Published in final edited form as:

J Virol Methods. 2012 January ; 179(1): 116–126. doi:10.1016/j.jviromet.2011.10.009.

Efficient generation and rapid isolation via stoplight recombination of Herpes simplex viruses expressing model antigenic and immunological epitopes

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Abstract

Generation and isolation of recombinant herpesviruses by traditional homologous recombination methods can be a tedious, time consuming process. Therefore, a novel stoplight recombination selection method was developed that facilitated rapid identification and purification of recombinant viruses expressing fusions of immunological epitopes with EGFP. This “traffic-light” approach provided a visual indication of the presence and purity of recombinant HSV-1 isolates by producing three identifying signals: 1) red fluorescence indicates non-recombinant viruses that should be avoided; 2) yellow fluorescence indicates cells co-infected with non-recombinant and recombinant viruses that are chosen with caution; 3) green fluorescence indicates pure recombinant isolates and to proceed with preparation of viral stocks. Adaptability of this system was demonstrated by creating three recombinant viruses that expressed model immunological epitopes. Diagnostic PCR established that the fluorescent stoplight indicators were effective at differentiating between the presence of background virus contamination and pure recombinant viruses specifying immunological epitopes. This enabled isolation of pure recombinant viral stocks that exhibited wildtype-like viral replication and cell-to-cell spread following three rounds of plaque purification. Expression of specific immunological epitopes was confirmed by western analysis, and the utility of these viruses for examining host immune responses to HSV-1 was determined by a functional T cell assay.

Keywords

Herpes Simplex Virus; Epitopes; Recombination; Fluorescent; Ova; CD8

1. Introduction

Herpes simplex virus type 1 (HSV-1) is a common human pathogen that is quite prevalent within the population (75–90% infected) and is generally associated with infections of mucosal surfaces and skin (Hill et al., 2008; Liesegang, 2001). Occasionally, more severe

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and sometimes lethal sequelae result from these infections, including sporadic encephalitis, neonatal HSV-1, and ocular blindness (Whitley, 1996). Indeed, HSV-1 infection of the eye is the leading cause of non-traumatic corneal blindness in the developed world (Pepose, 1996). Ocular infection of immunologically normal adults generally presents as conjunctivitis, involving a primary infection of the corneal epithelium (Kaye and Choudhary, 2006; Liesegang, 2001). Viral replication causes epithelial cell loss and corneal damage; however, the initial infection is generally controlled and HSV-1 establishes latency within the trigeminal ganglia (Cantin et al., 1992; Maggs et al., 1998; Toma et al., 2008). Sporadic reactivation of latent virus results in a recrudescence inflammatory disease within the corneal stroma called herpetic stromal keratitis (Kaye and Choudhary, 2006; Liesegang, 2001; Toma et al., 2008). With each subsequent recurrent episode, corneal damage and T cell infiltration increases. These cellular infiltrates clear the virus but also cause the cornea to become cloudy and eventually opaque, resulting in both blindness and a requirement for corneal transplantation (Carr et al., 2001; Doymaz and Rouse, 1992a; Doymaz and Rouse, 1992b; Ghiasi et al., 2000; Hendricks, 1997; Liesegang, 2001; Metcalf and Michaelis, 1984; Newell et al., 1989a; Osorio et al., 2004; Pepose, 1996; Streilein et al., 1997; Stuart et al., 2004; Williams et al., 1965).

Development of herpetic stromal keratitis lesions is an immunopathologic-associated process that is not a primary effect of HSV infection or toxicity (Carr et al., 2001; Doymaz and Rouse, 1992a; Doymaz and Rouse, 1992b; Metcalf and Michaelis, 1984; Newell et al., 1989a; Newell et al., 1989b; Niemialtowski et al., 1994; Stuart et al., 2004). Although virus titers are high following ocular infection, they rapidly decline in 6–7 days. In contrast, stromal opacity appears at 7 days and reaches peak intensity at 2 weeks when virus titers are no longer detectable (Pepose, 1996). This paradox was somewhat resolved when *Metcalf et al.* established that in T cell deficient mice, herpetic stromal keratitis did not develop following ocular HSV-1 infection (Metcalf, et al., 1979; Metcalf and Kaufman, 1976). Subsequent studies demonstrated that adoptive transfer of T cells prior to HSV-1 corneal infection could render T cell deficient animals susceptible to herpetic stromal keratitis, implicating T cells in the pathogenesis of herpetic stromal disease (Newell et al., 1989a; Newell et al., 1989b; Niemialtowski et al., 1994; Russell et al., 1984). Despite the recognized role of the immune response in development of herpetic stromal keratitis, there remain discrepancies between the roles of the various immune effectors in disease development, and as such, there is a need for viral reagents that may assist in dissecting the contributions by each of these effectors (Brandt, 2005; Brandt et al., 2003; Doymaz et al., 1991; Ghiasi et al., 2000; Hendricks and Tumpey, 1990; Lepisto et al., 2006; Osorio et al., 2004; Stuart et al., 2004).

Investigation of cellular processing and presentation of endogenous antigens in the context of infection is critical for understanding immune recognition of non-self. The use and development of model immunological epitopes has contributed to vaccine design, understanding of pathogen and tumor immune evasion mechanisms, and elucidation of mechanisms of immunopathologic diseases, such as herpetic stromal keratitis (Deshpande et al., 2001; Gangappa et al., 2000). However, generation, identification, and isolation of pure recombinant herpes simplex viruses via traditional homologous recombination methods have been a tedious and time consuming process. An adaptable system is described for the rapid and efficient isolation of recombinant HSV-1 that expresses exogenous proteins or model antigenic epitopes that may facilitate understanding of how HSV induces or evades host immune processes. The inclusion of a readily distinguishable stoplight recombination and identification system enables the investigator to ascertain the presence of a gene of interest, as well as the absence of contaminating non-recombinant viruses. Furthermore, a rapid system for quantitatively assessing MHC class I presentation and the subsequent CD8⁺ T cell responses is described using an HSV-1 recombinant that expresses an exogenous CD8

Ovalbumin (Ova) derived immunologic epitope. This recombination system and the generated viruses have utility for examining immune responses to HSV-1 infection of the eye and the roles of viral proteins in altering that response.

