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Virology. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

Virology. 2016 September ; 496: 186–193. doi:10.1016/j.virol.2016.06.006.

Production of Immunogenic West Nile Virus-like Particles using a Herpes Simplex Virus 1 Recombinant Vector

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Abstract

West Nile virus (WNV) is a flavivirus that swept rapidly across North America in 1999, declined in prevalence, and then resurged in 2012. To date, no vaccine is available to prevent infection in the human population. Herpes simplex virus (HSV) replication-defective vaccine vectors induce a durable immunity characterized by strong antibody and $CD8^+$ T cell responses even in HSV-immune animals. In this study, a WNV protein expression cassette was optimized for virus-like particle (VLP) production in transfection studies, and the cassette was recombined into an HSV-1 *d*106-WNV virus vector, which produced extracellular VLPs, as confirmed by immunoelectron microscopy. Immunization of mice with the *d*106-WNV recombinant vector elicited a specific anti-WNV IgG response. This study highlights the flavivirus coding sequences needed for efficient assembly of virus-like particles. This information will facilitate generation of additional vaccine vectors against other flaviviruses including the recently emerged Zika virus.

Keywords

West Nile virus; vaccine vector; Flavivirus; Zika virus; HSV recombinant vector

Introduction

The North American distribution of the flavivirus West Nile virus (WNV) expanded dramatically after its introduction into New York in 1999. It is now found throughout the

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United States, southern Canada, Central and South America, and the Caribbean (Cruz et al., 2005; Dauphin et al., 2004; Estrada-Franco et al., 2003; Komar, 2003; Reisen et al., 2004). WNV, which is transmitted primarily through *Culex* mosquito bites (Kulasekera et al., 2001), is spread to new areas *via* infected birds. WNV has caused significant human disease in the United States with estimates of 780,000 illnesses from 1999–2010 (Petersen et al., 2013), and elderly individuals are at greatest risk of neuroinvasive disease (Carson et al., 2012; Hayes et al., 2005). Neuroinvasive disease cases peaked in 2002–2003 and then declined until 2012 when WNV re-emerged with over 2800 neuroinvasive cases in the United States (Centers for Disease Control and Prevention, 2013). With the wide distribution and significant human morbidity associated with WNV, it continues to be an important target for vaccine development.

Despite extensive efforts, no effective WNV vaccine is approved to protect the susceptible human population (Heinz and Stiasny, 2012; Volz et al., 2016); however, several vaccine approaches for animals or humans have been pursued, including inactivated virus (Samina et al., 2005); chimeric attenuated flavivirus viruses expressing WNV premembrane (prM) and envelope (E) proteins (Dayan et al., 2012; Monath et al., 2006); WNV virus-like particles (VLPs) (Qiao et al., 2004); pox vectors (Heinz and Stiasny, 2012; Siger et al., 2006); a lentivirus vector (Iglesias et al., 2006); a subunit vaccine (Chu, Chiang, and Ng, 2007; Watts et al., 2007); and DNA vaccines (Yang et al., 2001). The WNV proteins prM and E are necessary and sufficient for VLP production. When prM and E are expressed together within a cell, they self-assemble into VLPs that are released into the extracellular environment (Allison et al., 1995). VLPs derived from the co-expression of prM and E in cell culture systems independent of other viral factors are structurally and antigenically similar to genuine West Nile virions and have been shown to elicit neutralizing antibody titers in immunized mice. The protease required for cleaving E from prM is cellular, so E is released as a separate protein when expressed along with prM.

We have previously generated replication-defective HSV vaccine vectors expressing simian immunodeficiency virus (SIV) proteins that successfully protected non-human primates against mucosal challenge with virulent SIV (Kaur et al., 2007; Watanabe et al., 2007). Replication-defective HSV vectors are attractive because of their safety, as demonstrated in animal models (Hoshino et al., 2008), and ability to induce durable immune responses that include both B and T cell responses (Brehm et al., 1999; Brehm et al., 1997; Brockman and Knipe, 2002; Brubaker et al., 1996; Da Costa et al., 2000; Da Costa, Jones, and Knipe, 1999; Dudek and Knipe, 2006; Jones, Taylor, and Knipe, 2000; Kaur et al., 2007; Morrison, Da Costa, and Knipe, 1998; Murphy et al., 2000; Watanabe et al., 2007), and they are immunogenic even in the face of pre-existing HSV immunity (Brockman and Knipe, 2002). The replication-defective HSV-1 *d*106 virus contains multiple deletions that remove the coding sequences of the immediate-early proteins ICP4 and ICP27 and the promoter regions of the *ICP22* and *ICP47* genes (Samaniego, Neiderhiser, and DeLuca, 1998). The loss of ICP4, ICP27, and ICP22 results in a dramatic decrease in the number of HSV proteins expressed during infection on non-complementing cells; however, *d*106 expresses the immediate-early ICP0 protein, which stimulates heterologous protein expression from transgenes encoded within the viral genome through its effects on the chromatin associated with the HSV genome (Cliffe and Knipe, 2008; Lee, Raja, and Knipe, 2016). We have

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therefore applied the HSV vector technology to the design of a WNV vaccine, and in this study we describe a recombinant replication-defective herpes simplex virus (HSV) vector, *d*106-WNV, which expresses the WNV structural proteins prM and E and part of the capsid (C) protein.

Results

With the continued prevalence of WNV, there is renewed interest in developing a safe and effective human WNV vaccine. To determine if a replication-defective HSV vaccine vector could serve as a candidate for flavivirus vaccine development, we constructed a recombinant HSV vaccine vector that expresses the WNV structural proteins premembrane (prM) and envelope (E).

