**The Simian Virus 40 Late Viral Protein VP4 Disrupts the Nuclear Envelope for Viral Release**

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Simian virus 40 (SV40) appears to initiate cell lysis by expressing the late viral protein VP4 at the end of infection to aid in virus dissemination. To investigate the contribution of VP4 to cell lysis, VP4 was expressed in mammalian cells where it was predominantly observed along the nuclear periphery. The integrity of the nuclear envelope was compromised in these cells, resulting in the mislocalization of a soluble nuclear marker. Using assays that involved the cellular expression of VP4 or the treatment of cells with purified VP4, we found that the central hydrophobic domain and a proximal C-terminal nuclear localization signal of VP4 were required for (i) cytolysis associated with prolonged expression; (ii) nuclear envelope accumulation; and (iii) disruption of the nuclear, red blood cell, or host cell membranes. Furthermore, a conserved proline within the hydrophobic domain was required for membrane perforation, suggesting that this residue was crucial for VP4 cytolitic activity. These results indicate that VP4 forms pores in the nuclear membrane leading to lysis and virus release.

Viruses navigate the complex network of membrane organelles that compartmentalize cellular processes in eukaryotic cells. They bind and deliver their genetic material to the host cell in order to produce and eventually release their progeny to propagate the infection. Enveloped viruses exploit cellular membranes and exit infected cells by utilizing a budding or membrane fusion process (3, 10). In contrast, nonenveloped viruses must be liberated from the cell without being encapsulated by lipids. The nonenveloped viral release process generally involves timely cytolysis or host cell perforation (8, 16, 56). Membrane disruption observed at the end of the viral life cycle is a highly tuned and temporally controlled step that maximizes viral dissemination (57). However, little is known about how viral release is initiated and controlled for nonenveloped viruses.

A growing number of viruses have been shown to produce small viral membrane proteins termed viroporins that modulate the permeability of membranes to ions or small molecules (25). These small proteins contain an amphipathic or hydrophobic stretch that supports membrane insertion. Once integrated or associated with the lipid bilayer, viroporins are proposed to oligomerize to form pores that traverse the membrane to assist in the propagation of both enveloped and nonenveloped viruses. Viroporins have been hypothesized to aid in the exit of viruses from host cells by supporting the movement of small molecules across the membrane barrier, leading to cell destabilization and lysis (9, 25). The analysis of the activity of viroporins is crucial for understanding of the viral release process.

Since the sequencing of the simian virus 40 (SV40) viral genome over 30 years ago, it has served as a model to explore fundamental processes including nuclear import, cell transformation, and virus structure (24, 29, 33, 51). After infection and replication of the viral genome, the late viral proteins are synthesized, including the minor structural proteins VP2 and VP3, which are produced from successive in-frame Met residues. Viral progeny assemble in the nucleus and are released from their host cell by a process that appears to involve the activity of a newly identified later-expressed protein, VP4 (16). VP4 is expressed from a third downstream Met residue found in the 19S viral transcript that codes for VP2 and VP3; therefore, VP2, VP3, and VP4 share their C-terminal residues. Recently, VP4 has been shown to perforate synthetic and biological membranes, and this membrane perturbation was proposed to aid in viral release (42); however, where VP4 acts in the cell and its mechanism of action are uncertain.

In the present study, the intracellular targeting of VP4 was explored and important domains were identified that assist in its localization and membrane perforation activity. The cytolytic properties of VP4 were controlled by using an inducible expression strategy in permissive host cells. VP4 accumulated along the nuclear envelope and disrupted the localization of nuclear proteins. VP4 was found to be peripherally associated with nuclear membranes using biochemical extraction procedures. A central hydrophobic domain (HD) and nuclear localization signal (NLS) were critical regions that dictated the targeting of VP4 to nuclear membranes. These domains were also required for the membrane disruption activity of VP4. Biochemical fractionation studies and characterization of site mutations provided valuable insights into the mechanism of action of VP4. Altogether, this study demonstrated that VP4 acts as a viroporin by targeting and perforating the nuclear envelope to promote viral release.

**MATERIALS AND METHODS**

**Reagents.** The T7 expression system RNaseasy kit was purchased from Qia-gen (Valencia, CA). The RNasin, components of the rabbit reticulocyte cell-free translation system, and the CytoTox 96 cytotoxicity assay kit for quantification of lactate dehydrogenase (LDH) release were purchased from Promega (Madison, WI). [35S]methionine-cysteine was acquired from PerkinElmer (Waltham, MA). The following antibodies were obtained as indicated: myc, lamin A/C, and emerin (Cell Signaling, Danvers, MA); GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Millipore,

