositol Triphosphate (IP_3) Accumulation

The following protocol was designed for the study of HCMV US28 stimulated IP_3 accumulation (a.k.a. PIP_2 hydrolysis, PLC activity, inositol triphosphate accumulation) in HCMV infected fibroblasts or in transiently transfected HEK-293 cells, but can easily be adapted for use in a number of different cell types and can be modified to study other cytomegalovirus GPCRs in conditions of virus infection or transient transfection [7, 9, 10, 32, 42–44]. In the case of virus infection, the methodology described uses the primary human fibroblast cell line, HS68 (ATCC, CRL-1635) and in the case of transient transfection, the methodology described uses the embryonic kidney cell line HEK-293 (ATCC, CRL-1573). $G_{q/11}$ stimulated PLC-β activity cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3)

and diacylglycerol (DAG). Accumulation of IP_3 is easily measured in the lab with standard equipment and reagents.

1. Plate the cells into 12-well culture plates so that they will be ~75 % confluent at time of plating. Incubate in humidified incubators at 37 °C and 5 % CO_2.
   (a) For infection of HS68 fibroblasts with HCMV, the suggested cell number is ~100,000 cells per well in a total volume of 1 ml of medium.
   (b) For transient transfection of HEK-293 cells, the suggested cell number is ~250,000 cells per well in a total volume of 1 ml medium. HEK-293 cells and their derivatives are not tightly adherent, and care should be taken to facilitate adherence, such as coating the culture wells with 5 mg/ml collagen prior to plating cells.
2. Let the cells adhere overnight.
3. Infect with virus or transfect with appropriate viral GPCR expression construct. The length of time to let the infection or transfection proceed prior to harvesting should be determined empirically depending on timing of viral GPCR expression, etc. In the case of HCMV infection experiments, US28 reaches maximal expression at approximately 48 h post-infection (hpi), and therefore, 48 h would be an appropriate time to analyze US28 dependent IP_3 accumulation. Similarly, 48 hpi is a typical time at which to analyze transfection experiments as this is the time at which most transient gene expression peaks.
   (a) Infection of HS68 fibroblasts with HCMV. Adsorb virus to cells at appropriate multiplicity of infection (MOI) for 3–6 h. Both wild-type and ΔUS28 strains should be used to ascertain the specific effects of US28 on driving the activation of this signaling pathway. To achieve roughly 95–99 % infection, an MOI of 3–5 should be chosen. At the end of the adsorption period, remove the medium containing virus and feed with fresh medium.
   (b) Transient transfection of HEK-293 cells. The following describes the amount of DNA and lipid required for each well of a 12-well plate, although the amounts can be scaled up or down depending on the scale of transfection required. 250 ng of plasmid DNA is diluted in 50 μl serum free medium, supplemented with 1 μl of Mirus TransIT® LT1 transfection reagent and incubated for 15 min at room temperature. The 50 μl transfection reaction is then transferred to the appropriate wells of a 12-well plate and the transfection is allowed to proceed for 6 h. At the end of the 6 h incubation, remove the medium containing DNA/ transfection reagent and feed with fresh medium. The TransIT® LT1 transfection reagent is highly efficient and

exhibits low toxicity and therefore can be left on the cells overnight if desired. The viral GPCRs themselves are somewhat toxic in nature and thus should be tested at various concentrations (i.e., 10, 50, and 250 ng of DNA per well). All transfections should contain a total of 250 ng plasmid DNA, so in cases where less than 250 ng of viral GPCR DNA is used, the transfection cocktail should be supplemented with an empty vector. It is recommended that the experiments be performed in duplicate or triplicate.

4. The next day (18–24 h post-infection/transfection), aspirate the medium and add 1 ml/well of fresh medium containing 1.0 μCi/ml [2-^{3}H(N)]-myo-inositol. The concentration of myo-inositol can be increased if necessary and the cells can be labeled in either serum free medium or serum containing medium (*see* **Note 4**).
5. The following day (approximately 40–48 h post-infection/transfection, wash the cells 1× with 1 ml serum free medium.
6. Feed the cells with 1 ml serum free medium containing 20 mM LiCl. If using chemokines or other potential agonists, add simultaneously with serum-free medium containing LiCl. The LiCl inhibits endogenous inositol phosphatase activity and enables newly produced, receptor stimulated IP_3 to accumulate.
7. Let inositol phosphates accumulate for 2–3 h.
8. Stop the reaction by aspirating medium, adding 1 ml of 0.4 M perchloric acid per well, and incubating for 15 min in the cold room. The perchloric acid will not cause the cells to lift off, but the perchloric acid at this point will contain the accumulated IPs.
9. Transfer 800 μl of perchloric acid from each well to a microfuge tube containing 400 μl of 0.72 M KOH/0.6 M $KHCO_3$. This will form a white fluffy precipitate.
10. Vortex and centrifuge for 1 min at 15,000×*g*.
11. Transfer 50 μl of supernatant to scintillation vials, add 10 ml scintillation fluid, and count. (This step is optional and may be used to internally control for the relative labeling and cell number used.)
12. Transfer 1 ml of the supernatant to Fisherbrand 12×75 mm glass tubes containing 3 ml dH_2O.
13. Prepare dowex columns by adding 1 ml of dowex slurry (described in Subheading 2) and let settle. A variety of reusable columns can be used for this step.
14. Pour sample from **step 12** over column. Let sample flow through.
15. Wash the columns with 2 bed volumes (~25 ml) of dH_2O.

16. Wash the columns with 1 bed volume of 60 mM sodium formate/5 mM disodium tetraborate.
17. Elute bound IPs. Each of the following elution steps should be performed by transferring the chromatography column into a fresh scintillation vial prior to elution (*see* **Note 5**).
    (a) Elute IP_1 with 4 ml of 0.2 M ammonium formate/0.1 M formic acid. Wash the column with 1 bed volume of the same buffer.
    (b) Elute IP_2 with 4 ml of 0.4 M ammonium formate/0.1 M formic acid. Wash the column with 1 bed volume of the same buffer.
    (c) Elute IP_3 with 4 ml of 0.8 M ammonium formate/0.1 M formic acid.
18. Add 10 ml scintillation fluid to each eluted sample. Count in scintillation counter.

3.2 Measuring Viral GPCR Stimulated Protein Kinase Activation

The following protocol is specifically designed for the detection of US28 or M33 stimulated p38-MAPK kinase activation in transiently transfected HEK-293 cells, but can easily be adapted for use in a number of different cell types or for different protein kinases [38, 44, 45]. Moreover, the protocol can be modified to study cytomegalovirus GPCRs in conditions of virus infection or transient transfection. The protocol takes advantage of phospho-specific antibodies (which recognize activated forms of protein kinases) to assess viral GPCR mediated activation of the protein kinase in question.