Design of WNV expression constructs

The WNV structural proteins prM and E require a signal sequence for proper orientation in the host cell membrane (Lindenbach, Thiel, and Rice, 2007). During natural infection, this signal sequence is derived from the C-terminal transmembrane region of the capsid (C) protein (Figure 1A). We first generated plasmid expression constructs expressing WNV E protein that lacked or contained a leader sequence for comparison with the WT C-prM-Env plasmid containing the coding sequences for the structural region starting with the methionine of the C protein (Figure 1B): 1. The EnvAss construct lacked a signal sequence. 2. The E-3XFlag construct expressed a modified WNV E protein containing the preprotrypsin leader sequence for proper membrane orientation and three consecutive FLAG epitope tags (Figure 1, Table 2).

The constructs were evaluated for levels of protein expression and the ability to release virus-like particles (VLPs) into the supernatant in Vero cells transfected with the plasmids. The C-prM-E plasmid showed the highest levels of full-size E protein (Figure 2A, lane 4). The E-3XFlag and Env ss proteins showed a ladder of bands on Western blots, possibly due to proteolytic cleavage (Figure 2A, lanes 2 and 3, respectively), compared to the E protein produced by C-prM-E (Figure 2A, lane 4). Therefore, the natural signal sequence seemed to be the best for stable expression of full-size E protein.

Because of the potential stability issue described above, we next determined the optimal sequences from the C coding sequences necessary for VLP production. We therefore constructed the prM-E1, prM-E2, and prM-E3 plasmids expressing prM and E fused to varying portions of C, residues 1–123, 94–123, 78–123, or 106–123 (Figure 1B and Table 2), to determine which region provided the best expression and protein maturation. The properties of the various constructs are shown in Figure 2B and summarized in Table 2. The prM-E1–3 constructs (Figure 2B, lanes 4–6) expressed more E protein than the E-3XFlag and C-prM-E constructs (Fig, 2B, lanes 2 and 3, respectively), as measured by Western blotting for E. While including most of the amino acid residues found at the end of the capsid sequence dramatically increased expression levels (Figure 2B), the highest production of VLPs from the cells was observed with only residues 106–123 of C fused to prM-E in prM-E3 (Figure 2B, lane 12), as measured by the presence of E in the extracellular media.

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We therefore used the prM-E3 construct to generate a recombinant HSV strain expressing WNV proteins.

Construction of an HSV-1 d106-WNV recombinant vector expressing WNV proteins

We next constructed a recombinant HSV strain expressing the prM and E proteins of WNV. Homologous recombination was used to introduce the prM-E3 expression cassette into the U_L54 region of *d*106 virus to generate the *d*106-WNV virus, as described in Materials and Methods (Figure 1C). We examined the kinetics of E protein expression and VLP production by infecting Vero cells with the *d*106-WNV virus at various MOIs for 24–72 h. The highest level of E expression were observed in cell lysates at 72 h post-infection (hpi) in cells infected at an MOI of 10 (Figure 3, lanes 2–4). Even with this high MOI infection, most of the Vero cells were viable at 72 hpi, as detected by the lack of visible CPE (results not shown), likely due to the limited HSV gene expression of the *d*106 virus in non-complementing cells (Watanabe et al., 2007). Virus-like particles were measured by the presence of sedimentable E protein in the extracellular supernatant (Figure 3A, lanes 6–14). The highest level of VLP accumulation was observed at an MOI of 10 at 72 hpi (Figure 3A, lane 14). Therefore, the *d*106-WNV recombinant vector expressed high levels of WNV E protein both intra- and extracellularly.

The structure of the extracellular particles containing E protein was determined by electron microscopy of VLP preparations stained with anti-E mAb (Figure 3B). Most of the particles were stained by anti-E antibody and were approximately 28 nm inner diameter surrounded by glycoproteins and bound antibodies (Figure 3B). The presence of E protein in VLPs was further confirmed by mass spectrometry analysis. Proteins in a VLP preparation were resolved by SDS-PAGE, and a Coomassie blue-stained band in the estimated size range of E (not shown) was excised from the gel and subjected to mass spectroscopic analysis. Multiple peptides specific to E protein were identified (Figure 3C), thus confirming the presence of E in extracellular particles in the medium.

Elicitation of anti-WNV antibodies in immunized mice—We examined the ability of the *d*106-WNV recombinant vector to elicit a specific anti-WNV response in immunized Balb/c mice. Mice were immunized subcutaneously with doses of 2×10^6 PFU of the *d*106-WNV recombinant vaccine vector at days 0, 21, and 42. At the times shown, serum samples were drawn to measure antibody responses. While control mice and mice immunized with *d*106-WNV showed no WNV-specific IgG at 7 days, 3 of 8 mice immunized with *d*106-WNV showed significant titers at 28 days and 7 of 8 showed significant titers at 49 days (Figure 4). Qualitative neutralization assays showed the presence of WNV neutralizing antibodies in the sera from the immunized mice (results not shown). Therefore, the *d*106-WNV recombinant vector elicited a specific anti-WNV IgG antibody response in the immunized mice.