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Billerica, MA; VP1 and VP2/3 (Abcam, Cambridge, MA; VP2/3 was also obtained from A. Oppenheim, Jerusalem, Israel); calnexin (Enzo Life Sciences Inc., Ann Arbor, MI); large T antigen (LT; Merck KGaA, Darmstadt, Germany); and glutathione S-transferase (GST; Abmart, Arlington, MA). The ERp57 antibody was obtained from H. Taira (Iwate, Japan) (54). Cos7 and BS-C-1 cells were obtained from ATCC (Manassas, VA). All tissue culture reagents contained fetal bovine serum (FBS) lacking detectable tetracycline and Lipofectamine 2000 were purchased from Life Technologies (Grand Island, NY). The pSV40 plasmid, which encodes wild-type (WT) SV40 (strain 776) was a generous gift from H. Kasamatsu (Los Angeles, CA) (27). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Construction of plasmids.** The pGEX-6P-1 (GE Healthcare, Piscataway, NJ) plasmid backbone was modified to include a tobacco etch virus (TEV) protease site and a C-terminal 6xHis epitope upstream and downstream of the multiple cloning site, respectively, to create pGEX-6P-1-TEV-His. VP2, VP3, and VP4 were subcloned using PCR into pcDNA4/TO/myc-His A (Life Technologies) using standard techniques to create VP2-myc-His, VP3-myc-His, and VP4-myc-His, respectively. To ensure that VP2 and VP3 were synthesized individually, the internal ATG start sites for VP3 and VP4 within the VP2 reading frame or VP4 within VP3 were mutated to ATA (Ile). VP4 was also subcloned into pGEX-6P-1-TEV-His (GST-TEV-VP4-His) for bacterial expression. The QuikChange mutagenesis primer design program (Agilent Technologies, Santa Clara, CA) was used to delete the HD, amino acids 65 to 83, of VP4 (PQWMMP LLLGLYSVTSAL) to create VP4ΔHD. Site-directed mutagenesis of the NLS (KKKKR) to QAGGE was used to create VP4ΔNLS. The NLS sequence KKKKR was introduced at residues 51 to 55 or 113 to 117 in the VP4ΔNLS backbone to create NLS1-5, NLS13-17, and NLS13-117, respectively. Phusion site-directed mutagenesis (New England BioLabs, Ipswich, MA) was used according to the manufacturer’s recommendations to generate HD point mutants L71K, L71D, L75E, L75D, L71K/L75E, L71D/L75D, and P70A. Mutagenesis was confirmed by sequencing. Finally, VP1 was subcloned by PCR into pcDNA3.1(−)/myc-His A (Life Technologies) with the native stop codon included so that VP1 lacked an epitope tag.

**In vitro translations and ER membrane binding.** [35S]methionine-cysteine-radio labeled in vitro translation reactions were carried out using rabbit reticulocyte lysate as previously described (14). Briefly, the viral proteins were synthesized by in vitro translation and incubated posttranslationally with rough endoplasmic reticulum (ER)-derived microsomes. Microsomes were recovered by ultracentrifugation through a sucrose cushion (500 mM sucrose, 50 mM TEA [pH 7.5], and 1 mM diethiothreitol [DTT]) for 10 min at 72,000 × g at 4°C. Globin nonspecifically binds [35S]methionine-cysteine and displays a mobility similar to that of polyvinylidene difluoride membrane and immunoblotted. Expression of VP2 or VP3 using the inducible expression system was carried out as described for VP4. For coexpression of VP1 and VP4, Cos7 cells were transfected at a VP1:VP4:tetracycline repressor plasmid molar ratio of 1:1:1.5. Transfections were incubated for 15 h before VP4 protein expression was induced with the addition of 10 μg/mL tetracycline. Cell lysates were prepared 6 h after induction.

**Confocal immunofluorescence microscopy.** Following transfection and induction of protein expression for 6 h, cells were fixed and permeabilized with either ice-cold methanol for 10 min at −20°C or fixed with 4% paraformaldehyde in PBS for 1 min and permeabilized with ice-cold methanol for 10 min at −20°C. Cells were incubated with primary antibody followed by the appropriate secondary antibodies in immunostaining buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, and 2% bovine serum albumin) according to the manufacturer’s instructions. Slides were rinsed with water and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired with a confocal microscope (Fluoview, Olympus Inc.) and processed using Adobe Photoshop.

**Viral genome transfection and protein expression levels.** To determine the relative level of protein expression produced from induction of VP4 alone compared to the SV40 viral genome, fixed cells were prepared for immunofluorescence and nuclear pellets were analyzed by immunoblotting. VP4 transfection and microscopy were carried out with Cos7 cells after 9 h of induction. Wild-type SV40 viral genome preparation was performed as previously described (14). BS-C-1 cells used for viral genome transfection were cultured and maintained as previously described (14). To quantify the transfection level, VP4-myc fixed cells were scored for VP4 expression after 9 h of induction, whereas the transfection level of the SV40 genome was quantified based on LT-positive fixed cells after 168 h of transfection. For immunoblot analysis of VP4 concentration, nonadherent and adherent cells were collected in lysis buffer (1% NP-40, 50 mM HEPES [pH 7.5], 200 mM NaCl) and protease inhibitors as previously described (14). The insoluble nuclear pellet was retained and analyzed by immunoblotting. VP4-myc from induction was compared to SV40-transfected cells, and VP4 was detected using VP2/3 antisera.

**Preparation of viral envelopes and chemical extractions.** The nuclear membrane insertion of VP4 was determined as previously described (6, 32). Following transfection and induction of protein expression for 9 h, cells were plated on ice, washed twice with PBS, and lifted in homogenization buffer (10 mM HEPES [pH 7.4], 1 mM EDTA, and 250 mM sucrose) with protease inhibitors. Cells were centrifuged at 250 × g for 10 min at 4°C. The cell pellet was suspended in ice-cold hypotonic buffer (10 mM HEPES [pH 7.4], 5 mM MgCl₂, 10 mM NaCl, and 1 mM DTT) with protease inhibitors at a ratio of 280 μL of buffer per 1 million cells and incubated on ice for 10 min to swell cells. After swelling, the cells were lysed using a ball bearing homogenizer with 10 passes on ice. To prevent lysis of nuclei, a 1:10 volume of high-sucrose buffer (2.2 M sucrose, 50 mM HEPES [pH 7.4], 25 mM KCl, 5 mM MgCl₂, and 1 mM DTT) with protease inhibitors was immediately added. Samples were centrifuged at 2,000 × g for 20 min at 4°C. The cytoplasmic supernatant fraction was concentrated with trichloroacetic acid precipitation. A sample of the total nuclear pellet was retained and the remainder was divided and extracted with either alkaline (0.1 M NaOH and 1 mM DTT) or chaotropic (0.1 M Na₂CO₃, 4 M urea [pH 11.5]) buffer for 15 min on ice. Extracts were centrifuged at 100,000 × g for 20 min at 4°C. Supernatant and pellet fractions were analyzed by immunoblot analysis.