1. Plate HEK-293 cells into 12-well culture plate so that they will be ~75 % confluent at time of plating. Incubate in humidified incubators at 37 °C and 5 % CO_2. The suggested cell number is ~250,000 cells per well in a total volume of 1 ml medium. HEK-293 cells and their derivatives are not tightly adherent, and care should be taken to facilitate adherence, such as coating the culture wells with 5 mg/ml collagen prior to plating cells.
2. Let the cells adhere overnight.
3. Transfect with appropriate viral GPCR expression construct as described above in Subheading 3.1 **step 3b**.
4. Forty-eight hour post-transfection, lyse the cells and prepare protein extracts for gel electrophoresis. Protein extracts can be prepared using several different lysis buffers, depending on the preference of the investigator (see Subheading 2). Extracts prepared directly in Laemmli sample buffer (**step 4a**) maintains the phosphorylation status of most kinases. However, the use of this buffer eliminates the possibility of quantifying protein concentrations and therefore requires accurate cell counts prior to preparation of the extracts. When extracts are prepared

in standard lysis buffer (**step 4b**), one must ensure that phosphatase activity does not affect the results of the experiments. In particular, it is important to use NaF and activated Na_3VO_4 in lysis buffers to inhibit serine/threonine and tyrosine phosphatases respectively (*see* **Note 2**).

(a) To prepare whole cell extracts directly in Laemmli sample buffer, the medium is aspirated from the 12-well plates, 250 μl of Laemmli sample is added directly to the wells, the wells are scraped briefly with a cell scraper, and the extracts are transferred to microcentrifuge tubes. The extracts are sonicated briefly to disrupt chromosomal DNA.

(b) To prepare whole cell extracts in standard lysis buffer, the medium is aspirated from the 12-well plates, the wells are washed 1× with 1× PBS, and 250 μl of standard lysis buffer is added directly to the wells. The wells are scraped briefly with a cell scraper, the extracts are transferred to microcentrifuge tubes, and incubated on ice for 15–30 min. The extracts are clarified by centrifugation at 12,000 × *g* and supernatant is transferred to a fresh tube. Protein concentration is then quantified by standard protein assays (Bradford, Bio-Rad Protein Assay, etc.).

5. The extracts prepared by either procedure in **step 4** are then subjected to SDS-PAGE using standard protocols for gel electrophoresis.
6. Transfer resolved proteins to supported nitrocellulose membranes and block nonspecific reactivity with Tris-buffered saline containing 0.1 % Tween 20 (TBST) and 5 % nonfat dried milk. In some cases, blocking with 5 % nonfat dried milk can increase nonspecific reactivity due to the presence of phosphoproteins present in the milk. In this case, 1 % bovine serum albumin (BSA) can be used as a substitute for the milk.
7. Antibody directed against the phosphorylated/activated form of the protein kinase of interest is then used to probe western blots. In the case of p38-MAPK, the anti-phosphospecific p38 antibody is diluted 1:1,500 in TBST. Bound primary antibody is then detected using the appropriate secondary antibodies using enhanced chemiluminescence or infrared fluorescence systems.

3.3 Measuring Viral GPCR Stimulated Transcription Factor Activity

The cytomegalovirus GPCRs have been reported to activate a number of transcription factors including NFκB, CREB, and NFAT [7, 12, 16, 18, 38]. The following protocol is specifically designed for the detection of US28 or M33 stimulated transcriptional reporter activity in transiently transfected HEK-293 cells, but can easily be adapted for use in a number of different cell types or transcription factors and can be modified to study other cytomegalovirus GPCRs in conditions of virus infection or transient transfection. Two important considerations should be taken into

account when assessing viral GPCR stimulated transcription factor activity in infected cells. First, it is important to use wild-type and viral GPCR null mutants (i.e., ΔUS28 mutants) to differentiate between viral GPCR effects and those due to either the virion itself or other cytomegalovirus proteins. Many of the transcription factors stimulated by the viral GPCRs are in fact activated during cytomegalovirus infection, but it is clear that the viruses use multiple mechanisms to activate transcription factors at different stages of infection. Such is the case for NFκB, which is activated within minutes after virion binding, presumably due to virus engagement of NFκB linked cell surface receptors and also during the IE and E phases of infection. Second it is also important to use internal controls such as the pHRG-TK Renilla luciferase control reporter. This will allow the investigator to control for generalized effects of cytomegalovirus infection on the basal transcription machinery itself, which can lead to artifactual conclusions regarding specific changes in transcription factor activity.

1. Plate HEK-293 cells into 12-well culture plate so that they will be ~75 % confluent at the time of plating. Incubate in humidified incubators at 37 °C and 5 % CO_2. The suggested cell number is ~250,000 cells per well in a total volume of 1 ml medium. HEK-293 cells and their derivatives are not tightly adherent, and care should be taken to facilitate adherence, such as coating the culture wells with 5 mg/ml collagen prior to plating cells.
2. Let the cells adhere overnight.
3. Transfect with appropriate viral GPCR expression constructs and reporter genes. For transient transfection of HEK-293 cells in one well of a 12-well culture plates, 250 ng of plasmid DNA is diluted in 50 μl serum free medium, supplemented with 1 μl of Mirus TransIT® LT1 transfection reagent and incubated for 15 min at room temperature. The 50 μl transfection reaction is then transferred to cells plated as described in **step 1** above and the transfection is allowed to proceed for 6 h. At the end of the 6 h incubation, remove the medium containing DNA/transfection reagent and feed with fresh medium. The TransIT® LT1 transfection reagent is highly efficient and exhibits low toxicity and therefore can be left on the cells overnight if desired. The viral GPCRs themselves are somewhat toxic in nature and thus should be tested at various concentrations (i.e., 10, 50, and 250 ng of DNA per well). The concentration of the reporter gene DNA per well is as follows: for assessing NFκB activity (15 ng of pGL3-3X MHC-Luc), for assessing NF-AT activity (15 ng of pGL3-9X NFAT-Luc) and for assessing CREB activity (30 ng pFR-LUC/10 ng pFA2-CREB) (*see* **Note 6**). The control pHRG-TK renilla luciferase plasmid should be included in all transfections at a concentration of 15 ng per well. All transfections should contain a total of 250 ng plasmid DNA, so in cases where less than 250 ng of

viral GPCR DNA is used, the transfection cocktail should be supplemented with an empty vector. To control for transfection variability, it is recommended that the experiments be performed in duplicate or triplicate.

4. Forty-eight hour post-transfection, aspirate medium and wash wells with PBS.
5. Add 200 μl of 1× Passive Lysis Buffer (PLB) per well (*see* **Note 7**). Incubate for 15–30 min at room temperature. Transfer lysate to microcentrifuge tubes. The samples can be stored at −80 °C at this point.
6. Experimental luciferase (firefly) and control luciferase (renilla) can be examined on a luminometer using Luciferase Assay Reagent II (LAR II) and Stop&Glo Reagent according to the manufacturer's instructions (*see* **Note 7**).