2. Materials and Methods

2.1. Viruses and cells

African green monkey kidney (Vero) cells were obtained from ATCC (Manassas, VA) and were maintained in DMEM supplemented with 5% FCS. MC57G murine fibroblasts were originally obtained from ATCC and were maintained in a 50/50 mix of DMEM and RPMI supplemented with 10% FCS, 50 μ M 2-mercaptoethanol, and 1mM pyruvate. The murine B3Z T cell hybridoma (Karttunen et al., 1992) specific for the CTL epitope from amino acids 257–264 of chicken ovalbumin (Shastri and Gonzalez, 1993) in the context of K^b, was a generous gift of Dr. N. Shastri (Univ. of Calif., Berkeley, CA). FLP-IN CV-1 cells were acquired from Invitrogen (Carlsbad, CA) and utilized as a non-complementing control and for generating the gK-null complementing cell line, CV1-HSV1gK. Isolation of a stable FLP-IN CV-1 cell-line that expressed HSV-1 gK, CV1-HSV1gK cells, was performed essentially according to the manufacturer's directions (Invitrogen) and as described previously (Melancon et al., 2005) by first cloning a PCR amplified HSV-1 gK gene into the pcDNA5-FRT TOPO vector (Invitrogen). The RE strain of HSV-1 (HSV-1(RE)), a known corneal keratitis inducing strain of HSV-1 (Hendricks et al., 1989; Hendricks and Tumpey, 1990), was the parental wildtype strain utilized in these studies and was initially obtained from Dr. James Hill, Louisiana State University Health Sciences Lions Eye Center. HSV-1(RE) virus stocks were propagated in Vero cells and stored as infectious cell preparations at –80°C.

2.2. Construction and generation of a gK-null HSV-1(RE) strain virus

An HSV-1(RE) strain virus that specifies a deletion within the UL53/gK gene, while maintaining the entire UL52 open reading frame, was generated by homologous recombination and isolation on CV1-HSV1gK cells. In order to facilitate homologous recombination with viral genomes, the CMV-tdtomato gene cassette was PCR amplified from the ptdtomato-C1 plasmid (Clontech, Mountainview, CA) and inserted in place of the UL53/gK gene flanked downstream by a UL52 gene fragment and upstream by the UL53 3' UTR and a UL54 gene fragment. gK-complementing CV1-HSV1gK cells were transfected with the resulting plasmid and subsequently infected with wildtype HSV-1(RE) virus. Recombinant viruses that exhibited bright red fluorescence when observed by fluorescent microscopy were plaque purified on gK-complementing cells and confirmed by differential growth and genetics for purity as has been described previously for the generation of the KOS strain Δ gK/EGFP virus (Foster, Rybachuk, and Kousoulas, 1998). The resultant gK-null HSV-1(RE) strain virus, designated RE Δ gK/tomato, was propagated on CV1-HSV1gK cells.

2.3. Fluorescent microscopy of viral plaques

Cell monolayers in 12 well plates were infected via limiting dilution starting at a multiplicity of infection (MOI) of 0.01. After 1 hour (h) adsorption, media containing virus was removed and cells were overlaid with DMEM containing 2% FCS and 0.5% methylcellulose. Infected cells were subsequently incubated at 37°C for the times indicated. Representative isolated viral plaques were directly visualized and photographed through either a GFP (for green fluorescence) or propidium iodide (for red fluorescence) filter set on a Zeiss Axio Observer Z1 inverted microscope. Phase contrast microscopy was utilized to visualize plaque and cellular morphology.

2.4. One step growth kinetics and viral yield assays

Analysis of one-step growth kinetics and infectious virus yield was described previously (Foster et al., 1999; Foster and Kousoulas, 1999; Foster et al., 1998). Briefly, an MOI of 5 for each virus isolate was adsorbed to Vero cells in 6 well plates at 4°C for 2 h. Thereafter, virus was removed, pre-warmed media was added, and virus was allowed to penetrate for 2 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (PBS, pH 3.0). Total virus (cells and supernatants) or extracellular virus (cell supernatants) were harvested immediately thereafter (0 h) or after 6, 12, 24, 30, 36, or 48 h incubations. Total virus samples were frozen and thawed three times to release cell associated virus prior to endpoint titration plaque assays. For one step growth kinetics, virus titers for each time point were determined in triplicate by endpoint titration of virus stocks on gK-complementing CV1-HSV1gK cells. Viral yield was determined as described above, except that only the 24 and 48 h time points were assessed for total and extracellular virus production and virus stocks were titrated on Vero cells.