**LT mislocalization assay.** Cos7 cells were transfected, induced, and stained with anti-myc and LT sera. Cells positive for VP4 or mutant protein expression were manually scored for the localization of LT either as solely nuclear (see Fig. 3, asterisk) or as nuclear and cytoplasmic (see Fig. 3, arrowhead). Consequently, for cells without induction (0 h) and mock transfections, random fields of view were scored since VP4 was not detected. Nuclear envelope breakdown during mitosis causes LT to localize...
throughout the cytoplasm. These cells were omitted from the analysis by excluding dividing cells, which were easily observed by DNA staining. The localization of LT was evaluated in at least three independent experiments for each time point to determine the average and standard deviation. Thus, in total, more than 6,200 cells were evaluated.

**Bacterial expression and purification of GST-tagged proteins.** Bacterial expression and purification of GST-VP4 and its mutants were performed as described previously (42). All GST-VP4 mutants in this study were created by mutagenesis as described above.

**RESULTS**

**VP4 does not target to ER-derived microsomes.** Previously, we found that SV40 VP2 and VP3 integrate posttranslationally into ER-derived membranes or microsomes (14). As VP4 is an N-terminal truncation of VP2 and VP3, the targeting of VP4 to ER membranes was characterized to gain insight into where VP4 acts in infected cells. A cell-free membrane-targeting assay was employed that involved the in vitro translation of the [35S]methionine-cysteine-radiolabeled viral proteins and the posttranslational addition of rough ER-derived microsomes. Membrane-associated or integrated proteins were isolated by centrifugation through a sucrose cushion and separated from unbound proteins found in the supernatant.

As previously reported (14), VP2 and VP3 efficiently associated with the ER membranes at levels of 25 and 31%, respectively (Fig. 1B and C). Sedimentation was due to membrane association, as in the absence of membranes only minimal levels of the late viral proteins sedimented (Fig. 1B, lanes 3 and 7). Interestingly, VP4 did not associate with ER membranes regardless of whether it was translated from the VP2, VP3, or VP4 transcripts. These results demonstrated that despite containing the C-terminal HD also found in VP2 and VP3 (Fig. 1A), VP4 did not associate with ER-derived membranes.

**VP4 localizes to the nuclear membrane.** To determine the localization of VP4 in permissive host cells (Cos7), an expression system that permitted the cellular expression of the late viral protein was developed. A variety of overexpression strategies were explored; however, these strategies did not permit protein expression due to the viroporin and cytolytic properties of VP4 (16, 42). Therefore, a tetracycline-on inducible protein expression system that supported the expression of a bolus of VP4 with temporal induction due to the viroporin and cytolytic properties of VP4 (16, 42). Therefore, a tetracycline-on inducible protein expression system that supported the expression of a bolus of VP4 with temporal induction was utilized. Cos7 cells were cotransfected with tetracycline repressor and VP4 possessing a C-terminal myc tag. Following transfection, protein expression was induced by the addition of tetracycline and monitored by immunoblotting.

The expression of VP4 was initially observed 3 h postinduction (Fig. 2A, lane 4). VP4 expression peaked after 9 h of induction, and after 16 h VP4 was no longer observed. This suggested that the cells subjected to prolonged induction or expression of VP4 for times greater than 9 h were not viable, an observation consistent with the cytolytic properties of VP4. Protein overexpression might induce deleterious cellular effects, so the VP4 expression level observed with the induction system was compared to viral genome transfection level (see Fig. S1 in the supplemental material). The expression level of VP4 after 9 h of induction was much lower than that observed by viral genome transfection. This indicated that the apparent cytolytic properties of VP4 were not the result of overexpression.

This inducible expression system supported the analysis of the cellular localization of VP4 using indirect immunofluorescence confocal laser scanning microscopy (Fig. 2B). VP4 localization was compared against ER (ERP57) and nuclear staining after 6 h of induction. VP4 predominantly localized to the nuclear and nucle-
olar periphery as it completely encircled both the nucleus and the nucleoli (Fig. 2B, top overlay image). Since VP2 and VP3 contain the VP4 sequence, their cellular localization was also analyzed after they were individually expressed (see Fig. S2 in the supplemental material). Both VP2 and VP3 were localized mainly to the nucleoplasm and notably were only weakly concentrated at the nuclear rim. Thus, despite containing the same sequence as VP4, when expressed individually VP2 and VP3 were localized to the nucleoplasm (see Fig. S2).

To verify the peripheral nuclear profile observed for VP4, the localization of VP4 was compared to that of two nuclear membrane-associated proteins, lamina and emerin. Lamina forms the meshwork of the nucleoplasm along the nuclear envelope that gives the nucleus structural support and serves as a scaffold for chromatin and other nuclear components (1, 20, 21, 36, 39). Based on their binding to inner nuclear membrane (INM) proteins and chromatin, the lamin proteins are concentrated along the nuclear envelope. VP4 partially colocalized with lamin A/C along the nuclear periphery (Fig. 2B, middle panels). Furthermore, VP4 also colocalized with the integral INM protein emerin (Fig. 2B, bottom) (4, 37). These results verified the localization of VP4 to the nuclear periphery in Cos7 cells.