These techniques provide the basis from which to examine proximal (IP_3 accumulation), intermediate (p38-MAP kinase), and distal (transcription factor) signaling activity emanating from cytomegalovirus encoded GPCRs. The number of antibodies that recognize phosphorylated and thus activated protein kinases is increasing at a rapid pace, thus enabling researchers to continue to explore a variety of signaling pathways that lie downstream of the viral GPCRs. There are numerous other methodologies that have been used to examine viral GPCR signaling; however, space constraints simply prevent us from covering each of these techniques in detail. For example, Smit and colleagues have used limited microarray analyses to uncover genes upregulated in response to US28 expression, and data mining approaches could easily be combined with large scale gene expression studies to identify networks of signaling pathways downstream of the viral GPCRs [40]. Finally, several investigators have used pharmacological inhibitors of signaling proteins such as PLC-β, PKC, PI3-K, etc. to identify additional signaling proteins downstream of the viral GPCRs [9, 18, 38–40, 46]. It is important to note that most of the signaling information that we currently have regarding the viral GPCRs has been generated in in vitro transfection/overexpression systems and it will be essential to use this current knowledge and extend these studies to identify what signals are truly generated in cytomegalovirus infected cells.

3.4 Generating and Analyzing Recombinant Cytomegaloviruses with Mutant GPCRs

Although significant advancements in our understanding of cytomegalovirus GPCRs have been made using the transient systems described above, recombineering methodologies have made possible the ability to investigate the CMV GPCRs in the context of viral infection.

3.4.1 BAC Recombineering of GPCR Genes

Early work aimed at generating recombinant viruses with mutations in viral GPCR genes took advantage of homologous recombination in mammalian cells, whereby a selectable marker was introduced by site-directed mutagenesis (Table 1). This method proved laborious and inefficient, and for some genes, impossible. Open reading frames (ORFs) that were essential for growth or those that conferred a severe growth defect when compared to wild-type, could only be mutated by this method if they were generated on complementing cell lines, which expressed the ORF of interest in *trans* [47]. Fortunately RCMV, RhCMV, MCMV, guinea pig CMV (GPCMV), and a variety of laboratory and clinical HCMV strains have been cloned into bacterial artificial chromosomes (BACs). Original protocols for BAC recombineering generated recombinant viral BACs that remained “marked” with either an insertion cassette or partial sequence from the shuttle plasmids used for RecE/T-mediated recombineering techniques [48]. Thus, this procedure does not yield seamless recombinants, which complicates the generation of viral BACs with multiple mutations and/or tags as well as revertants.

The field of bacterial recombineering has advanced greatly over the recent years, and researchers have adapted these methods for generating recombinant CMV BACs to study the function of the CMV GPCRs (Table 1). In particular, two methods including *galK* [49] and I-SceI [50] recombineering have proved extremely useful, as each of these protocols results in recombinant viral BAC DNA that is seamless at the site of recombination. The advantage of seamless recombineering is such that one can generate multiple site-specific mutations, epitope tags, fusion proteins, gene insertions, or whole ORF deletions within a single background. Additionally, these recombineering protocols are more efficient than previous BAC-mediated methods or site-directed mutagenesis in mammalian cells, have lower rates of off-site spontaneous recombination, require less time to generate mutants, permit reversion of the mutation, and unlike homologous recombination in mammalian cells, support the mutagenesis of essential ORFs. *GalK* and I-SceI recombination each take advantage of the Red recombinase system [49, 50]. The I-SceI method has previously been described for the study of HCMV GPCRs, and thus this chapter focuses on the utilization of the *galK* recombineering system [46]. Recombineering by *galK* uses a straightforward methodology, which involves a positive selection of the *galK* insertion cassette, followed by homologous recombination of either a double-stranded oligonucleotide or a purified PCR product by counterselection.

1. PCR amplify the galactokinase (*galK*) gene using no more than 2 ng of pGalK plasmid as the template with primers that contain a minimum of 50 bp of homologous sequence to the intended site of mutagenesis within the BAC. The underlined sequences below are complimentary to the pGalK cassette:

Table 1
Cytomegalovirus GPCR recombinant virus constructs

Viral GPCR	Host	Strain	Recombination method
M33	Murine	K181	Homologous recombination in mammalian cells [15–17, 65]
M33		K181	Kan-*frt* BAC [18]
M78	Murine	K181	Homologous recombination in mammalian cells [22]
R33	Rat	Maastricht	Homologous recombination in mammalian cells [19, 74]
R78	Rat	Maastricht	Homologous recombination in mammalian cells [23, 74]
UL33	Human	AD169	Kan-*frt* BAC [12, 21], homologous recombination in mammalian cells [20], *galK* BAC [21]
		FIX	Kan-*frt* BAC, *galK* BAC [21]
		TB40/E	Kan-*frt* BAC, *galK* BAC [21]
UL78	Human	AD169	Kan-*frt* BAC [21], *galK* BAC [21], pST shuttle vector BAC [25]
		FIX	Kan-*frt* BAC [24], *galK* BAC [24]
		TB40/E	Kan-*frt* BAC [24], *galK* BAC [24]
US27	Human	AD169	Homologous recombination in mammalian cells [28]
		AD169	Kan-*frt* BAC [21], *galK* BAC [21]
		FIX	Kan-*frt* BAC [26], *galK* BAC [26]
		TB40/E	Kan-*frt* BAC [26], *galK* BAC [26]
US28	Human	AD169	Homologous recombination in mammalian cells [28]
		Toledo	Homologous recombination in mammalian cells [31]
		Towne	Homologous recombination in mammalian cells [29]
		AD169	Kan-*frt* BAC, *galK* BAC [21], pST shuttle vector BAC [10, 42]
		FIX	Kan-*frt* BAC [32, 43], *galK* BAC [21]
		TB40/E	Kan-*frt* BAC [21], *galK* BAC [75]
		Titan	Kan-*frt* BAC [35, 40]
US27:US28 multiple deletion mutant	Human	AD169	Homologous recombination in mammalian cells [28]
UL33:UL78:US27:US28 multiple deletion mutant	Human	AD169	I-SceI BAC [46]
		FIX	*galK* BAC [21]
		TB40/E	*galK* BAC [75]

Forward Primer: 5′–50′ bp homology-CCTGTTGACAATTAATCATCGGCA-3′

Reverse Primer: 5′–50′ bp complimentary strand homology-TCAGCACTGTCCTGCTCCTT-3′

(a) To remove the pGalK template, digest the PCR product by adding 1 μl of DpnI directly into the PCR reaction and incubate at 37 °C for 1 h.

(b) Use PCR cleanup columns to purify the PCR product and elute the PCR product in 25 μl of dH_2O.

2. This cassette is then inserted into the CMV BAC genome by homologous recombination mediated by heat shock induced Red recombinase enzymes. It is critical to maintain the BAC at 32 °C in a recombination competent bacterial strain, such as SW102, SW105, or SW106 all of which are *galK*⁻.