2.5. Construction of epitope-expressing recombination plasmids

A recombination plasmid that enables the simple insertion and expression of amino terminal EGFP fusions was constructed by inactivational cloning of a CMV-EGFP gene cassette into the unique BamHI restriction site within the intergenic region between UL53 and UL54 in a manner that maintained utilization of the viral UL52/53 poly-adenylation signal sequence. The CMV-EGFP gene cassette was constructed to enable in-frame insertion of immunological epitopes in a newly generated unique BamHI site. This base recombination vector was designated pHSV1-epirec. In order to illustrate the utility of this vector, three immunological epitopes were generated as three times (3x) tandem repeats and inserted into the pHSV1-epirec recombination vector: a 3x FLAG epitope (3xFLAG: MDYKDHGDDYKDHIDYKDDDDK), designated pHSV1-3xFLAG; a preprotrypsin secretion signal leader sequence followed by a 3x FLAG for directed secretion of expressed products (ss3xFLAG: MSALLILALVGAADVADYKDHGDDYKDHIDYKDDDDK), designated pHSV1-ss3xFLAG; and a 3x tandem repeat of the SL8 CTL T cell epitope from chicken ovalbumin amino acids 257–264 (Shastri and Gonzalez, 1993) (3xCD8Ova: SIINFEKLGGSIINFEKLGGSIINFEKL), designated pHSV1-3xCD8Ova. The 3xFLAG and ss3xFLAG epitopes were generated by PCR amplification of the epitopes from plasmids p3xFLAG-CMV10 and p3xFLAG-CMV9 (Sigma, St. Louis, MO), respectively. A tandem repeat of three CD8 Ova epitopes (3xCD8Ova) was generated by annealing and extending complementary synthetic oligonucleotides synthesized by IDT (Coralville, IA) followed by PCR amplification.

2.6. Generation, isolation and plaque purification via stoplight recombination of specific immunological epitope-expressing HSV-1 viruses

The recombination plasmids that encoded specific immunological epitopes were individually transfected into Vero cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. 12 h post transfection cells were infected with the REΔgK/tomato virus at an MOI of 3 and incubated at 37°C for 36 h. Virus stocks containing recombinant viruses were plated onto Vero cells for plaque purification. 24 h post infection (p.i.) viral plaques were observed by fluorescent microscopy on a Zeiss Axioobserver Z1 inverted microscope. Isolated green fluorescent plaques, which showed no signs of red fluorescent non-recombinant REΔgK/tomato virus, were marked, picked by pipettor, and infected by limiting dilution to new Vero cells for further plaque purification. Virus was considered pure after two rounds of plaque purification exhibited no signs of contaminating red background virus. Diagnostic PCR was utilized on each round of plaque purification to monitor and ensure viral isolate purity.

2.7. Viral DNA preparation and diagnostic PCR

To isolate viral DNA, infected Vero cells that exhibited 100% cytopathic effect at each stage of plaque purification were lysed with 1% NP-40 in 10 mM Tris and 1 mM EDTA. The lysates were treated with RNase (10 µg/ml) for 10 minutes at 37°C, followed by the addition of sodium dodecyl sulfate (1% final concentration) and incubation with proteinase K (10 µg/ml) at 55°C. DNA was purified with two phenol-chloroform extractions and precipitated with 3 M sodium acetate and isopropanol. Differential diagnostic PCR was performed to determine both the presence of the rescued recombinant viruses and the absence of contaminating non-recombinant REΔgK/tomato viruses. The forward primer for all reactions was P5', which hybridized within the HSV-1 UL52/UL53 overlapping sequences. For purity diagnostics, the P5' forward primer was paired with either the P1 reverse primer, which hybridizes within the CMV promoter sequence, or the P3 reverse primer, which hybridizes within the EGFP or tomato open reading frame. Due to the absence of UL53 sequences in the gK-null REΔgK/tomato virus, PCR would amplify the smaller UL53/gK deleted products more preferentially and thereby detect any non-recombinant REΔgK/tomato contamination sensitively. PCR analysis for the presence of specific immunological epitopes was performed using the P5' forward primer paired with an epitope sequence specific P2(3xFLAG) or P2(3xOva) reverse primer. All PCR amplifications were performed using the Failsafe PCR system reagents and buffer E (Epicentre, Madison, WI). PCR products were sequenced to confirm the presence of the specific immunological epitopes within each virus isolate.

2.8. SDS-PAGE and Western blot analysis

Vero cells in 6 well plates were infected at an MOI of 5 with the indicated virus. At 24 h p.i. cell supernatants were collected for analysis and infected cell lysates were prepared by direct lysis for 7 minutes in Mammalian Protein Extraction Reagent (MPER, Pierce Chemical, Rockford, IL) supplemented with 0.1% SDS and complete protease inhibitor cocktail (Roche, San Francisco, CA). Cell supernatant and cell lysate preparations were clarified by centrifugation, normalized for protein concentration, and prepared for SDS-PAGE analysis in NuPage LDS sample loading buffer containing NuPage sample reducing agent (Invitrogen). Samples were separated on NuPage 4–12% Bis-Tris gradient gels (Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. Blots were blocked with 5% nonfat dry milk (BioRad, Hercules, CA) for 1 h and probed overnight at 4°C with primary antibody either to GFP (rabbit, 1:4000; Abcam; cat# ab290) or FLAG (mouse, 1:5000; Sigma; M2). Proteins were visualized by autoradiography using HRP conjugated rabbit (1:200,000) or mouse (1:50,000) secondary antibodies, respectively and Femto Supersignal chemiluminescent detection (Pierce Chemical). All antibody dilutions and washes were performed in TBS-0.05% Tween 20.

2.9. In vitro quantitative assays for CD8⁺ T cell recognition of MHC class I presentation of the CD8 Ova epitope

K^b restricted MC57G fibroblasts in 24 well plates were infected in triplicate with the indicated viruses at an MOI of 5. 6 h p.i. cells were washed in medium and cultured with 5×10⁵ cells of the B3Z CD8⁺ T cell hybridoma for 24 h. B3Z T cells express LacZ in response to recognition of the OVA CTL SL8 immunodominant epitope in the context of H-2K^b MHC class I molecules. Cells were lysed in passive lysis buffer (Promega, Madison, WI) and cell lysates were quantified for LacZ activity using chemiluminescent detection of Gal-Screen Reagent (Applied Biosystems, Carlsbad, CA) following 1 h incubation at 25°C. Chemiluminescence was quantified on a Zylux FB12 (Berthold Detection Systems, Huntsville, AL) tube luminometer and the results were expressed as a fold increase relative to wildtype HSV-1(RE) virus infected cells after uninfected cell control background subtraction.