Of special note was the observation that the nuclear periphery appeared to be deformed or disrupted in many of the VP4-expressing cells.
expressing cells. This was visualized by analyzing the lamina and emerin staining in VP4-infected cells (Fig. 2B, arrows in the two bottom panels). In comparison to what was seen for untransfected cells, lamina and emerin were not as tightly juxtaposed to the nucleus and there was increased lamina and emerin staining in the nucleoplasm in VP4-expressing cells. Furthermore, in some cases the nuclear envelope integrity appeared to be compromised in VP4-expressing cells (Fig. 2B, overlay; lamin arrows). Altogether, this suggested that VP4 localized to the nuclear envelope, where it appeared to perturb nuclear membrane integrity.

**VP4 expression supports the cytoplasmic localization of LT.** The effect of VP4 expression on the integrity of the nuclear membrane was probed by determining its ability to act as a barrier to the leakage of soluble nuclear localized proteins into the cytoplasm. The Cos7 cell line used to study the expression and localization of VP4 was immortalized with SV40 large T antigen (LT) (24). NLS was first defined using SV40 LT as a substrate (29, 33). Furthermore, the diffusion rate of LT is rapid, supporting its utility as a useful bulk nucleoplasmic marker (45).

In both mock- and VP4-transfected cells, LT was localized to the nucleus prior to the induction of VP4 expression (Fig. 3B). After 6 h of induction, LT was found in the cytoplasm in 45% of the VP4-expressing cells (Fig. 3A, arrowheads, and B). In cells where LT leakage was observed, the nuclear periphery often appeared to be irregular as found by VP4 localization or nuclear staining (Fig. 3A, arrowheads). The proportion of LT localized to the cytoplasm increased in VP4-expressing cells with time (Fig. 3B). These results are consistent with the hypothesis that VP4 localization to the nuclear envelope supports the disruption of this membrane barrier.

VP4 contains the C-terminal portion of VP2 and VP3 found to bind to VP1 pentamers (2, 11). As VP2 and VP3 expression has been shown to change the localization of VP1 upon coexpression in mouse and insect cells (18, 22), it was of interest to determine the localization of VP4 when coexpressed with VP1. Whole-cell lysate immunoblot analysis indicated that VP1 and VP4 were expressed at similar levels when produced individually and in combination (see Fig. S3A in the supplemental material). Localization of VP1 and VP4 in fixed cells demonstrated that VP1 localized to the nucleoplasm in the absence and presence of VP4 expression (see Fig. S3B). However, VP4 was detected to a greater extent in the nucleoplasm of VP1-coexpressing cells (see Fig. S3B). As VP4

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**FIG 3** VP4 expression disrupts the localization of LT. (A) Cos7 cells were transfected with VP4-myc, and protein expression was induced with the addition of tetracycline for 6 h. Subsequently, cells were fixed and stained for the myc (VP4-myc), a soluble nuclear localized protein large T antigen (LT), and Hoechst. Representative fluorescence images are displayed, with the bar representing 10 μm. Cells positive for VP4 expression with LT detected solely in the nucleoplasm were scored as nuclear (asterisk), whereas cells with LT strongly localized in the cytoplasm were scored as cytoplasmic (arrowheads). (B) Quantification from three independent experiments of the percentage of cytoplasmic localized LT in cells transfected with empty vector (Mock) or VP4-myc at various times after induction. Error bars represent the standard deviation of more than 3,800 cells evaluated.
is not incorporated into viral particles, it appears that the timing of expression is an important factor in VP1 binding and localization (16). VP4 is expressed 24 h after VP2 and VP3 upon genome transfection of BS-C-1 cells, providing a substantial period for the VP1 pentamers to bind VP2 and VP3, thereby inhibiting VP4 binding to VP1 pentamers. It should be noted that the temporal expression profile of the late SV40 proteins is likely dependent upon whether expression was initiated by infection or transfection, and the host cell type.

**VP4 does not integrate into the lipid bilayer.** To biochemically verify and further define the microscopy results that found VP4 localized to the nuclear periphery, isolated nuclei were subjected to chemical extractions to determine if VP4 was inserted into the lipid bilayer (Fig. 4). This approach has been used to identify inner nuclear membrane proteins (19, 32, 49). The periphery-associated membrane protein and the integral membrane protein lamin and emerin were used as controls for the characterization of the separation procedure.

The alkaline extraction of membranes combined with centrifugation has been widely used to separate integral membrane proteins from soluble or periphery-associated membrane proteins (23, 40). Regardless of whether the alkaline extractions were performed in the presence or absence of the denaturing agent urea, the peripheral membrane proteins lamin A/C were found in the supernatant while the integral membrane protein emerin localized to the membrane pellet (Fig. 4, compare lanes 3 and 4 to 5 and 6). These results confirmed the effectiveness of the extraction procedure. The localization of VP4 after the individual extraction steps was similar to that of lamin, as VP4 was found in the supernatant after alkaline extraction. Interestingly, these results indicated that VP4 did not appear to be integrated into the hydrophobic core of the nuclear membrane bilayer.

**The HD and the NLS of VP4 are required for its nuclear membrane localization.** VP4 possesses two domains that likely play a role in its cellular localization: a central HD and a C-terminal NLS (Fig. 1A). The involvement of these domains in its localization was explored by deletion of the HD (VP4ΔHD) or the mutation of the NLS (VP4ΔNLS). The expression of mutant forms of VP4 was induced for various times, and the expression levels were monitored by immunoblotting. In contrast to wild-type VP4, the cytolytic properties of VP4ΔHD and VP4ΔNLS were disrupted, demonstrated by detection of the mutant proteins even at 24 h postinduction (compare Fig. 2A and 5A).