Discussion

In this study, we used a transfection assay to define and optimize a WNV expression cassette that efficiently expresses WNV E protein and forms VLPs. We then constructed and characterized a replication-defective HSV recombinant virus, *d*106-WNV, which expresses

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the WNV prM and E proteins fused to C-terminal residues of the C protein. Vero cells infected with *d*l06-WNV virus secreted VLPs that contained E, and immunization of mice with *d*l06-WNV virus induced a specific anti-WNV IgG response. We will discuss each of these points individually and how these results will provide important information for future vaccine vectors Optimization of WNV Expression Cassettes for VLP Production. We used a transfection approach to define the sequences from the C protein needed to serve as a secretion signal for the prM and E proteins and to optimize WNV protein expression and VLP release from infected cells. Previous studies of the flavivirus proteins (Allison et al., 1999; Chang, Hunt, and Davis, 2000; Fonseca et al., 1994; Ishikawa et al., 2007; Kojima et al., 2003; Konishi et al., 2000; Takahashi et al., 2009) have found that a sequence from the C-terminus of the C protein is needed to serve as a signal sequence for PrM and for efficient E protein accumulation and assembly into VLPs, but the optimal sequence had not been defined. Our analysis showed that the 18 C-terminal residues of C protein were the most efficient for E protein accumulation and VLP formation. This expression cassette also generated VLPs in an HSV *d*l06 recombinant virus. As in cells transfected with plasmids encoding the WNV proteins, cellular proteases cleave E from prM so E is released as a separate protein, and the HSV vector did not inhibit this process.

Long-term Expression of WNV proteins by the *d*l06 vector. We observed that *d*l06-WNV-infected Vero cells accumulated high levels of E protein through at least 72 hpi. The *d*l06 virus does not express several major immediate-early regulatory proteins, ICP4, ICP27, and ICP22, resulting in a limited HSV gene expression profile with few HSV proteins detected post-infection. Although the *d*l06 virion contains the virion host shut off (U_L41) protein, vhs, ICP27 is also needed for shutoff (Song et al., 2001). The limited HSV expression profile also decreases the cytopathogenicity of the vector allowing.prolonged expression of the transgene.. Because Vero cells do not express type I interferons (Desmyter, Melnick, and Rawls, 1968; Mosca and Pitha, 1986), it is conceivable that type I interferon production in normal cells would limit *d*l06S viral gene expression. However, ICP0 expressed by *d*l06 virus can inhibit interferon-β by several mechanisms (Melroe, DeLuca, and Knipe, 2004; Melroe et al., 2007; Orzalli, DeLuca, and Knipe, 2012). Therefore, this is unlikely to be a factor affecting *d*l06-encoded transgene expression in normal cells.

Immunogenicity of the HSV-1 *d*l06 Vector. The HSV-1 *d*l06S-WNV virus was immunogenic in that mice immunized with three doses of the *d*l06S-WNV recombinant virus nearly all seroconverted. Although this could appear to be less efficacious than other immunogens that used less viral vector or fewer immunizations to achieve complete seroconversion (Dayan et al., 2012; Iglesias et al., 2006; Monath et al., 2006), it is hard to compare these results because the sensitivities of the ELISA assays may be different. Furthermore, some of the vaccine constructs, such as the ChimeriVax viruses, are replication-competent, so they will spread and therefore be more immunogenic than a replication-defective virus. Complete head-to-head comparisons of the immunogenicity and safety of the various WNV vaccine candidates are needed.

Protective Immune Responses Against WNV. It is known that a neutralizing antibody response is required to protect against WNV infection and that CTL activity is critical for WNV clearance of infected animals (Chu, Chiang, and Ng, 2007; Diamond et al., 2003a;

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Diamond et al., 2003b; Mehlhop et al., 2005; Shrestha and Diamond, 2004; Wang et al., 2003). Replication-defective HSV strains induce durable immune responses with induction of both antibody and CD8+ CTL responses (Brehm et al., 1999; Brehm et al., 1997; Brubaker et al., 1996; Da Costa et al., 1997; Kaur et al., 2007; Morrison, Da Costa, and Knipe, 1998; Morrison and Knipe, 1997; Murphy et al., 2000). Also, antigens expressed by replicative-defective HSV recombinants are acquired by dendritic cells at the site of inoculation that then traffic to the lymph nodes for antigen presentation (Watanabe et al., 2007; Zhao et al., 2003). We observed that expression of the E protein without prM or a signal sequence resulted in proteolytic cleavage of the expressed E protein. The internal expression of E protein may enhance presentation of E epitopes on class I MHC; therefore, HSV vectors that express E protein that assembles into VLPs or E protein that accumulates intracellularly should be tested for induction of E-specific CD8+ CTLs. Therefore, one or both of these vectors should induce a broader immune response than just administering VLPs alone. In this study, *d*106-WNV-immunized mice produced anti-WNV antibody, and further studies will be needed to determine the breadth of immune responses and protection of animals against lethal WNV challenge induced by different vectors.

Because HSV vectors induce a Th1 response in immunized animals (Brubaker et al., 1996; Nguyen, Knipe, and Finberg, 1994), they may provide a useful prime immunization in prime-boost protocols. We have observed that optimal protection against SIV infection was observed with *d*106 vectors providing the priming immunization followed by a heterologous boost (Kaur et al., 2007; Kaur et al., in preparation). Therefore the HSV-1 *d*106 WNV vector might be a useful and safe prime for the more well-developed, but replication-competent chimeric flavivirus WNV vaccine (Dayan et al., 2012) or vesicular stomatitis virus vector (Marzi et al., 2015).