3. Results

3.1. Generation and characterization of gK-null HSV-1 viruses incompetent for replication and cell-to-cell spread

Rescue of a deleted gene that conveys a replication defect while simultaneously transferring in a marker gene or expression cassette is an efficient method for generating recombinant viruses. It has previously been shown that deletion of the UL53/gK gene imparts a severe replication defect upon HSV-1 and other herpesviruses (Foster and Kousoulas, 1999; Foster et al., 1998; Hutchinson and Johnson, 1995; Jayachandra et al., 1997; Klupp et al., 1998; Mo et al., 1999) and that the UL53/UL54 intergenic region is a viable and stable region for insertion of exogenous expression cassettes (Foster et al., 1999). Therefore, a replication incompetent red fluorescent gK-null HSV-1(RE) virus was constructed to facilitate rapid and efficient isolation of wildtype-like recombinant viruses that specify gene expression cassettes from the UL53/UL54 intergenic region. A plasmid that specifically replaced the UL53/gK gene with a CMV-tdTomato gene cassette was constructed with HSV-1 sequences flanking 5' and 3' to facilitate deletion of the UL53/gK gene by homologous recombination (Fig. 1A). Recombinant viruses that exhibited bright red fluorescent plaques were isolated and plaque purified on gK-complementing CV1-HSV1gK cells. Viruses were plaque purified at least five times and tested by diagnostic PCR for the purity of the gK-null virus stock by assaying for the absence of the gK gene. Only singly infected cells were exhibited when the resulting tdTomato-expressing gK-null virus, REΔgK/tomato, was plaqued on non-complementing CV1 cells. (Fig. 1B: column 1). In contrast, REΔgK/tomato infection of CV1-HSV1gK cells, which complement gK *in trans*, displayed efficient cell-to-cell spread and egress similar to that of the wildtype virus (Fig. 1B: columns 2 and 3). Similar to what has been described previously for other herpesvirus gK-null mutants (Foster and Kousoulas, 1999; Foster et al., 1998; Hutchinson and Johnson, 1995; Jayachandra et al., 1997; Klupp et al., 1998; Mo et al., 1999), the REΔgK/tomato total viral yield was reduced by approximately 3 logs (Fig. 1C), while extracellular virus was reduced by nearly 4 logs relative to wildtype HSV-1(RE) virus (Fig. 1D). The inability of the REΔgK/tomato virus to replicate and spread in non-complementing cells provides a viable target vector system for marker rescue/marker transfer homologous recombination reactions. The large replication and plaquing differential between gK-null non-recombinant viruses and wildtype-like recombinant viruses has the potential to enable efficient transfer and rapid isolation of viruses that express model immunological antigens.

3.2. Construction of a marker rescue/marker transfer recombination plasmid that expresses exogenous proteins or model immunological epitopes

To facilitate rescue of the deleterious UL53/gK deletion, while simultaneously transferring in a gene cassette that constitutively expresses exogenous immunological epitope/EGFP chimeras, a marker rescue/marker transfer recombination plasmid was engineered (pHSV1-epirec), which contained the HSV-1 genomic regions of UL52, UL53 and UL54, as shown in Figure 2. A CMV-EGFP expression cassette was inserted within the UL53 and UL54 intergenic region. This marker cassette was constructed to specify a unique BamHI cloning site that enabled in-frame insertion of exogenous proteins or immunologic epitopes fused to the amino terminus of EGFP (Fig. 2B). Three well characterized immunological epitopes were created as 3x tandem repeats and inserted into the BamHI restriction site of the base recombination vector: 1) the 3x FLAG antibody epitope; 2) the 3x FLAG antibody epitope with a preprotrypsin signal leader sequence for directed secretion from cells; 3) a 3x tandem repeat of the SL8 chicken ovalbumin CTL epitope encompassing amino acids 257–264 (Shastri and Gonzalez, 1993). Each of these plasmid constructs exhibited bright green fluorescence when transfected into cells (data not shown) and were utilized for rescuing the replication incompetent REΔgK/tomato virus (Fig. 2E) while simultaneously deleting the

red fluorescent gene cassette and transferring into the viral genome the immunological epitopes/EGFP chimera expression cassette (Fig. 2D). As illustrated by the insertion of three model antigenic peptides, the base recombination pHSV1-epirec plasmid exhibits broad adaptability for insertion of foreign genes or model antigenic epitopes that are fused to the amino terminus of EGFP. Furthermore, the plasmid enabled real-time visualization of antigenic epitopes that were targeted for either constitutive intracellular or secreted expression.