Morphological studies using immunofluorescence microscopy showed that after 6 h of induction VP4ΔHD efficiently localized to the nucleus. Furthermore, it was not concentrated along the nuclear periphery as observed with wild-type VP4 but was found throughout the nucleoplasm (Fig. 5B). In contrast, alteration of the NLS (VP4ΔNLS) resulted in the protein being observed throughout the cell, displaying no distinct membrane association pattern. These results indicated that the hydrophobic domain and the NLS of VP4 play important roles in the targeting of VP4 to the nuclear periphery or membrane.

**The HD and the NLS of VP4 are required for its membrane disruption activity.** The requirement of the HD and the NLS of VP4 for its membrane disruption activity was studied using a variety of membrane integrity assays. Initially, the effect on LT leakage to the cytoplasm was probed after expression of wild-type and mutant forms of VP4 in Cos7 cells. In the presence of VP4ΔHD or VP4ΔNLS, LT remained in the nucleus regardless of the time of induction (Fig. 6A). Consistent with the prolonged viability of the cells expressing the VP4 HD and the NLS mutants (Fig. 5A), the nuclear membrane remained intact after VP4ΔHD or VP4ΔNLS expression.

We have previously characterized the membrane disruption activity of bacterially expressed and purified VP4 (42). GST and His tags were added to the N and C termini of VP4, respectively, to optimize its solubility and facilitate purification. Bacterially expressed VP4-mediated hemolysis was monitored by determining the fraction of hemoglobin released into the supernatant after centrifugation by measuring the absorbance of the supernatant. In agreement with previously reported results (42), the wild-type but not VP4ΔHD supported the perforation of bovine red blood cell (RBC) membranes (Fig. 6B). Furthermore, VP4ΔNLS was also found to be ineffective at disrupting the RBC membranes. This indicated that the five positively charged residues that constitute the NLS (KKKKK) not only have a role in the localization of the protein in the cell but were also required for the membrane perforation activity of VP4.

The binding of VP4 to RBCs was analyzed using a membrane sedimentation assay to determine if the defects in hemolysis were due to the inability of the mutant proteins to bind to the RBCs. In the absence of membranes, all the purified proteins were found in the supernatant after centrifugation, consistent with the proteins being soluble (Fig. 6C, lane 2). Wild-type VP4 efficiently sedimented with the RBCs, and this association was completely inhibited by the removal of the HD as observed previously (Fig. 6C) (42). Almost half of the VP4ΔNLS was in the supernatant after incubation with the RBCs and centrifugation. Therefore, the alteration of the positive charge cluster in VP4 diminished membrane binding but did not completely ablate the interaction with RBC membranes.

As the lipid composition of membranes is organism dependent, a membrane disruption assay that used the SV40 host Cos7 cells as the membrane source was employed (42, 55). Cell lysis was monitored by following the release of cytoplasmic LDH upon the addition of purified VP4 (42). Cos7 cells were efficiently disrupted.
by wild-type VP4, supporting the release of LDH (Fig. 6D). Consistent with the hemolysis results, the HD and the NLS of VP4 were required for the perforation of Cos7 cells. Thus, both the HD and the proximal NLS of VP4 were required for proper cellular localization and its membrane perforation activity.

The hydrophobicity, proximal spacing of basic amino acids, and Pro are required for VP4 membrane disruption. To further explore the requirements for the HD and the NLS in the perforation activity of VP4, bacterially expressed and purified VP4 mutants were used to screen for disruption of RBCs and Cos7 cells. To identify important residues or features within the HD and NLS utilized for membrane disruption, a sequence alignment of the minor structural protein VP2 from the Orthopolyomavirus genus was investigated (see Fig. S4 in the supplemental material) (28). As deletion of the HD abolished membrane binding and disruption, it was of interest to determine if the overall hydrophobicity of this domain was critical for membrane perforation (Fig. 6 and 7A and B; also see Fig. S5). Individual or pairs of charged residues were incorporated within the HD (L71K, L71D, L75E, L75D, L71K/L75E, or L71D/L75D) by altering hydrophobic amino acids and the mutant proteins were tested for their membrane perforation activity.

Similar to VP4, the single-charge mutants (L71K, L71D, L75E, or L75D) retained full membrane disruption activity against RBC and Cos7 cell membranes (Fig. 7A and B, respectively), indicating that a single charged residue was tolerated. However, the addition of two charged residues (L71K/L75E or L71D/L75D) abolished membrane disruption activity, suggesting that the overall hydrophobicity of the domain was important. Membrane binding was tested to uncover the cause for changes in membrane disruption activity; proteins were incubated in the absence or presence of RBC membranes, and the membranes were recovered by low-speed centrifugation as previously described (42). Since the double-charge mutants were detected solely in the unbound supernatant fraction, this indicated that they were defective in membrane binding compared to wild-type and single-charge mutants (Fig. 7C, compare lanes 5 and 6). This implied that the net hydrophobicity of the HD was required for membrane binding and subsequent disruption.