In conclusion, this study provides the blueprint for the design of expression cassettes expressing flavivirus proteins that efficiently form virus-like particles. Inclusion of the 18 C-terminal residues of the C protein provides an effective signal sequence that promotes membrane insertion and formation of VLPs. This strategy should be readily adaptable to other flaviviruses including Zika virus. This study also demonstrates that a replication-defective HSV vector can express WNV structural proteins that form extracellular virus-like particles. This vector can induce a specific anti-WNV IgG antibody response in immunized mice. Further research is required to optimize the dose and route of inoculation to determine what combination is required to optimally protect against lethal challenge with WNV or other flaviviruses.

Materials and Methods

Cells and viruses

African green monkey kidney (Vero) cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with 5% fetal bovine serum-5% newborn calf serum. The E11 cell line, which expresses ICP27 and ICP4 and complements replication of HSV-1 *d*106 virus (Samaniego, Neiderhiser, and DeLuca, 1998), was maintained in DMEM 5% fetal bovine serum-5% bovine calf serum + G418 (400 (μg/ml).

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Plasmids

To construct the *d*106-WNV recombinant vaccine vector, we first cloned WNV coding sequences into the CMV expression vector pCI AflII (Murphy et al., 2000). DNA copies of WNV coding sequences were derived from RNA preparations of infected cells provided by Sharon Isern and Scott Michael (Florida Gulf Coast University), using the One-Step RT-PCR process (Qiagen) with the primers shown in Table 1, and were cloned into the pCR2.1 TA TOPO vector (Invitrogen). Plasmids pCR2.1prM-E1, prM-E2, and prM-E3 were generated using the indicated forward primers (Table 1) and the reverse primer R-Sal. Subcloning of the *Eco*RI fragments from the pCR2.1-based plasmids into the pCI AflII plasmid generated the plasmids pCMVprM-E1, prM-E2, and prM-E3. pCMVEnv ss and pCMV-C-prM-E were constructed in a similar fashion using primer pairs F2-Eco/R-Sal and CF/R-Sal, respectively. The plasmid used to generate the recombinant *d*106-WNV virus via homologous recombination was derived from the pPs27pd1 plasmid (Rice, Su, and Knipe, 1989), which contains the HSV genomic sequences surrounding the U_L54 *(ICP27)* gene. The *Sal*I site in pPs27pd1 was converted to a *Bgl*II site by inserting a linker (New England Biolabs). The *BamHI/BglII* fragment from pCMVprM-E3 was then subcloned into the *Bgl*II site to generate the pd27-WNV plasmid, which contained the prM-E3 expression cassette in the opposite orientation of U_L54. The plasmid pCMV-E-3XFlag expressing WNV E with a preprotrypsin leader was the kind gift of K.G. Kousoulas (Louisiana State University).

Isolation of the *d*106-WNV recombinant virus vector

Infectious *d*106 viral DNA purified from infected E11 cell lysates by sodium iodide gradient centrifugation (Walboomers and ter Schegget, 1976) was co-transfected with varying amounts of linearized pd27-WNV into E11 cells using Lipofectamine 2000 (Invitrogen). The resulting progeny viruses were expanded before plaque purification. Potential recombinants were screened for by the loss of the green fluorescent protein (GFP) signal in individual plaques using an inverted fluorescence microscope (Nikon). Recombinants were confirmed by the detection of WNV E protein in cell lysates by Western blot analysis. *d*106-WNV was triple-plaque purified, and a stock was grown on E11 cells.

Western blot analysis

For analysis of transfected cells, confluent Vero cells in 6-well plates were transfected with 2 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen). For analysis of infected cells, Vero cells in 6-well plates were infected with *d*106-WNV at the indicated multiplicity of infection (MOI). Supernatants from 6-well plates were clarified by centrifugation at 15,000 rpm for 30 minutes at 4°C. Proteins were resolved in 12% polyacrylamide gels and analyzed by western blotting as described previously (Watanabe et al., 2007), using an anti-E monoclonal antibody (Chemicon) as the primary antibody.

Mice and immunizations

Animal studies were performed in accordance with Harvard University and National Institutes of Health guidelines. BALB/c mice (Jackson Laboratories) were immunized with 2×10^6 PFU of *d*106-WNV (8 mice) subcutaneously followed by two booster immunizations at days 21 and 42. Immunizations consisted of virus stock diluted into a volume of 20 μl of

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sterile 0.9% NaCl solution (Sigma). At 0, 7, 28, and 49 days, 100 μl of blood was drawn from each mouse via the tail vein. Sera were prepared using microtainer serum separators (Beckton Dickinson) and stored at −70 °C until use. Enzyme-linked immunosorbent assays (ELISA) were conducted to measure WNV-specific IgG as described previously (Brown et al., 2007) using WNV-infected Vero cell lysates (WNV antigen) or mock-inoculated Vero cell lysates (control antigen) for coating ELISA plates. Serum samples were tested at a dilution of 1:100 in the same assay. The relative OD values were calculated by dividing the mean optical density (OD) value from the WNV antigen-coated wells by the OD value from control antigen-coated well. Samples were considered positive if the relative OD value was greater than 2.