3.3. Rapid isolation of pure recombinant HSV expressing immunological epitopes via stoplight purification

Traditional homologous recombination systems for generation and isolation of herpes viruses that encode an exogenous expression cassette can be a tedious and time consuming process. The incorporation of a stoplight recombination system facilitated rapid identification and purification of recombinant viruses that expressed specific exogenous immunological epitopes. Vero cells transfected with marker rescue/marker transfer plasmids encoding specific epitopes were infected with the red gK-null virus, REΔgK/tomato, to enable homologous recombination between the plasmid and virus. As depicted in Figure 2(D & E), rescue of the gK-null associated replication deficiency from the REΔgK/tomato virus simultaneously replaces the red CMV-tdtomato marker gene cassette with the CMV-epitope-EGFP cassette. This recombination event rescued wildtype-like replication and cell-to-cell spread, while enabling their identification by green fluorescence. 24 h p.i. infected cell supernatants were plated by limiting dilution on confluent Vero cells. Viral plaques were observed 24 to 48 h later by fluorescent microscopy to identify isolated recombinant viruses. On the first round of plating, four conditions were observed during stoplight purification as shown in Figure 3: 1) Non-recombinant gK-null viruses were observed as singly infected red fluorescing cells (Fig. 3, column 1). These viruses were avoided since no recombinant viruses were present. 2) The formation of yellow fluorescent viral plaques containing cells that were co-infected with both the red non-recombinant gK-null virus and the green recombinant virus (Fig. 3, column 2). Selection of these viruses should be avoided if possible or used with caution as these plaques contained both recombinant and non-recombinant viruses and require further purification. 3) Bright green fluorescent plaques that were not co-infected with REΔgK/tomato virus but had these viruses present in close proximity (Fig. 3, column 3). Although these viruses have the potential to be separated further, invariably, these viral plaques were contaminated with unwanted REΔgK/tomato virus. 4) Isolated large recombinant viral plaques that exhibited bright green fluorescence without the presence of any red non-recombinant virus (Fig. 3, column 4). Due to the three log increase in replicative ability of these recombinant viruses relative to the REΔgK/tomato virus, these large green plaques were often present in wells where the dilution factor was such that no red non-recombinant viruses were observed in the same well. This green “signal” without any intervening red “signals” instructs the investigator to proceed with isolation of pure recombinant viruses. Viral plaques that exhibited conditions as shown in column 4 and, where unavoidable, column 3 were picked and re-infected to Vero cells by limiting dilution for subsequent rounds of plaque purification. During round two of plaque purification, in very rare cases, red non-recombinant viruses were observed with green recombinant viruses (Fig. 3, panel A). These rare events were observed only if the titrated plaques were picked from conditions that had contaminating non-recombinant viruses nearby as seen in Fig. 3 column 3. However, the majority of round two plaques and all plaques observed by round three of purification exhibited only green recombinant virus with no indications of contaminating red non-recombinant virus (Fig. 3, panel B).

To confirm the ability of the stoplight recombination selection system to identify pure recombinant viruses, DNA from each round of viral isolation was tested by diagnostic PCR

for the presence of both the CMV-EGFP cassette specifying the exogenous immunological epitopes and contaminating non-recombinant REΔgK/tomato viruses (Fig. 4). The diagnostic PCR was engineered with a single forward primer (P5') and varying reverse primers utilized either to determine viral purity (P1 and P3) or to assay for the presence of specific immunological epitopes [P2(3xFLAG) and P2(3xOva)]. The relative positioning of binding sites for each of these primers were as depicted in Figure 2. The P5' forward primer hybridizes within the UL52/UL53 overlapping region enabling amplification of both recombinant and non-recombinant viruses and was utilized in all diagnostic PCR reactions (Fig. 2A & D). The P1 reverse primer hybridized within the CMV immediate early promoter (Fig. 2B & D); whereas the P3 reverse primer hybridized within either the EGFP or tomato gene cassettes (Fig. 2B, D, & E). The P5' forward primer paired with either the P1 (Fig. 4A) or P3 (Fig. 4B) reverse primer sets were employed separately to confirm both the presence of the recombinant epitope containing viruses and the absence of contaminating non-recombinant REΔgK/tomato virus after one or three rounds of plaque purification. Following direct plating of recombinant viruses (Fig. 4A & B, Round 1 Purification) the predominant viral DNA detected using both primer sets was the smaller PCR product specific for non-recombinant REΔgK/tomato viral DNA (solid arrowhead). The presence of the UL53/gK gene within recombinant viruses shifts the amplified PCR product upward by approximately 1350 additional nucleotides depending on the size of each specific immunological epitope as shown for the RE/EGFP control virus (unfilled arrowhead). In round one of purification, except for the slight amplification of the larger product in the RE/3xFLAG virus (Fig. 4A & B, asterisk), the presence of recombinant viral DNA was not identified readily. This is because PCR amplification has a predilection for amplification of smaller products rather than that of the recombinant viruses that contain an additional 1350 nucleotides inclusive of the UL53 coding region with its regulatory 3'UTR. However, if the reverse primer utilized did not bind within the REΔgK/tomato viral DNA and was specific for either the 3xFLAG or 3xCD8Ova epitopes (Fig. 4C & D, respectively) then the presence of specific recombinant viruses could be observed readily. Taken together, these data indicate that the virus stocks at round one of purification contained contaminating REΔgK/tomato virus as was observed by fluorescent microscopy analysis prior to viral DNA extraction. In contrast, by round three of plaque purification there was no longer any contaminating REΔgK/tomato viral DNA present and all amplified viral DNA appeared as the higher molecular weight recombinant viral DNA bands (Fig. 4A & B, Round 3 Purification). In concordance, only green recombinant virus was observed by fluorescent microscopy analysis by round 3 of viral DNA extraction. Therefore, the engineered stoplight recombination selection and identification system was effective at identifying the genetic presence and purity of the viruses specifying immunological epitopes.

3.4. Characterization of replication and spread of recombinant HSV isolates

In addition to a change in fluorescent markers from red to green, a primary indicator that the gK-null defect had been rescued is the ability of recombinant viruses to replicate and spread. As shown in Figure 1, the gK-null REΔgK/tomato virus demonstrated a defect in cell-to-cell spread (Fig. 1B, column 1), as well as a 3 log reduction in total viral yield (Fig. 1C) and a 4 log decrease in release of extracellular virus (Fig. 1D). In contrast, all immunological epitope-expressing recombinant viruses exhibited a plaque phenotype similar to that of wildtype HSV-1(RE) (Fig. 5B), indicating that the gK-null cell-to-cell spread and replication defects had been repaired. In addition, all of the immunological epitope-expressing viruses exhibited bright green fluorescence (Fig. 5C; E; F) with the exception of the RE/ss3xFLAG virus, which exhibited dim fluorescence (Fig. 5D). This difference in fluorescent intensity was most probably the result of the preprotrypsin signal peptide inducing the directed secretion from cells of the 3xFLAG/EGFP chimera. Quantitation of total and extracellular viral yield for all viruses at 24 and 48 h were similar to the parental RE wildtype virus yield

at the same time points (Fig. 6). This further demonstrated that these recombinant viruses exhibit characteristics of wildtype-like replication and cell-to-cell spread. Taken together, these results indicate that the recombination system efficiently repairs the UL53/gK deletion and transfers a functional expression cassette within the UL53/54 intergenic region without deleterious effects to HSV-1(RE) viral replication.