The proximal spacing of the basic cluster of amino acids that comprise the NLS appeared to be conserved in orthopolyomaviruses (see Fig. S4 in the supplemental material). This led us to question whether this orientation and spacing of the NLS and HD were crucial for membrane binding and disruption. Since the mutation of the NLS reduced RBC binding and perturbed membrane disruption (Fig. 6), the NLS (amino acids, KKKRK) was introduced eight residues upstream of the HD (VP4-NLS<sub>51-55</sub>) or 29 residues carboxyl to the HD (VP4-NLS<sub>113-117</sub>). Purified proteins were tested for plasma membrane disruption of RBC or Cos7 cells. Interestingly, neither mutant rescued the membrane disruption activity on RBCs or Cos7 cells (Fig. 7A and B, respectively). However, both VP4-NLS<sub>51-55</sub> and VP4-NLS<sub>113-117</sub> bound RBC membranes with affinity similar to that of wild-type VP4 (Fig. 7C). This implied that the basic cluster of residues was able to rescue mem-

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FIG 5 The HD and NLS are critical for intracellular targeting of VP4. (A) Cos7 cells were transiently transfected with either empty vector (−), VP4ΔNLS or VP4ΔHD (+). Tetracycline was added for the indicated times. Whole-cell lysates were immunoblotted with antisera against myc to detect mutant VP4. (B) Confocal immunofluorescence localization of VP4-myc, VP4ΔHD-myc or VP4ΔNLS-myc in Cos7 cells after 6 h of induction. Samples were labeled with myc or ERp57 antisera, and DAPI. Bars, 10 μm.
brane binding but could not form a functional structure capable of perforating membranes.

Finally, the high conservation of Pro at positions 65 and 70 suggested that these residues might be critical (see Fig. S4 in the supplemental material). To test the role of the central HD Pro for membrane disruption activity of VP4, Pro 70 was substituted with Ala (VP4-P70A). VP4-P70A displayed minimal membrane disruption activity toward both RBC and Cos7 cells (Fig. 7A and B, respectively). However, VP4-P70A exhibited membrane-binding activity consistent with wild-type VP4 (Fig. 7C). Together, this suggested that a step downstream of membrane binding was impaired in VP4-P70A, indicating that the central HD Pro was required for the membrane perforation activity of VP4.

VP4 has been shown to be sensitive to alkaline extraction, implying that VP4 does not fully integrate into the lipid bilayer (Fig. 4) (42). To analyze this property further, wild-type and mutant proteins that were capable of binding RBC membranes were alkali extracted after incubation with the RBCs to separate integral membrane and peripherally associated proteins (Fig. 7D). Interestingly, wild-type VP4 and mutants of VP4 were readily removed by alkaline extraction, except in the case of VP4-P70A that displayed a 30% alkaline-resistant fraction. Since VP4-P70A was inactive against membrane disruption, this suggested that the Ala residue might impart a transmembrane topology that is inactive. To uncover if the same changes in topology might hold true for its association with nuclear envelopes, myc-tagged VP4-P70A was expressed in Cos7 cells and nuclear envelopes were alkaline extracted (Fig. 7E). As lamin A/C (soluble protein) and emerin (membrane protein) proteins were separated into the supernatant and pellet fractions, respectively, this demonstrated the efficacy of the extraction procedure. As predicted, VP4-P70A displayed an increased alkaline extraction-resistant fraction compared to wild-type VP4 (Fig. 7E, compare lanes 3 and 4). This indicated that VP4-P70A expressed in cells adopted a transmembrane topology in nuclear envelopes. Altogether, this suggested that the hydrophobicity of the HD along with the spacing of the basic charged NLS influenced the membrane perforation activity of VP4 and that other factors including the central Pro were essential for membrane disruption.

The hydrophobicity and C-terminally adjacent positive charges are required for nuclear membrane targeting of VP4. By utilizing purified proteins to screen the membrane binding and disruption activities of several VP4 mutants, the inactive mutations were then selected for further analysis of their expression and localization in Cos7 cells. A single-charge mutant that displayed wild-type activity (VP4-L75E) was also included to validate the
FIG 7 The hydrophobicity, spacing of the basic amino acid cluster, and Pro in the HD are required for membrane disruption by VP4. (A) Bacterially expressed and purified VP4, its mutants, and GST were incubated with RBCs, and the percent hemolysis was determined as in Fig. 6B. Error bars represent the standard deviation of three independent experiments. (B) Bacterially expressed and purified VP4 and its mutants were incubated with Cos7 cells and the release of LDH into the medium was determined as in Fig. 6D. Error bars represent the standard deviation of three independent experiments. (C) RBC binding was determined as in Fig. 6C. Samples were immunoblotted with anti-GST sera. (D) The membrane pellets of VP4 or its mutants that bound RBC membranes (Fig. 6C and 7C) were obtained after low-speed centrifugation. The total membrane fraction (T) was subjected to alkaline extraction (Na$_2$CO$_3$, pH 11.5), and the supernatant (S) and pellet (P) fractions obtained after ultracentrifugation were analyzed by immunoblotting using anti-GST sera. Anion exchanger 1 (AE1), an abundant integral membrane protein found in RBC membranes, was used to demonstrate the robustness of the extraction and was detected by Coomassie staining. (E) Cos7 cells were transfected with VP4-myc (VP4) or VP4-P70A-myc (P70A), and nuclear envelopes were purified and extracted as in Fig. 4, except urea was excluded from the reaction mixture to facilitate direct comparison of the nuclear envelope extraction and the RBC extractions. Fractions were analyzed by immunoblotting using anti-myc sera to detect VP4 or lamin and emerin antisera as indicated.
utility of purified proteins to recapitulate the activities observed in cells. VP4 or mutant protein expression was monitored by immunoblot analysis after 6 h of induction to confirm protein expression (Fig. 8A).