   (a) Following overnight culture of the BAC-containing bacteria at 32 °C in 5–10 ml of medium containing 12.5 μg/ml chloramphenicol, inoculate 25 ml of medium containing 12.5 μg/ml chloramphenicol with a 1:50 dilution of the overnight culture. Grow the bacteria to an OD_{600} of 0.5–0.6. Heat-shock the cells at 42 °C for 15 min in a shaking water bath.

   (b) Quickly cool the bacteria in an ice bath slurry, with shaking, then transfer 10 ml of the culture to a pre-chilled conical and pellet the bacteria at 4 °C. Gently resuspend the pellet in 1 ml ice-cold ddH_2O and transfer to a microcentrifuge tube. Wash the cell pellet three additional times in ice-cold ddH_2O, and resuspend the pellet after the final wash in 100 μl of ice-cold ddH_2O.

   (c) Use 2.5 μl of the PCR product to transform 50 μl of the bacteria by electroporation in a pre-chilled 2 mm gap electroporation cuvette. Recover for 1 h in 1 ml of medium without antibiotic at 32 °C, and then wash the pellet three times in M9 salts taking care not to vortex or pipette too harshly. Resuspend the final pellet in 1 ml M9 salts.

3. Plate 100 μl of undiluted cells and 100 μl of a 1:10 dilution onto M63 minimal medium plates and incubate at least 3 days at 32 °C. BACs that recombine to express *galK* are chosen by positive screen for growth on minimal medium containing galactose as the sole carbon source. Although all colonies that grow on these plates should ideally contain an integrated *galK* cassette, these plates screen for the preferential growth of potentially successful recombinants.

4. Successful *galK* recombinants are further selected using MacConkey's indicator plates containing galactose, on which *galK*-positive clones will grow as single red colonies, while *galK*-negative clones will grow as white colonies. This step is

critical to ensuring that the clones used in the counter-selection step do indeed contain the *galK* cassette.

5. Patch single red colonies from **step 4** onto LB/chloramphenicol plates and confirm the insertion of the *galK* cassette at the proper location of interest by PCR using flanking primer sets.
6. Next, counterselect against *galK*, by substituting either a PCR product or a double stranded oligo that contains the mutation or epitope tag of one's choice, all of which also contain flanking arms to the region in the BAC DNA being mutated. In this step the PCR product or double stranded oligo is inserted into the CMV BAC genome by homologous recombination mediated by red recombinase as described in **step 2**, with slight modification.
   (a) Prepare competent bacteria as described above (*see* Subheading 3.4.1, **steps 2a** and **2b**).
   (b) Mix 10 mg of each oligo in a volume of 100 ml 1× PCR buffer. Boil for 5 min and cool slowly to room temperature. EtOH-precipitate the annealed oligos and resuspend the final pellet in 100 μl ddH_2O to yield a final concentration of 200 ng/ml. Use 1 μl per transformation. For reversion using PCR products, PCR amplify the desired insert with at least 50 bp of flanking sequence to the site of recombination, generating a PCR product of ~1,000 bp. If the product is larger, increase the size of the flanking sequence. For example, use 500 bp for products >2 kb.
   (c) Following transformation, recover the bacteria in 10 ml medium without antibiotic in a 100 ml baffled flask for 4.5 h in a 32 °C shaking incubator. Remove 1 ml of the culture and wash with ice-cold ddH_2O in a refrigerated microcentrifuge as above in **step 2c**. The cells are diluted and plated (as described in **step 3**) on 2-deoxy-D-galactose (2-DOG) plates. Incubate the plates for 3–4 days at 32 °C.
7. Selection against *galK* involves resistance to 2-DOG on minimal plates with glycerol as the carbon source. 2-DOG is harmless to bacteria, unless phosphorylated by functional *galK*. As a result, 2-DOG becomes 2-deoxy-galactose-1-phosphate, which bacteria cannot metabolize, and thus it is a toxic intermediate to those clones that still harbor the *galK* cassette. The resulting 2-DOG-resistant colonies are recombinant clones that have no residual foreign DNA sequences as a result of the recombineering protocol.
8. Patch colonies on both 2-DOG and M63 minimal plates to ensure for the absence of *galK*. Confirm clones for the absence of *galK* by PCR and finally sequence the recombinants to ensure genomic integrity at the site of the recombination. One can now use this clone to generate additional recombinants within the same background, or alternatively, reconstitute infectious virus (*see* Subheading 3.4.2).

3.4.2 Reconstitution of Infectious Virus

Following the successful generation of recombinant BACs for the GPCR(s) of interest, one can easily reconstitute infectious virus. The HCMV clinical strain TB40/E [51], for example, yields high titers following reconstitution, providing ample virus with which to perform a multitude of experiments. Additionally, as none of the GPCR mutants, including those recombinants that harbor multiple GPCR deletions, show a particle to PFU defect, each recombinant will indeed yield a stock with a titer that is sufficient [21]. The first step in reconstituting recombinant viruses is purifying the BAC DNA by either alkaline lysis/precipitation or column purification kit. The protocol described here involves purification of BAC DNA by alkaline lysis and we have modified the protocol to also include an additional step to remove endotoxins. Compositions of the buffers used in this protocol are given in Subheading 2 of this chapter.

1. Grow 10 ml overnight (~16–18 h) culture of bacteria containing the BAC of interest.
2. Pellet the bacteria, resuspend in 200 μl CMPS1 w/RNAseA solution, lyse with 400 μl alkaline SDS solution, and neutralize with 300 μl potassium acetate. Pellet debris and treat clarified supernatant with the Endotoxin Removal solution (Sigma) according to the manufacturer's instructions (*see* **Note 8**). Precipitate DNA with 1.0 ml isopropanol.
3. Dissolve the resulting pellet in 500 μl TEN buffer at room temperature. Once the pellet has dissolved (roughly 10 min) centrifuge briefly to remove any remaining cellular debris, and precipitate the DNA from the supernatant with two volumes ethanol. The resulting BAC DNA is resuspended in 10.1 TE buffer, and should be used within 24 h for transfection. Importantly, one should refrain from freezing BAC DNA that is slated for transfection, as this greatly reduces the efficiency. Additionally, when manipulating BAC DNA, one should take care not to shear the DNA by rapid pipetting or using standard pipette tips (wide-bore tips are optimal).
4. To reconstitute virus, transfect low-passage, primary fibroblasts (1.7×10^6 cells) in a 4 mm cuvette by electroporation (960 μF, 0.26 V) in 500 μl of Opti-MEM with 1 μg of pCGN-pp71 (or equivalent pp71-expressing plasmid) and the BAC DNA of interest. Plate transfected cells in either T75 flasks or 100 mm dishes.
5. Feed transfected cells every 2–3 days until significant cytopathic effect (CPE) is observed.
6. To generate TB40/E stocks proceed to **step 7**, to generate FIX stocks proceed to **step 10**.
7. When fibroblasts have reached 100 % CPE, scrape cells into the infectious supernatant and collect by low-speed centrifugation.
8. Reserve the supernatant and bath-sonicate the cells to release the cell-associated virus, spin the cell debris as before, and combine the supernatants.