3.5. Confirmation of immunological epitope expression by recombinant HSV isolates

Immunoblot analysis of both cell lysates (Fig. 7A) and cell supernatants (Fig. 7B) was utilized to confirm expression of the exogenous immunological epitopes as a chimeric fusion with the EGFP protein. Two independent isolates of each recombinant virus were assessed to ensure that expression levels were similar between isolates and that the results were reproducible. As expected, no proteins were detected in either uninfected or parental RE wildtype (wt) infected cells, since they did not express either the EGFP protein or immunological epitopes. The RE/EGFP control virus expressed the EGFP protein in cell lysates at the expected molecular weight of 27 kDa, as detected using an anti-GFP antibody (Fig. 7A). Infection of cells with viruses that specified specific 3x tandem repeats of each immunological epitope fused to EGFP induced an increase in the observed apparent molecular weight when probed with the anti-GFP antibody (Fig. 7A). Infection of cells with the RE/3xCD8Ova virus revealed a band with an apparent molecular weight to 31 kDa. This increase in apparent molecular weight of approximately 3.5 kDa relative to the RE/EGFP control virus corresponded to the predicted molecular weight of the 3xCD8Ova immunological epitope. Similarly, a single band with an apparent molecular weight of approximately 33 kDa was observed in RE/3xFLAG infected cell lysates probed with either anti-GFP (Fig. 7A, top panel) or anti-FLAG (Fig. 7A, bottom panel) antibodies, corresponding to a shift in apparent molecular weight due to the presence of the 3xFLAG epitope. The RE/ss3xFLAG recombinant viruses expressed a similar 33 kDa protein; as well as, a second protein species of approximately 34 kDa; however, the intensity of detection was decreased significantly compared to other recombinant viruses. The two bands observed in RE/ss3xFLAG infected cell lysates and the relative decrease in signal intensity are due most likely to the cleavage of the 1 kDa preprotrypsin secretion signal peptide, and thereby the directed secretion of the 3xFLAG/EGFP chimera to cell supernatants, respectively. Accordingly, the presence of the 3xFLAG/EGFP chimeric protein was detectable in RE/ss3xFLAG infected cell supernatants but was absent in either parental RE wildtype or RE/3xFLAG infected cell supernatants (Fig. 7B).

3.6. Functional expression of CD8 OVA T cell epitopes

Although no specific antibody was available to demonstrate expression of the 3xCD8Ova immunological epitope in a manner similar to the FLAG epitope, western blot analysis indicated that the RE/3xCD8Ova virus expressed what appeared by molecular weight shift to contain a chimeric EGFP with a 3x tandem repeat of the CD8 Ova epitope (Fig. 7A). To assess functional CD8 Ova epitope expression and presentation, a T cell response assay was employed. B3Z CD8⁺ T cells, which express LacZ in response to recognition of the OVA CTL SL8 immunodominant epitope, were utilized to quantify presentation of the Ova epitope by H-2k^b MHC class I molecules (Karttunen et al., 1992; Shastri and Gonzalez, 1993). The parental HSV-1(RE) wildtype, RE/EGFP control and RE/3xFLAG viruses exhibited similar activation of B3Z T cells following infection of H-2k^b MHC class I restricted MC57G fibroblasts, signifying that there is only basal activation of LacZ expression in the absence of presentation of the CD8 Ova epitope (Fig. 8). In contrast, the RE/3xCD8Ova infected MC57G fibroblasts induced a nearly ten-fold increase in LacZ activity from B3Z T cells (Fig. 8), indicating that there was efficient MHC class I presentation of the CD8 Ova peptide following viral infection. These data demonstrate the

quantitative capacity of the CD8 Ova epitope-expressing virus for analysis of antigen presentation and subsequent T cell activation.

4. Discussion

This study describes a rapid and efficient method for generating and isolating recombinant herpesviruses that express exogenous immunological epitopes. Traditional homologous recombination methods are plagued by tedious and time consuming recombinant virus identification, isolation and plaque purification. Alternative methods for generating recombinant herpesviruses, such as the use of herpes viral genomes within a bacterial artificial chromosome (BAC), are limited in availability to only a couple of HSV-1 strains (Gierasch et al., 2006; Horsburgh et al., 1999). For the study of herpetic stromal keratitis, specific HSV-1 strains result in different disease phenotypes with HSV-1(RE) being associated with a high efficiency of corneal keratitis formation (Hendricks et al., 1989; Hendricks and Tumpey, 1990). Furthermore, BAC-systems still require several series of confirmation following both recombination and virus generation (Gierasch et al., 2006; Horsburgh et al., 1999). Therefore, a stoplight homologous recombination method for generation of HSV-1(RE) recombinant viruses was engineered that takes advantage of a “traffic-light” approach to provide a visual indicator of the presence and purity of recombinant HSV-1 isolates expressing immunological epitopes. Importantly, the expression cassette inserted within the HSV-1 UL53/UL54 intergenic region did not interfere with either viral replication or cell-to-cell spread and resulted in a phenotype that was virtually indistinguishable from wildtype HSV-1(RE). Although it still must be determined if the epitope expressing viruses retain their pathogenic profile, insertion of an expression cassette within this region of other HSV-1 strains did not adversely affect their pathogenic phenotypes (Bhattacharjee et al., 2008; David et al., 2008).