Purification and analysis of WNV virus-like particles

Vero cells in 150-cm^2 flasks were infected with the *d*106-WNV virus at an MOI of 10. At three days post-infection, the supernatant was clarified by centrifugation in a Sorvall SA-600 rotor at 4 °C for 30 minutes at 15,000 rpm. The VLPs were pelleted from the clarified supernatant by centrifugation in a Beckman 50Ti rotor at 4 °C overnight at 50,000 rpm. The pellet was resuspended in 1 ml of phosphate-buffered saline. VLPs were stored at −70 °C until use. The structure of VLPs was determined by visualization in a JEOL 1200EX transmission electron microscope following incubation with anti-E mAb and goat anti-mouse antibody complexed with protein A-gold and negative staining. VLP proteins were analyzed by western blotting for E protein and by mass spectrometry at the Taplin Biological Mass Spectrometry Facility, Harvard Medical School by microcapillary liquid chromatography-tandem mass spec with an LCQ DECA ion-trap mass spectrometer (Thermo Finnigan).

Acknowledgments

We thank Kim Appler for her technical assistance with the antibody analysis, Maria Ericsson for her assistance with electron microscopy, Gus Kousoulis for plasmids, and Patrick T. Waters for assistance with preparation of the manuscript. This research was supported by NIAID grants U54-AI057159 (New England Regional Center for Excellence in Biodefense and Emerging Infections) and U54-AI057158 (Northeast Biodefense Center) and R01 AI057552 to DMK and U19-AI109740 to SPJW.

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Research Highlights

- Defined optimal conditions for West Nile virus virus-like particle formation.
- Optimal signal sequence was 18 C-terminal residues of capsid (C) protein for E protein expression.
- Expression cassette of signal sequence-PrM-E was recombined into an HSV-1 vector.

HSV-1 recombinant vector expressed E protein that was assembled into VLPs and was immunogenic.

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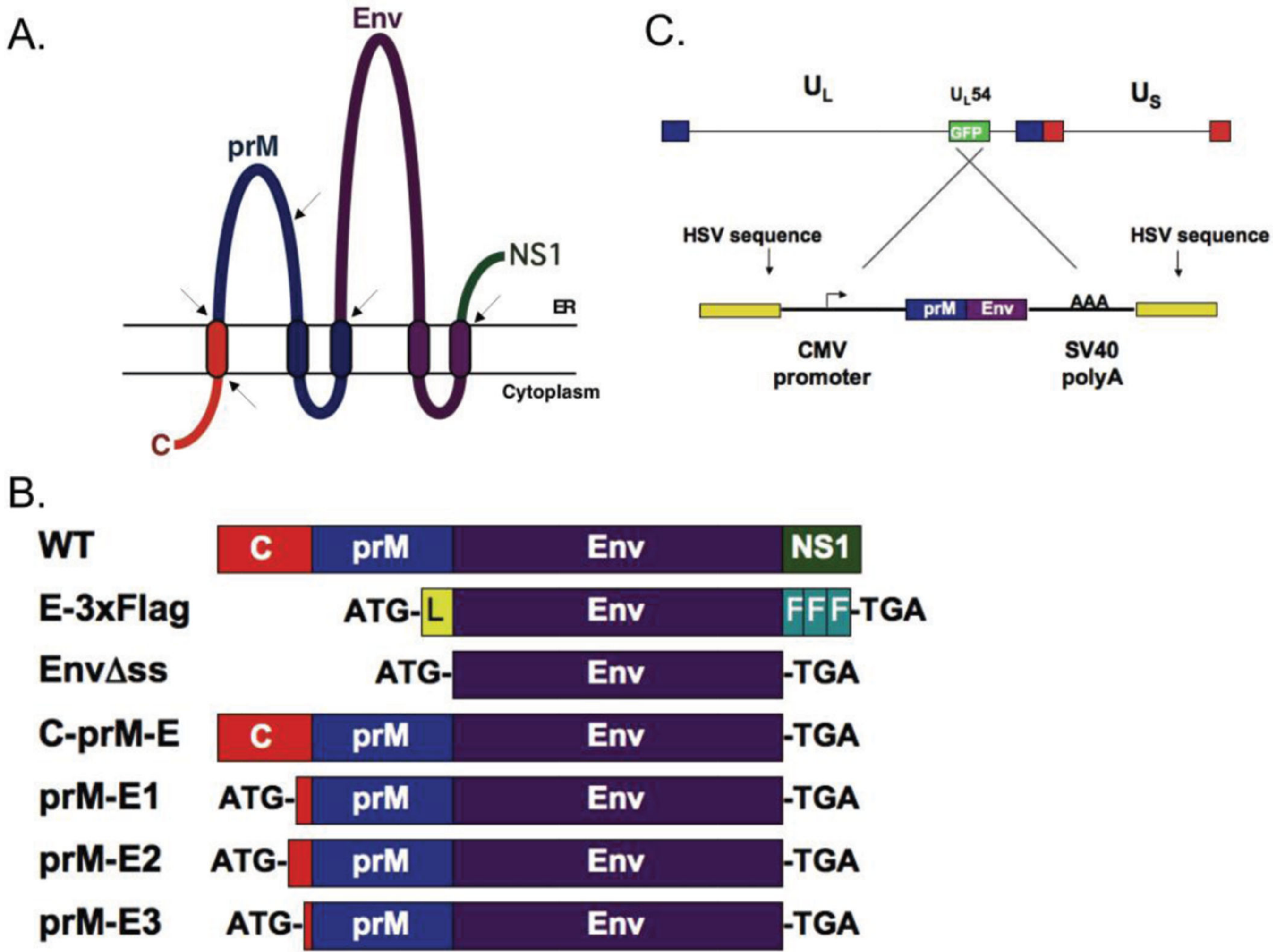