9. This combined supernatant can now be used to generate high titer stocks by further expansion.
   (a) For generating TB40/E stocks, only 1/10th of the infectious supernatant from **step 8** is necessary to infect at least 5×10^7 cells. The remaining supernatant can be stored at −80 °C in 1–2 ml aliquots and used to generate additional future stocks at another time.
10. For generating FIX stocks, when fibroblasts have reached 100 % CPE, remove the medium and trypsinize the cells. Seed the infected cells onto approximately 4×10^7 to 5×10^7 cells. FIX is highly cell-associated, and thus seeding the cells rather than the medium from the transfection plate is critical.
11. For both FIX and TB40/E, generating a high titer usable stock may require one to concentrate the viral stock. To concentrate virus, harvest cells and medium from the expanded stock by scraping cells into the infectious media, as described above in **step 7**. Pipette the cleared medium into ultracentrifugation tubes, and underlay with 20 % D-sorbitol, containing 50 mM Tris–HCl, pH 7.2 and 1 mM $MgCl_2$. Concentrate infectious virus by ultracentrifugation at $72{,}128 \times g$ for 90 min at 25 °C. Virus stocks can be stored at −80 °C in complete medium containing 1.5 % BSA for long-term storage. Store sterile-filtered 3 % BSA in 1× PBS at 4 °C and resuspend virus 1:1 in complete media: 3 % BSA.

3.4.3 Assessment of Viral Growth Properties

Many of the early assessments of the HCMV ORFs' necessities for viral replication in tissue culture were performed using fibroblasts [52, 53]. Although fibroblasts are invaluable to the study of HCMV lytic replication, they do not afford the ability to uncover functions of HCMV genes that are essential for growth in other clinically relevant cell types and tissues. Such is the case for the viral GPCRs that are not required for HCMV replication in fibroblasts. Thus, many investigators have taken advantage of clinical strains of HCMV that exhibit a broader cell tropism. The use of these clinical strains (i.e., TB40/E, TR, or FIX) allows for studies in an expanded repertoire of cell types including, but not limited to fibroblasts, hematopoietic progenitor cells, monocytes, macrophages, epithelial and endothelial cells. Assessing the growth properties of a mutant virus in a range of cell types is critical, as CMV pathogenesis in vivo is complicated and involves a plethora of different cells and tissues.

Assessing Production and/or Spread of Virus Occurring Via the Extracellular Route

1. Plate cells in 6-well plates (~5×10^5 to 1×10^6 cells, depending on cell type), designating two wells for each virus being tested. These two wells will serve as duplicate infections. Note that infection of ARPE19 cells does not result in efficient extracellular spread of HCMV (e.g., FIX and TB40/E) therefore quantifying viral replication is performed by cell-associated viral assays (see below).

2. For multi-step growth curves in fibroblasts or endothelial cells use a low MOI (e.g., 0.01–0.1 PFU/cell). For single-step growth curves, a high MOI between 1.0 and 3.0 PFU/cell is recommended.
   (a) Dilute the viral stock in the medium specific to the cell type being used. Ensure that enough inoculum is prepared to cover each well in addition to some that is reserved to assess the input titer. This is important when comparing viral growth between wild-type and recombinants, as one needs to ensure an equal amount of virus was used in the initial infection. Thus, reserving some inoculum that was not put onto cells is important when titering the growth curve.
   (b) Remove the medium and wash one time with 1× PBS. Add the inoculum to the cells in low volume (750 μl for a well of a 6-well plate) to ensure sufficient contact of the virus with the cells. Incubate at 37 °C/5 % CO_2 for 1 h, rocking the plate every 15 min.
   (c) Remove the inoculum, wash three times with 1× PBS, add fresh medium to the cells, and return to the incubator.
3. For low MOI infections, suggested time points include 0, 4, 8, 12, and 15 days post-infection (dpi). For high MOI infections, suggested time points include 0, 24, 48, 72, 96, and 120 hpi. The investigator should adjust these, as necessary. At each time point, remove a portion of the supernatant. This will vary depending on the assay being used to titer the viral growth curve. One should reserve enough supernatant from the cells such that the titering assay can be performed in triplicate. Replenish the cultures with the same volume of fresh medium that was removed for the time point. Store all of the collected samples at −80 °C until the time course is completed.
4. Once all of the time points have been collected, thaw the samples in a 37 °C water bath, and assess the titers by plaque assay, $TCID_{50}$ analyses, or modified IFA for IE protein expression. Each time point for each virus should be measured in triplicate.

Assessing Production and/or Spread of Virus Occurring Via the Cell-Associated Route

1. Plate cells in 6-well plates (~5×10^5 to 1×10^6 cells, respectively). Dedicate one well for each time point for every virus being assessed.
2. Both endothelial cells and fibroblasts support infection with low MOIs of 0.01–0.1 PFU/cell or high MOI infections of at least 1.0 PFU/cell. Viral infection of ARPE19 cells spreads exclusively by cell-to-cell contact following either FIX or TB40/E infection. For ARPE19 cells, it is advisable to use a MOI of approximately 0.1 PFU/cell for multi-step growth curves and a MOI of at least 1.0 PFU/cell for single-step growth curves (*see* **Note 9**).

3. Prepare the inoculum as above, diluting virus in the appropriate medium.
   (a) Remove the medium from the cells, and wash the cells with 1× PBS as described in **step 2c** of Assessing Production and/or Spread of Virus Occurring Via the Extracellular Route.
   (b) Add the inoculum to the appropriate wells as above reserving an aliquot of the inoculum, and infect the cells for 1 h at 37 °C/5 % CO_2, rocking the plates every 15 min. Although not required, infection of ARPE19 cells is increased by centrifugal enhancement at 1,000 × *g* for 30 min at room temperature. If this step is performed, the cells should next be incubated at 37 °C/5 % CO_2 for an additional 1 h with rocking every 15 min.
   (c) Remove the inoculum and wash the cells three times with 1× PBS to remove any residual virus that had not entered the cells. Replenish the cultures with fresh medium and return to the incubator.
4. For either fibroblasts or endothelial cells, collect the cell-associated virus at the times described above in **step 3** of Assessing Production and/or Spread of Virus Occurring Via the Extracellular Route for low and high MOIs. For ARPE19 cells, infection progresses at a slower rate, and thus, low MOI time points include 0, 10, 20, and 30 dpi. Additionally, the medium on ARPE19 cultures should be changed every 5 dpi to ensure cell health over the time course of infection. For single-step growth analyses at high MOI, suggested time points include 0, 4, 8, and 12 dpi.
5. To collect cell-associated virus at each time point, remove the medium from the cultures, and wash 2–3 times with 1× PBS. Add back at least 1 ml of fresh medium, and scrape the cells into the medium. Samples should be stored at −80 °C until the time course is completed.
6. Evaluating the titer of the cell-associated virus requires three freeze-thaw cycles to disrupt the cells thereby releasing the virus. Thaw samples in a 37 °C water bath, ensuring that the samples completely thaw, and then quickly re-freeze in liquid nitrogen. Following the third thaw, spin down cellular debris and transfer the medium to a fresh tube for use in a titering assay as described above.