As expected VP4-L75E, a mutant that displayed wild-type membrane perforation and binding activities toward RBC and Cos7 cell membranes was expressed at a level similar to that of wild-type VP4 (Fig. 8A). Furthermore, VP4-L75E was concentrated along the nuclear periphery, similar to wild-type protein (Fig. 8B). In contrast, the double-charge mutants (VP4-L71K/L75E and VP4-L71D/L75D) were highly expressed after 6 h of induction, suggesting that they did not elicit cytolytic effects (Fig. 8A). In addition, both mutants were diffusely localized in the nucleoplasm similar to VP4ΔHD (compare Fig. 8B to 5B), supporting the membrane-targeting deficiency of the proteins. This suggested that while a single charged residue was tolerated without affecting membrane targeting, two charges significantly inhibited binding and consequently membrane disruption.

Both VP4-NLS51-55 and VP4-NLS113-117 were expected to be highly expressed, since bacterially expressed and purified proteins did not disrupt RBC or Cos7 cell membranes; however, these proteins were expressed at a level similar to that of wild-type VP4. Microscopy revealed that both mutants were localized throughout the cell with a preference for the nucleoplasm (Fig. 8). In comparison to VP4ΔNLS, VP4-NLS51-55 and VP4-NLS113-117 appeared to be partially rescued for nuclear localization but were not concentrated along the nuclear envelope (compare Fig. 5B and 8B). Altogether, these results indicated that the positioning of the NLS slightly C terminal to the HD was required for nuclear membrane targeting.

VP4-P70A was efficiently expressed in Cos7 cells. This was expected, since bacterial purified VP4-P70A did not disrupt RBC or Cos7 cell membranes (Fig. 8A and 7A and B). Strikingly, VP4-P70A was also found to localize in the nucleoplasm and along the nuclear envelope, though not to the same extent as wild-type VP4 (Fig. 8B). In light of the apparent binding to RBC membranes (Fig. 7C), this indicated that VP4-P70A bound membranes but was disruption incompetent. This suggested that VP4-P70A might be unable to adopt the conformation required to disrupt membranes. VP4-P70A associated with membranes and displayed an increased alkaline-resistant fraction compared to wild-type VP4 in RBC and nuclear envelopes (Fig. 7D and E). This suggested that removal of the Pro residue in the case of VP4-P70A supported the formation of a transmembrane topology that is resistant to alkaline extraction and inactive for membrane perforation.

**DISCUSSION**

SV40 viral release appears to be initiated by the VP4-mediated perforation of the nuclear envelope. An inducible VP4 expression system was used to demonstrate that VP4 was localized to the nuclear membrane prior to cytolysis. This resulted in the mislocalization of nuclear membrane proteins and the release of a soluble nucleoplasmic marker protein, indicative of a compromised permeability barrier. The central HD and the C-terminal NLS of VP4 were both required for nuclear membrane localization and the lethality observed with

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**FIG 8** The hydrophobicity and position of the NLS are required for localization at the nuclear envelope. (A) Cos7 cells were transiently transfected with mutant or wild-type forms of VP4 as indicated. Tetracycline was added for 6 h, and whole-cell lysates were immunoblotted with antisera against myc to detect mutant VP4 or the loading control GAPDH. (B) Confocal immunofluorescence microscopy localization of wild-type or mutant VP4-myc as indicated in Cos7 cells after 6 h of induction. Samples were labeled with myc and ERp57 antisera, and DAPI. Bars correspond to 10 µm.
prolonged expression. Cos7 cells were able to support the efficient expression of both VP4/H9004HD and VP4/H9004NLS even after 24 h of induction (Fig. 5A). Furthermore, purified VP4/H9004HD and VP4/H9004NLS, unlike wild-type VP4, did not disrupt RBCs or Cos7 cells (Fig. 6).

The outer nuclear membrane (ONM) is contiguous with the ER membrane. INM proteins in yeast and mammalian cells, such as emerin and lamin B receptor, first integrate into the ER/ONM and diffuse laterally to the INM, in part, using a basic membrane proximal targeting sequence (NLS) recognized and transported by a karyopherin-mediated process (31, 46, 47). VP4 appears to use a different route to reach the nuclear envelope, as unlike VP2 and VP3, it did not integrate into ER membranes. The deletion of the HD supported the nucleoplasmic targeting of the protein, without membrane association (Fig. 5B). In addition, mutation of the NLS resulted in diffused localization of VP4, whereas classical INM proteins lacking a NLS accumulate in the ER. Therefore, VP4 likely entered the nucleus through the nuclear pore as a soluble protein bound by karyopherins before associating with the nuclear membrane.

Results from biochemical extractions of VP4-containing nuclear membranes were consistent with VP4 associating with nuclear membranes but not integrating across the lipid bilayer. VP4 alkaline membrane extraction profiles resembled that of lamina, a peripherally associated INM protein (Fig. 4 and 7E) (21, 32). As purified VP4 created pores with an inner diameter of 3 nm in biological membranes (42), the important question becomes how a peripherally associated protein can perforate membranes.