Figure 1. Diagrams of the constructs used in this study

A. Graphical representation of the membrane orientation of the structural proteins in WNV including the initial portion of the nonstructural NS1 protein. Transmembrane regions are denoted by ovals passing through the lipid bilayer. Arrows indicate where cellular or viral proteases cleave the polyprotein to free the individual proteins: capsid (C), premembrane (prM), and envelope (Env). B. Comparison of the various plasmid constructs to the wildtype (WT) structural protein coding sequences. All plasmids used the CMV promoter/enhancer to drive transcription. The addition of start (ATG) and stop (TGA) codons are indicated. E-3xFlag contains the preprotrypsin leader sequence (L) and three sequential FLAG epitope tags (F). Env ss expresses the E protein lacking C and prM sequences. C-prM-E contains the WNV coding sequences for C-prM-Env-NS1. prM-E1-3 contain varying amino acid residues of C as described in Table 2. (C) Top: the *d*106 genome with the location of the U_L54 *(ICP27)* region containing the GFP transgene indicated. Bottom: the pd27-WNV plasmid used to generate the recombinant *d*106-WNV vaccine vector. The crossed lines show the homologous recombination event between the reversed plasmid sequences with the *d*106 genome that generated the *d*106-WNV recombinant virus.

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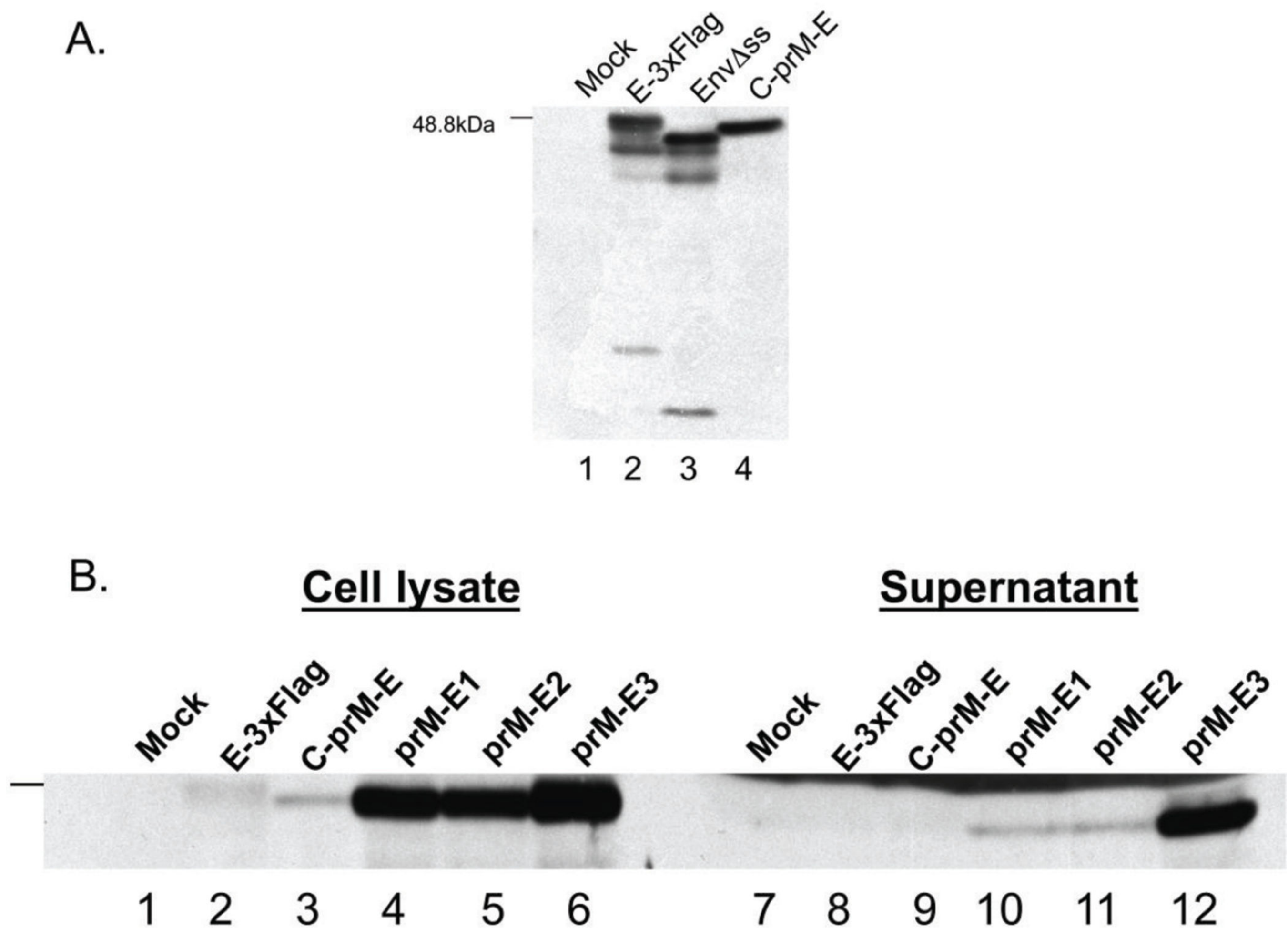

Figure 2. Expression of WNV proteins and VLP release in transfected cells

Vero cells mock-transfected or transfected with the plasmids expressing the indicated WNV construct were harvested at 72 hours post-transfection. An equal volume (4% of total sample) of either cell lysate or clarified supernatant was resolved by SDS-PAGE, transferred to a membrane, and the membrane was probed with an anti-E monoclonal antibody. The position of the 48.8 kDa ladder band is indicated. (A) Comparison of preprotrypsin leader (lane 2), leaderless (lane 3), and native E (lane 4) expressing constructs in transfected Vero cells. (B) Comparison of the VLP production by the indicated constructs as measured by E in the cells and supernatant.