Undoubtedly, the types of approaches described in this section will generate important information on the roles of viral GPCRs in viral replication and spread in clinically relevant cell types in vitro, and when combined with in vivo viral replication experiments in animal models, will provide clues as to how these proteins function to facilitate replication and pathogenesis during the natural course of cytomegalovirus infection.

3.5 Detecting Viral GPCR Proteins in Infected Cells

As mentioned above, bacterial recombineering techniques have afforded investigators the ability to epitope tag viral proteins, in particular the viral GPCRs. Previous studies assessing the expression and subcellular localization of the CMV GPCRs generally included the overexpression of individual GPCRs in cell types in which CMV infection is not supported. Although these studies yield important information about potential function, these were not performed in the context of viral infection. Moreover, the expression level of viral GPCRs in infected cells may be very different than that observed in transient assays and may result in qualitative and quantitative differences in signaling. Antibodies directed against several of the CMV GPCRs including US27 [54], US28 [55], UL33 [20, 56], and MCMV M78 [22] have been generated. However, construction of viral recombinants expressing epitope-tagged GPCRs allows investigators to utilize commercially available validated antibodies that work across a variety of techniques including immunofluorescence assay (IFA), immunoprecipitation/western blot, immuno-electron microscopy, and fluorescence activated cell sorting (FACS).

3.5.1 Detection and Localization of Viral GPCRs by Immunofluorescence Assay

Immunofluorescence assay (IFA) provides a useful platform for determining the cellular localization of a given GPCR and provides a convenient tool for determining the percentage of cells expressing the GPCR in question.

1. Grow cells on gelatin coated glass coverslips and infect at an MOI of at least 0.5 PFU/cell. Using an MOI of 3 PFU/cell will typically guarantee that >95 % of the cells are infected.
2. At the desired time post-infection, wash cells with 1× PBS, and fix with 2 % paraformaldehyde at 37 °C for 15 min. Alternatively, one can use cold 100 % EtOH to fix the infected cells, although it is important to note that this will destroy any color marker (e.g., eGFP or mCherry) that is expressed from the viral genome.
3. Following fixation, wash the cells three times with 1× PBS at room temperature, and then permeabilize with 0.1 % Triton X-100 for 15 min at room temperature.
4. Wash the cells with PBS containing 0.2 % Tween 20, and then block for at least 1 h at room temperature in 2 % BSA/0.2 % Tween 20 in 1× PBS. Alternatively, one can block the coverslips overnight at 4 °C. If multiple time points are necessary, the blocking step is an excellent step at which to stop until the remaining slides are harvested.
5. After blocking all of the coverslips, stain with primary antibody in blocking buffer for at least 1 h at room temperature.
6. After staining with primary antibody, wash the coverslips with 1× PBS containing 0.2 % Tween 20 at least three times at room temperature.

7. Stain the cells/coverslips with secondary antibody containing the appropriate conjugated fluorophore for at least 1 h in the dark at room temperature. A nuclear dye, such as 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst should also be included, as this serves as an excellent control.
8. Wash the cells/coverslips three times in 1× PBS containing 0.2 % Tween 20.
9. Mount and seal the coverslips onto slides using an appropriate anti-fade mounting medium.
10. View the cells using standard fluorescent or confocal microscopy techniques.

We have taken advantage of recombineering techniques to generate FLAG-tagged GPCR recombinants in the AD169, FIX, and TB40/E backgrounds. Using these recombinants coupled with IFA, we have shown cellular localization for each of the GPCRs and have determined the presence of each in the mature HCMV virion [21, 24, 26].

3.5.2 Detection and Localization of Viral GPCRs (and Interacting Partners) by FLAG Immunoprecipitation/Western Blot

Immunoprecipitation followed by western blotting is a very sensitive technique that can be used to detect viral GPCRs. This sensitivity is essential as future investigations designed to analyze vial GPCR expression function are likely to be performed in clinically relevant cell types that may not exhibit lytic expression patterns comparable to that observed in standard HCMV infected fibroblasts.

1. Plate cells in 100 mm dishes at 50–75 % confluent. Infect with viruses or expression constructs expressing FLAG-tagged GPCRs as described elsewhere in this report.
2. At the appropriate times post-infection or post-transfection, remove medium and wash cells 1× with PBS.
3. Add 1.0 ml of RIPA buffer containing protease and phosphatase inhibitors.
4. Transfer the lysate to microcentrifuge tubes and shear the DNA by passing lysates through a 22 G needle and syringe 15–20 times.
5. Clarify the supernatant by centrifugation at 15,000 × *g* for 15 min at 4 °C.
6. Transfer supernatant to a clean microcentrifuge tube and preclear lysates by adding 50 μl Sepharose 4B and rotating for 30–60 min at 4 °C.
7. Pellet Sepharose 4B by centrifugation at 15,000 × *g* for 15 min at 4 °C.
8. Transfer the supernatant to fresh tube and add 20 μl of anti-FLAG M2 beads. Rotate for ≥4 h at 4 °C.
9. Pellet M2 beads by centrifugation at 15,000 × *g* for 15 min at 4 °C. Wash beads four times with 1 ml RIPA buffer.

10. Resuspend washed beads in 50 μl of 3× sample buffer and incubate for 30 min at room temperature or 10 min at 42 °C. It is important to avoid boiling the immunoprecipitated samples as GPCRs have a tendency to aggregate and can form altered species that do not migrate at the predicted molecular weight on SDS-PAGE gels.
11. Separate immunoprecipitates by SDS-PAGE and analyze by western blot as described above in Subheading 3.2.
12. For western blot analyses of immunoprecipitated viral GPCRs and interacting proteins, it is important to use antibody reagents that are derived from a species different from that used in the immunoprecipitation step (in this case, the immunoprecipitating M2 antibody is mouse) to prevent cross reactivity between the immunoprecipitating and primary western antibody.

3.5.3 Detection and Localization of Viral GPCRs by Fluorescence Activated Cell Sorting

Fluorescence Activated Cell Sorting (FACS) analysis is a powerful and rapid tool for assessing the expression of a given viral GPCR. Investigators have successfully used this method to demonstrate cell surface expression of epitope-tagged CMV GPCRs [32, 43, 55, 57–62]. For example, Stropes and Miller generated a variety of FLAG-tagged US28 recombinants to study US28 signaling in infected cells and demonstrated that while wild-type and a N-terminal truncation mutant exhibited similar constitutive signaling activities, the N-terminal truncation mutant exhibited decreased cell surface accumulation in comparison to wild-type US28 [43].