A hallmark of this HSV-1 recombination and viral identification system is the engineered marker rescue/marker transfer vector that incorporates “stoplight recombination” indicator markers. These fluorescent markers produced three identifying signals that visually designated the presence and purity of recombinant HSV-1 isolates: 1) a red fluorescent “stop signal” that indicated the presence of non-recombinant viruses. 2) a yellow fluorescent “caution signal” that indicated co-purifying recombinant and non-recombinant viruses. 3) a green “proceed signal” that, when completely isolated, indicated a pure recombinant virus isolate that could be used for the preparation of virus stocks. As demonstrated by diagnostic PCR, these combined indicators yielded a rapid and efficient method for generating and isolating recombinant herpesviruses that express exogenous immunological epitopes. However, this system is not limited to immunological epitopes. The base recombination plasmid is flexible in that it can also be employed for the construction of recombinant viruses that express full exogenous proteins. In this regard, recombinant viruses can be engineered to express immune modulating factors such as cytokines and chemokines, and the effect of these immune modulators on host responses can be determined. In addition, the fusion of a protein of interest to the amino terminus of GFP can enable the real-time tracking, visualization and localization of that protein within infected cells.

The model immunological epitopes chosen to demonstrate the adaptability of the system will prove useful in studying host immune effectors, including but not limited to responses by B cells, dendritic cells, macrophages, CD4⁺ T cells and CD8⁺ T cells. It is of note that immunological epitopes were able to be targeted for expression to different cellular locations: 1) to the extracellular spaces, which may facilitate endosomal uptake, MHC class II presentation, and subsequent CD4⁺ T cell responses; 2) to the intracellular cytoplasmic compartment, which was established as advantageous for classical MHC class I presentation and CD8⁺ T cell responses. Infection of cells with the RE/3xCD8Ova recombinant virus,

which expressed the well characterized SL8 OVA CTL epitope, was utilized to assess MHC class I presentation and subsequent CD8⁺ T cell responses by an *in vitro* quantitative assay. Recognition of the 3xCD8Ova peptide by the B3Z T cell hybridoma, which expressed LacZ in response to Ova presentation, could be quantified both efficiently and sensitively by determination of relative LacZ enzymatic activity. This assay enabled the relative measurement of MHC class I antigen presentation and T cell responses in the context of an HSV-1 infection.

HSV-1 has been shown to encode several viral proteins that function to subvert both MHC class I and class II presentation (Brandt et al., 2003; Jugovic et al., 1998; Neumann et al., 2003; Smith et al., 2002; Tomazin et al., 1996). For example, the UL41 gene that encodes for the virion host shutoff (VHS) protein has been implicated in altered MHC class I presentation and is the only identified determinant of corneal virulence (Brandt, 2005; Brandt et al., 2003). However, if other human herpesviruses are any indication (Mocarski, 2004; Pinto and Hill, 2005; Yewdell and Hill, 2002), many additional HSV-1 viral proteins that alter MHC presentation remain to be identified. The above described B3Z T cell assay can be utilized for identifying viral genes that regulate MHC class I antigen presentation and/or CD8⁺ T cell responses. Viral genes suspected of contributing to altered MHC class I presentation can be deleted from the HSV-1(RE)/3xCD8Ova virus. Cells infected with these deletion viruses can be compared with the B3Z T cell responses to wildtype-like HSV-1(RE)/3xCD8Ova virus infection to determine if these deleted viral proteins contribute to evasion of MHC class I antigen presentation and/or subsequent CD8⁺ T cell responses.

These reagents and assays are particularly relevant for understanding the development of herpetic stromal keratitis. Herpetic stromal keratitis is an immunopathologic disease characterized by an inappropriate T cell response (Doymaz et al., 1991; Doymaz and Rouse, 1992a; Doymaz and Rouse, 1992b; Gangappa et al., 2000; Metcalf et al., 1979; Metcalf and Kaufman, 1976; Newell et al., 1989b; Niemialtowski et al., 1994; Russell et al., 1984). However, the mechanisms by which CD4⁺ and CD8⁺ T cells contribute to herpetic stromal keratitis remains an intense area of investigation (Gangappa et al., 2000; Ghiasi et al., 2000; Lepisto et al., 2006; Osorio et al., 2004; Stuart et al., 2004). Transgenic mice that have T cells responsive either to the immunodominant CD8 SL8 Ova epitope (OT-I mice) (Hogquist et al., 1994) or the immunodominant CD4 Ova epitope (OT-II mice) (Barnden et al., 1998) have become an essential component in characterizing T cell responses to viral infections (Deshpande et al., 2001; Gangappa et al., 2000; Thomas et al., 2010). The wildtype-like replication and cell-to-cell spread properties of the recombinant viruses enables their use in established herpetic stromal keratitis animal models that utilize these transgenic animals. The HSV-1(RE)/3xCD8Ova virus and/or a virus constructed to express the CD4⁺ T cell OVA immunological epitope would be powerful reagents for assessing T cell responses during HSV-1 infection and may assist in improving understanding of herpes-associated ocular disease development.

Acknowledgments

We would like to acknowledge Dr. Barry Rouse for the inspiration in creating the recombinant viruses specifying various immunological epitopes. We thank Drs. Ashok Aiyar and James Hill for insightful discussions on additional experiments and professional guidance. We especially thank Dr. Augusto Ochoa and the Stanley S. Scott Cancer Center for critical financial and professional development support. This work was supported by a Louisiana Board of Regents Research Competitiveness Award LEQSF-RD-A-13 and by a National Institutes of Health Award Number P20RR021970 from the National Center for Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

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Highlights

A system for producing a recombinant HSV-1 expressing exogenous epitopes was created.

Traffic light inspired fluorescent markers gauged the purity of recombinant viruses.

Adaptability was demonstrated by generating recombinants for 3 immunologic epitopes.

Recombinants were rapidly identified and isolated to purity via stoplight indicators.

Presentation of a CD8 epitope was quantified using a T cell hybridoma reporter cell.

Figure 1.