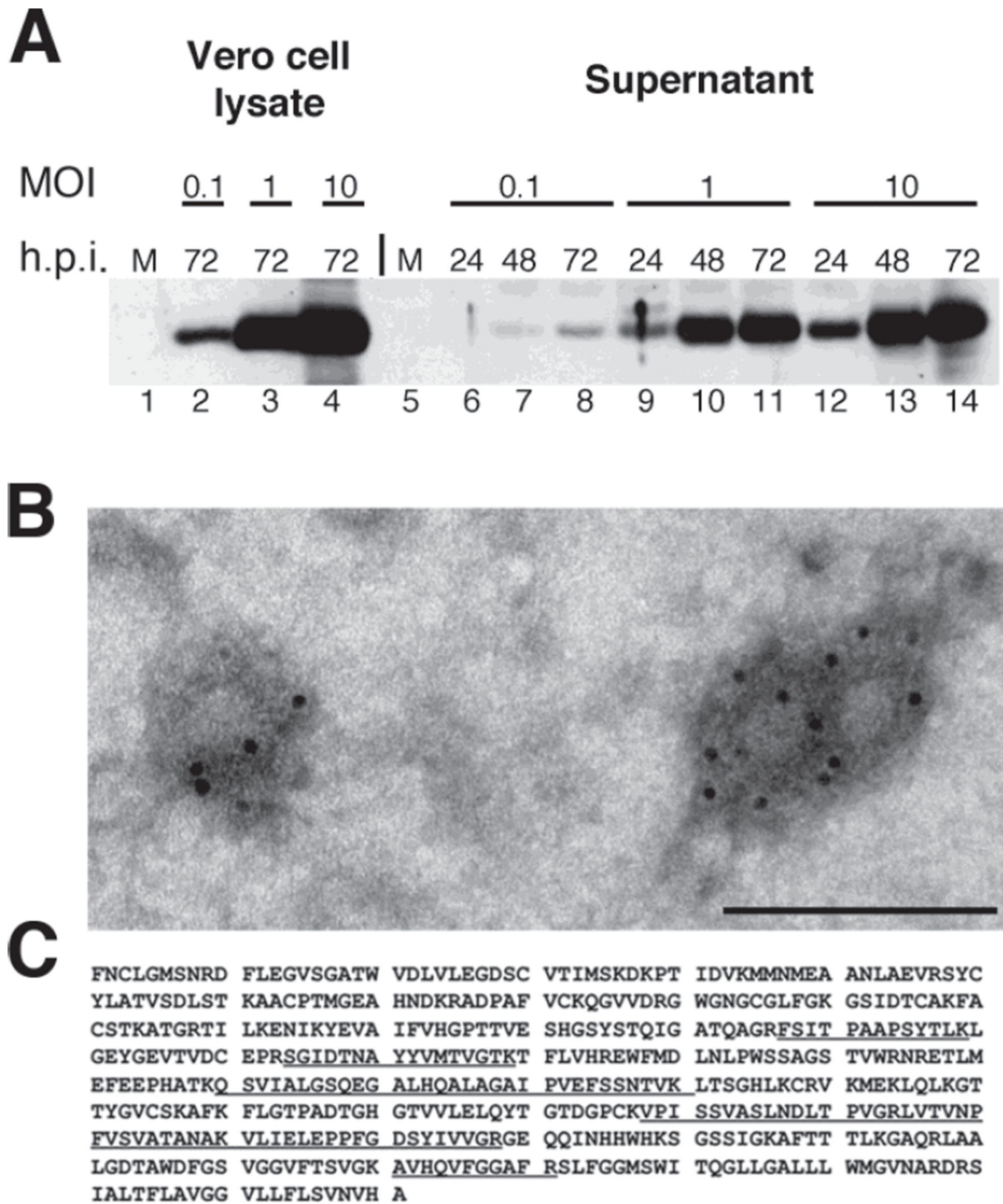

Figure 3. Assembly and properties of virus-like particles in *d*106-WNV virus-infected cells

A. Kinetics of WNV protein expression and VLP release. A. Vero cells were mock-infected or infected with *d*106-WNV virus at a range of MOIs (0.1, 1.0 or 10 PFU/cell) and harvested at 24, 48, or 72 hpi. Lysate (10% of sample total) and clarified supernatant (2% of sample total) were separated by SDS-PAGE, transferred to membrane, and probed with anti-E monoclonal antibody. The position of the 48.8 kDa ladder band is indicated. **B. Electron micrograph of VLPs**. WNV VLPs were diluted in Tris-buffered saline and adsorbed for 1 minute to a carbon coated grid that had been made hydrophilic by a 30 second exposure to a

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glow discharge. Samples were blocked with 1% BSA and incubated with a mouse anti West Nile Virus Envelope protein (Chemicon) for 30 minutes. After washing, samples were incubated with a rabbit anti mouse bridging antibody and protein A-gold (5nm) for 20 minutes. Samples were washed with PBS and water. Excess liquid was removed with a filter paper, and the samples were stained with 0.75% uranyl formate for 30 seconds. After removing the excess uranyl formate with a filter paper, the grids were examined in a JEOL 1200EX Transmission electron microscope, and images were recorded with an AMT 2k CCD camera. Scale bar: 100 nm. C. **Identification of Peptides from E Protein in Extracellular Particles**. Partially purified VLPs were resolved by SDS-PAGE, and the Coomassie blue-stained band corresponding to E was analyzed by mass spectrometry. The E-specific peptides identified by mass spectrometry are underlined.