1. Using as few as 1×10^5 cells, infect the cells with a recombinant virus of choice as described throughout this chapter. The cells can be infected with a wide range of MOIs as FACS can accurately detect a positive cell population as small as 2–3 %.
2. Harvest the infected cells by trypsinization Trypsin–EDTA (0.05 % Trypsin, 0.53 mM EDTA) and neutralize the trypsinized population by resuspending cells in complete medium containing serum.
3. Wash the cells two times with 1× PBS.
4. Stain the cells with a primary antibody directed at the epitope tag for ≥1 h at 4 °C. The antibody should be diluted in 1× PBS containing 0.5 % BSA. When using FLAG-tagged viral GPCRs, it may be beneficial to use biotinylated anti-FLAG antibody as this coupled with fluorophore conjugated streptavidin can enhance the signal significantly.
5. If the primary antibody used is not preconjugated with a fluorophore, wash the cells as above in PBS, and then stain the cells with the appropriate secondary antibody in the aforementioned buffer for ≥1 h at 4 °C.
6. After a final series of washes in PBS, analyze cells by FACS.

3.5.4 Other Potential Methodologies for the Detection and Localization of Viral GPCRs

Investigators have also utilized a variety of additional techniques, including enzyme-linked immunosorbent assay (ELISA) and immune-electron microscopy (immuno-EM) to assess the intracellular and/or surface expression of viral GPCRs [9, 20, 22, 24, 26, 42, 44, 54, 56, 58, 63, 64]. The finding that US27 also localizes to the membranes of the cells was demonstrated by immunoprecipitation of cell surface proteins following infection with a US27 FLAG-tagged, yet localizes as well to the perinuclear region as shown by IFA [26]. More recently, Tschische et al. described the heteromerization of HCMV UL33, UL78, and US27 each with US28 in transient transfection assays, and provided evidence of their colocalization using a combination of IFA, immunoprecipitation, and bioluminescence resonance energy transfer (BRET) analyses [64]. Additionally, Fraile-Ramos and colleagues utilized immuno-EM to discern the intracellular localization of HCMV UL33 and US27 [56]. Taken together, these methodologies provide useful tools in examining the expression and localization of the CMV GPCRs within infected cells.

3.6 Methods for Studying Viral GPCR Function in Animal Models

The experimental approaches and methodology described thus far enable a thorough examination of the biochemical and molecular signaling activities of the viral GPCRs and can be used to study the in vitro function of these interesting and conserved cytomegalovirus proteins. However, they fall short of addressing perhaps the most important fundamental questions regarding the CMV GPCRs: (1) What are the primary biological functions of these CMV GPCRs in vivo? (2) How do these functions affect pathogenesis? and (3) How does the signaling activity of the CMV GPCRs mediate their roles in pathogenesis? Therefore, it is essential to extend the biochemical and molecular genetic experiments described thus far with pathogenesis experiments performed in animal models. The results obtained from in vivo studies will provide the genesis for the rational design of experiments aimed at exploring the molecular functions of cytomegalovirus GPCRs in biologically relevant cellular models. Of the models available for cytomegalovirus research, the mouse model appears to be the best suited for studies on the role that the GPCRs play in pathogenesis in vivo. Both the M33 and M78 genes exhibit profound growth defects in organs important for viral persistence such as the salivary gland [15–18, 22, 65]. Moreover, the mouse is easily amenable to genetic manipulation such as transgenesis and gene knockout, thus allowing investigators to extend pathogenesis studies and potentially explore detailed mechanisms underlying pathogenic processes. Finally, the mouse is a cost-effective model in which one can functionally and mechanistically examine these cytomegalovirus encoded GPCRs before moving on to more complex primate models, if warranted. In this section, we will describe basic methodology to assess cytomegalovirus replication/dissemination in

the mouse using wild-type and M33 null MCMVs as an example. BACs containing the Smith and K181 strains have been generated and can be manipulated to delete entire GPCR ORFs or one can make more subtle mutations in signaling motifs, etc. using recombineering methodologies similar to that discussed above [66, 67].

There are a multitude of different strains of mice that have been used to study cytomegalovirus pathogenesis, many offering unique attributes that can be exploited to gain additional insight into the mechanisms of cytomegalovirus replication and spread in vivo. Briefly, strains such as Balb/C are relatively sensitive to MCMV infection, while other strains, such as C57BL/6 are much more resistant to MCMV infection [68, 69]. The nature of this difference lies in the *cmv1* locus which encodes the activating NK receptor LY49H in the resistant, but not sensitive strains. Severely immunodeficient mouse strains such as $CB17^{SCID}$ and NOD-SCID-gammaCnull (NSG) mice have emerged as useful models to explore CMV replication and trafficking in the absence of adaptive ($CB17^{SCID}$) or adaptive/innate NK (NSG) immune function [70–73]. Particular care must be given to dosage and duration of infection when using the immunodeficient animals, as these animals are particularly sensitive to cytomegalovirus and quickly succumb to the infection.

3.6.1 Examination of MCMV Replication and Spread in the Mouse Using Viral Recombinants with Deletions/Mutations in the GPCR Genes

1. Five- to six-week-old female mice are obtained from the appropriate vendor and housed under pathogen-free conditions in barrier-filtered SMI cages according to Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved guidelines. The mice are given water and chow ad libitum for the duration of the experiment.
2. Six- to twelve-week-old mice are infected with 1×10^5 to 1×10^6 PFU/animal of tissue culture derived wild-type or M33 null viruses. Salivary gland derived stocks of many MCMV strains can alternatively be used. However, mutants such the M33 null viruses do not exhibit strong salivary gland tropism and thus do not allow for the generation of salivary gland stocks. Thus, in the case of M33 null viruses or other mutants that do not grow in the salivary gland, one is limited to tissue culture derived virus.
3. Virus is injected into animals via one of several routes including intraperitoneal (i.p.), intravenous (i.v.), or subcutaneous (s.c.) into the rear footpad. The i.p. route is the most convenient for routine assessment of MCMV growth in various tissues and in this case a 28 gauge insulin syringe containing up to 300 μl of virus diluted in PBS is used for the infection.
4. At appropriate times post-infection (*see* **Note 10**) animals are sacrificed by CO_2 asphyxiation and blood is immediately obtained by cardiac puncture and placed into EDTA treated

blood collection tubes. The blood can be used to assess the number of MCMV infected blood leukocytes as described in **step 6**.