Construction and characterization of a red UL53/gK null virus expressing the tdTomato fluorescent protein. (A) Depiction of the prototypical arrangement of the HSV-1 genome with approximate map units. Shown below is an amplified representation of the UL52, UL53 and UL54 genomic region where the UL53/gK gene is deleted via insertion of the tdTomato expression cassette. The virus resulting from this homologous recombination event is the red fluorescent gK-null HSV-1(RE) virus (REΔgK/tomato). (B) Fluorescent micrographs of the red gK-null virus (columns 1 and 2) or an EGFP expressing wildtype virus (RE/EGFP, column 3). Virus was plaqued on Vero cells (columns 1 and 3) or on CV1-HSV1gK cells, which complemented *in trans* the growth defect associated with the UL53/gK gene deletion (column 2). (C and D) One step growth kinetics of total (C) or extracellular (D) viral yield for wildtype (open circles) or gK-null (open triangles) viruses.

Figure 2.

Construction of recombinant viruses constitutively expressing model exogenous immunological epitopes fused to the amino terminus of EGFP. (A) Schematic of the base marker rescue/marker transfer recombination plasmid containing the HSV-1 genomic regions for UL52, UL53 and UL54. (B) A CMV-EGFP expression cassette was inserted into the intergenic region of the rescue plasmid between the UL53/gK and UL54/ICP27 genes such as to not interrupt any viral gene expression. This cassette contained a unique BamHI cloning site for in-frame insertion of proteins at the GFP amino terminus. (C) Representation of the three tandem repeats of the described epitopes that were inserted into the unique BamHI restriction site to facilitate expression of the immunological epitopes/EGFP chimeras. (D and E) Depiction of homologous recombination between gK-null viruses (E) and the epitope-containing plasmids (D) that facilitates the rescue of the red replication deficient gK-null virus and simultaneously transfers into the viral genome the model immunological epitopes/EGFP chimera expression cassette. Relative binding sites and amplification direction for diagnostic primers are indicated by arrows.

Figure 3.

Rapid isolation of recombinant viruses that express immunological epitopes fused to EGFP via stoplight recombination. Representative fluorescent micrographs of dilutions from the initial plating following marker rescue/marker transfer recombination. Left panels depict the four conditions observed: Non-recombinant, all red gK-null viruses (column 1); cells co-infected with co-purifying red gK-null and green recombinant viruses (column 2); not co-infected but only partially isolated recombinant viruses with red gK-null viruses in close proximity (column 3); isolated and apparently pure recombinant viruses (column 4). Right panels depict representative fluorescent micrographs of recombinant virus plaques following rounds 2 and 3 of plaque purification.

Figure 4.

Diagnostic PCR of recombinant viruses after plaque purification rounds 1 or 3 to determine presence of immunological epitopes and absence of gK-null contaminating virus. (A) Primer pairs P5'/P1 were utilized to detect the rescue of the UL53/gK gene in the recombinant viruses (open arrowhead) and the absence of gK-null contaminating virus (filled arrowhead). (B) Primer pairs P5'/P3 detected the presence of the EGFP gene cassette within the recombinant viruses (open arrowhead) and the absence of gK-null contaminating viruses (filled arrowhead). Starred band in A & B depicts a recombinant virus isolate that exhibited simultaneous amplification of both the gK-null genomes and recombinant virus genomes. (C & D) Primer pairs P5'/P2(3xFLAG) detected the presence of the 3xFLAG epitope (C) while primer pair P5'/P2(3xOva) detected the presence of the 3xCD8Ova epitope within recombinant virus isolates.

Figure 5.

Comparison of the plaque phenotypes of isolated recombinant viruses to the parental HSV-1(RE) wildtype virus. Vero cell monolayers were mock infected (A) or infected with the parental HSV-1(RE) virus (B), the EGFP control virus that specified no immunological epitopes (C), the RE/ss3xFLAG virus (D), the RE/3xFLAG virus (E) or the RE/3xCD8Ova virus (F). 48 h p.i. viral plaques were visualized by phase and fluorescent microscopy and representative viral plaques were imaged and merged. (*) For the secretion signal containing 3x FLAG epitope virus, RE/ss3xFLAG (D) capture of the EGFP fluorescent image was twice as long as all other fluorescent exposures of viral plaques.

Figure 6.

Comparison of total (A) and extracellular (B) viral yields by one step growth analysis. The parental HSV-1(RE) wildtype, RE/ss3xFLAG, RE/3xFLAG, RE/3xCD8 Ova and RE/EGFP control viruses were infected to Vero cells and at 24 and 48 h p.i. viral titers were determined from cell supernatants (extracellular) or cell lysates and supernatants (total).

Figure 7.

Detection of immunological epitope expression by recombinant viruses in infected cell lysates (A) and/or cell supernatants (B) by Western Blot analysis. The specific expression of immunological epitopes by the indicated recombinant viruses was indirectly detected (α GFP) by a shift in GFP's apparent molecular mass (RE/EGFP control lane) caused by the fusion of the specified immunological epitope to the amino terminus of GFP. In addition, for the virus isolates that specified 3xFLAG immunological epitopes the presence of the FLAG epitopes were directly detected by an anti-FLAG monoclonal antibody (α FLAG).

Figure 8.

Quantitative assessment of OVA-specific CD8⁺ T cell responses to HSV-1(RE) viruses expressing immunological epitopes. MC57G fibroblasts were infected with either the wildtype HSV-1(RE) strain or the indicated immunological epitope expressing viruses. Infected cells were subsequently co-cultured with the LacZ-inducible, OVA/K^b-specific B3Z T cell hybridoma at an effector: target ratio of 1. Activation of B3Z T cells were quantified by a chemiluminescent beta-galactosidase assay and the fold induction of enzymatic activity was determined relative to wildtype HSV-1(RE) infected cells. The experiments were performed in triplicate and the mean $\pm$ standard deviation is reported.