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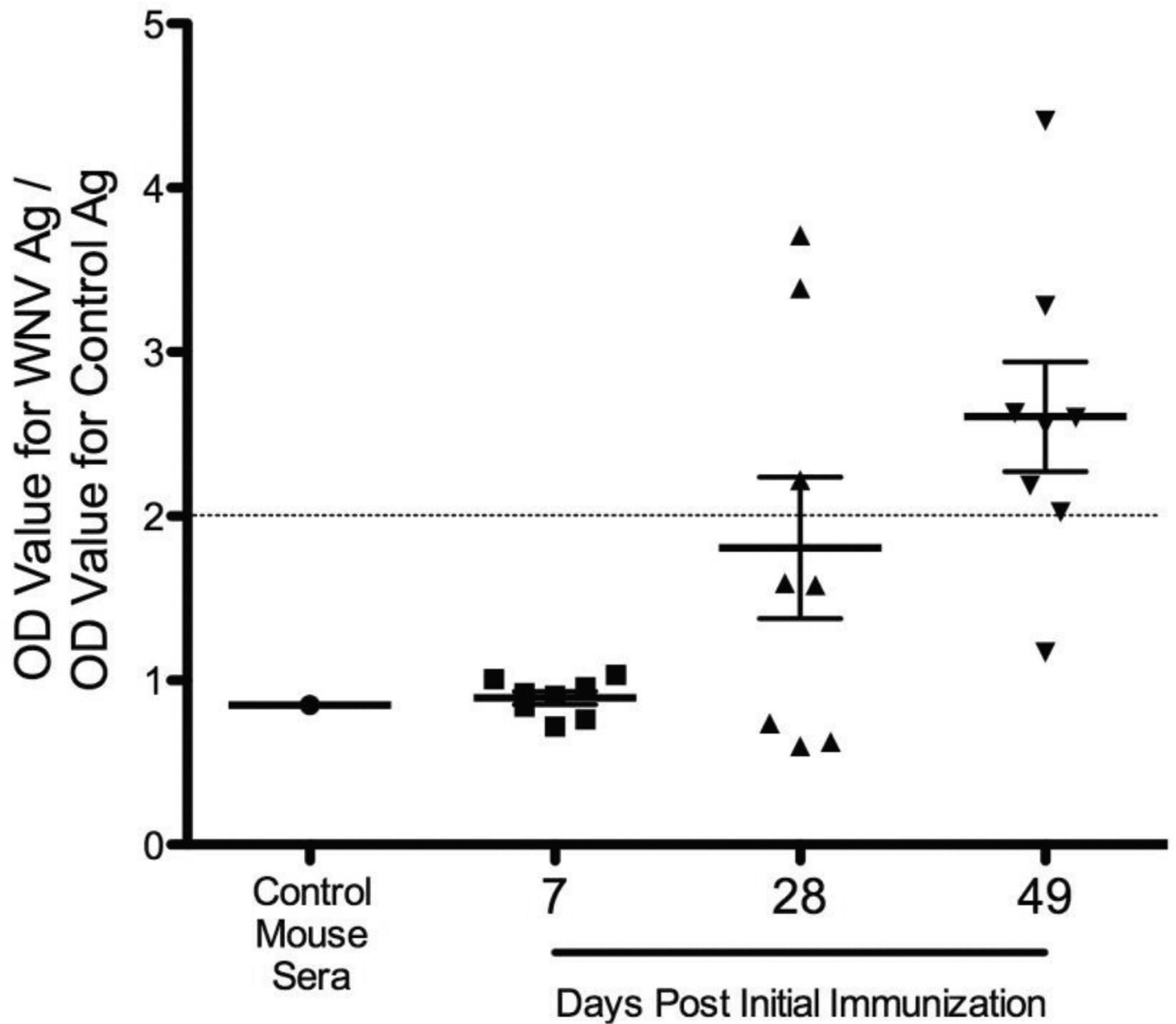

Figure 4. WNV-specific IgG responses in *d*106-WNV immunized mice

Sera were taken from 8 immunized mice at 7, 28, and 49 days post-initial immunization, and IgG levels were determined by indirect ELISA using a 1:100 dilution of serum. The dashed line represents the OD ratio threshold that is considered positive for this assay. The values shown are mean and standard error of the mean. The value for control mice was from a pool of sera from unimmunized mice.

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Table 1

Primers used in this study.

Primer
prM-E1: 5’ ATGGCTATCAATCGGCGGAGCTC
prM-E2: 5’ ATGAAACACCTTCTGAGT
prM-E3: 5’ ATGGCAGCAGGAGGCAAGACCGGAATTGC
F2-Eco: 5’ GAATTCATGTTCAACTGCCTTGGAATGAGC
CF: 5’ATGTCTAAGAAACCAGGAGG
R-Sal: 5’ GTCGACTCAAGCGTGCACGTTCACGGAGAG

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Table 2

Comparison of WNV envelope construct properties in transfected Vero cells.

Plasmid	Capsid Amino Acid Residues	Expression in transfected cells	VLP production
E-3xFlag	None	+	−
Env ss	None	+	−
C-prM-E	1–123	+	−
prM-E1	94–123	++	+/−
prM-E2	78–123	++	+/−
prM-E3	106–123	+++	++