5. Internal organs and/or tissues of interest (such as spleen, liver, and salivary gland) are removed via dissection and placed into 1 ml DMEM, flash-frozen, and stored at −80 °C until use. To assess virus titers in organs and/or tissues proceed to **step 7**.
6. Assess the number of infected leukocytes by infectious center assay.
   (a) Dilute 500 μl of blood in 5 ml RBC lysis buffer. Mix and incubate at room temperature until the RBCs lyse (the solution will change from opaque to clear, but remain deep red). Pellet WBCs for 10 min at 400 × *g*. Wash the WBCs three times with sterile 1× PBS to remove hemoglobin and platelets. Resuspend washed WBCs in 1 ml of 1× PBS.
   (b) Transfer 1×10^4 to 1×10^5 WBC to MEF monolayers (*see* **Note 11**), incubate for 3–4 h to allow WBCs to settle to bottom of well and come in contact with MEFs. Carefully remove the medium without disturbing settled WBCs and overlay with DMEM containing 0.75 % carboxymethyl cellulose (CMC). Incubate undisturbed for 6–7 days.
   (c) Remove the medium, fix monolayers with methanol, and stain with Giemsa diluted 1:5. Count plaques. Plaques that develop arise as a consequence of a single infected WBC that is productively shedding virus, and hence the term infectious center.
7. Assess virus titers in organs/tissues by plaque assay.
   (a) Thaw tissue that was suspended in 1 ml DMEM and flash-frozen as described in **step 5**. Transfer to Dounce and homogenize organ using 20–30 passes with the tight fitting glass pestle. Ensure visually that the tissue is completely homogenized—if not proceed with additional passes until the tissue is completely disrupted.
   (b) Centrifuge for 5 min in a microcentrifuge at 5,000 × *g* to pellet cellular and tissue debris. Transfer supernatant to fresh tube.
   (c) Transfer dilutions of tissue supernatant to MEF monolayers (*see* **Note 11**), and incubate for 3–4 h to allow virus adsorption. Carefully remove the medium and overlay with DMEM containing 0.75 % CMC. Incubate undisturbed for 4–5 days. Virus titers in organs vary dramatically depending on initial virus dose and dpi, so care should be taken to ensure that the dilutions of tissue supernatant used in the assay will allow for quantitation of plaques in each well.

(d) Remove the medium, fix monolayers with methanol, and stain with Giemsa diluted 1:5. Count plaques.

Using in vivo assays like the one just described, it is evident that the cytomegalovirus GPCRs confer important activities that facilitate viral replication in the whole organism. It is important to ensure that the observed phenotype is due to deletion/alteration of the targeted gene, and this can be accomplished by using "rescue" viruses in which the mutated region is reverted to wild-type. While it is clear that the GPCRs and their ability to signal through G-proteins are essential for replication in vivo, it is not clear what specific signaling pathways are involved or how activation of these signaling pathways facilitate replication. The power of mouse genetics combined with in vivo growth assessment of viruses with GPCR mutations should provide important answers to these questions. It is the answers to these questions that should be at the forefront of future investigations aimed at exploring molecular and biochemical properties of the viral GPCRs.

3.7 Conclusions and Discussion of Current State-of-the-art Techniques Useful for Studying Viral GPCR Signaling/Function

Techniques such as transient transfections and related gene delivery methodologies have proved to be invaluable in providing a basic understanding of the CMV GPCRs and how they function in vitro. However, continued vertical advancement of our understanding of the CMV GPCRs requires us as investigators to distance ourselves from standard in vitro techniques and begin to perform studies in the context of virus-infected cells using clinical strains of HCMV and cell types important for in vivo pathogenesis. Taking advantage of CMV GPCR mutants constructed by recombineering techniques is critical for the successful transition to these more sophisticated types of experiments. The tools and resources, including bacterial recombineering techniques, now exist for the cytomegaloviruses, therefore making such studies possible. BAC recombineering protocols provide efficient means to derive viral recombinants for use in both in vitro and in vivo studies. The benefit to generating mutants in BAC viral DNA as opposed to utilizing expression plasmids is that one can investigate the function of the GPCRs in the milieu of the remaining viral ORFs and at physiologically relevant expression levels. Finally, the tools now available for studying CMV GPCRs affords us as investigators the ability to perform high-throughput screens to search for novel viral GPCR therapeutics that may influence HCMV infection and/or replication. Over one third of marketed drugs target cellular GPCRs, and thus the CMV GPCRs are attractive targets. Both the gammaherpesvirus and betaherpesvirus subfamilies encode GPCRs, and in animal models, these proteins have been shown to aid in viral pathogenesis. Thus, it seems likely that herpesviruses have hijacked cellular GPCRs to promote viral replication and dissemination in

the host. Utilizing the current resources and technologies, we will undoubtedly uncover the function of these proteins, and perhaps exploit their activities in an effort to develop novel anti-viral therapies to combat HCMV infections.

4 Notes

1. Typical doubling times are 48–72 h for HS68 fibroblasts and 18–24 h for HEK-293 cells.
2. To activate the sodium orthovanadate, prepare a 200 mM stock solution, adjust the pH to 10 using NaOH/HCl and boil until colorless. Cool to room temperature, readjust to pH 10 and repeat until the solution stabilizes at pH 10 and remains colorless. Store the activated sodium orthovanadate in aliquots in the −20 °C freezer. The protease inhibitors aprotinin, leupeptin, and PMSF can be substituted for Complete Mini Protease tabs (Roche).
3. Biotin is degraded by light—make fresh and use plates ≤1 month.
4. The different medium formulations contain various amounts of unlabelled myo-inositol, and therefore, it may be beneficial to use MEM as the inositol concentration is lower and gives better labeling.
5. In many cases, simply eluting the total inositol phosphates will provide an extremely accurate measurement of receptor signaling. In this case after **step 15**, simply transfer the columns to scintillation vials and elute the total inositol phosphates with 4 ml 0.1 M formic acid/1.0 M ammonium formate.
6. The pFR-LUC and pFA2 plasmids are part of the PathDetect In Vivo Signal Transduction *trans*-reporting system available from Agilent Technologies™. More information about these plasmids and the *trans*-reporting system can be found at: http://www.genomics.agilent.com/files/Manual/219000.pdf.
7. Passive Lysis Buffer (PLB), Luciferase Assay reagent II (LAR II), and Stop&Glo are components of the Promega Dual-Luciferase® Reporter Assay System. More information about this system can be found at: http://www.promega.com/products/reporter-assays-and-transfection/reporter-assays.
8. The bacterial strains SW102, SW105, and SW106 produce endotoxins, which are co-purified with the BAC DNA and thus can be introduced into mammalian cells during the transfection process. Endotoxins stimulate components of the innate immune response in mammalian cells, and therefore are toxic to cells in tissue culture. Thus, adding a step within the alkaline lysis protocol to remove endotoxins will greatly improve the health of the mammalian cells post-transfection

of the BAC DNA and greatly improve the overall transfection efficiency.

9. Infections below a MOI of 0.1 PFU/cell in ARPE19 cells result in insufficient viral output for assessment by plaque assay and are therefore not recommended.
10. MCMV replicates in a large number of cell types and tissues. In particular the virus can be found at high levels during the acute phase in organs such as spleen and liver (3–5 dpi) and during the persistent phase in tissues such as the salivary gland (12–21 dpi).
11. 1 day prior to using for plaque or infectious center assays, plate 100,000 primary MEFs (passages 2–8) into each well of a 12-well plate.

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