Antimicrobial peptides are small cationic amphiphiles created by animals, invertebrates, and plants to act as a defense against microbial pathogens by perforating membranes. Several mechanisms of membrane disruption have been demonstrated for antimicrobial peptides (5). For the carpet mechanism, membrane disruption is initiated by peptide binding to phospholipid head groups and acting as a detergent at high concentrations by dissolving membranes into micelles. A carpet mechanism is ruled out for VP4, since it formed size-selective pores in RBC and Cos7 cell membranes, and the size of liposomes was not affected after VP4-mediated disruption (42). Alternatively, the barrel stave mechanism involves pore formation by integral membrane proteins creating an aqueous channel that crosses the lipid bilayer. Since VP4 was alkaline extractable from membranes, it does not appear to create pores using the barrel stave model. Finally, the toroidal mechanism is initiated by membrane binding and induced membrane curvature that supports the fusion of the outer and inner leaflets of the lipid bilayer. Antimicrobial peptides magainin 2 and melittin are proposed to form toroidal pores in membranes by connecting the outer and inner leaflets of the membrane through peptide-mediated bending of lipid monolayers (34, 38). The polar surfaces of the peptide associate with the polar head groups of the lipids. With the toroidal pore structure, the peptide is not integrated through the hydrophobic core of the lipid bilayer. VP4 membrane properties are consistent with the formation of toroidal pores in the nuclear membrane that support membrane perforation (Fig. 9).

Point mutations of VP4 also support a toroidal mechanism of

FIG 9 Proposed models for membrane perforation by VP4. VP4-induced membrane curvature results in fusion of the inner and outer leaflets of the lipid bilayer to form a toroidal pore. VP4 membrane binding requires a central HD (black rectangles) and proximal basic charged NLS (positive charges). Interactions with the lipid bilayer are mediated primarily through the phospholipid head groups and therefore disrupted upon alkaline extraction. A conserved central Pro residue (P) in the HD is required to support membrane curvature and is essential for membrane disruption. Proposed structures for VP4 membranes pores are as follows: a classical toroidal membrane topology, where VP4 projects across the bilayer but does not integrate into the hydrophobic core of the lipid bilayer (A); and an alternative disordered toroidal pore, where both the N and C termini face the same side of the membrane (B).
membrane disruption. The combined effect of the HD and NLS results in efficient membrane binding. The spatial organization of these domains is highly conserved, and increasing the spacing or changing the topology of this organization did not support membrane disruption. The central Pro requirement in the HD suggests that this residue is crucial for inducing membrane bending by acting as a hinge to cause bilayer fusion. Antimicrobial peptides commonly contain Pro which introduces a kink within a helix. The antimicrobial peptides maculatin 1.1 and alamethicin appear to use central Pro residues to create structures that perforate membranes (12, 17). In addition, computational modeling studies suggest that melittin pores do not adopt a helical structure in the membrane (26). Overall, these results are consistent with the central Pro in the HD of VP4 being required to introduce curvature in the hydrophobic domain to create toroidal pores in the membranes, leading to their perforation.

VP4 was expressed at the time of lysis (16), and it supported the disruption of both biological and synthetic membranes through the formation of membrane pores (42) (Fig. 6). The nuclear pore complex connects the cytoplasm and nucleus with an ~45-nm-diameter channel (52). Why then would positioning an ~3-nm-diameter pore in the nuclear envelope aid the viral life cycle? The formation of a pore in the nuclear membrane would compromise the barrier that separates nuclear and ER luminal contents. However, the destabilization of the nuclear membrane would support the release of the viral particles from the nucleus. Two scenarios can be envisioned for subsequent viral release from the cell. First, the nuclear membrane might act as the Achilles heel of the cell, and its destabilization could lead to subsequent cell lysis supporting viral release. SV40 infection has also been shown to trigger the activation of apoptotic, survival, and stress pathways, which might contribute to cell lysis (7). Alternatively, polyomaviruses express agnoprotein late in infection. Agnoprotein from JC polyomavirus was recently shown to act as a viroporin that created a pore in the plasma membrane of infected cells (53). Therefore, VP4 and agnoprotein might work in concert to disrupt the nuclear and plasma membrane, respectively, to permit efficient viral exit from the nucleus and the cell, respectively.

A long-standing paradox in regard to polyomavirus assembly is that SV40 viral particles are stabilized by calcium and interpenetrating disulfide bonds, yet the virus assembles in the nucleus where calcium levels are low and redox conditions are reducing (30, 35, 48, 51). Since the ER is rich in calcium and it provides an oxidizing environment, a pore in the nuclear membrane might result in the release of calcium or oxidizing agents such as oxidized glutathione into the nucleus or cytoplasm. Alterations in calcium or redox conditions to the nuclear milieu could potentially aid in the assembly of viral particles. Future studies will be required to determine if VP4 assists in optimizing the nuclear environment for capsid assembly prior to or during viral release.

The HD and NLS of VP2/3/4 appear to serve dual roles in the virus life cycle. Both domains are required for the membrane association and disruption activity found with VP4. In the context of VP2 and VP3, the HD from VP4 serves as the VP1 binding domain that supports virus assembly by binding to the hydrophobic central cavity within VP1 pentamers (2, 11, 41). The basic amino acid cluster, or the NLS, directs the trafficking of the late proteins to the nucleus (Fig. 5B) (13). The NLS is also required for the association and perforation of membranes by VP4 (Fig. 5 and 6), likely through the interaction with anionic lipid head groups. A similar requirement for membrane binding was recently uncovered for a basic cluster of amino acids located proximal to the hydrophobic stretch that is essential for the viroporin activity of the influenza virus M2 protein (44). The multiple functions of the late proteins ensure strict conservation of these critical functional domains found in VP2, VP3, and VP4. In addition, VP2 and VP3 possess the perforation domain found in VP4, and they have been found to insert posttranslationally into ER membranes (14, 15, 43). Therefore, in addition to VP4 acting as a viroporin to initiate cell perforation for viral release and dissemination, this perforation activity in the context of VP2 and VP3 might be exploited to aid in the viral penetration process. Further studies are required to understand the full scope of functions that the growing number of viroporins carry out to support the navigation of host cell membranes for the timely progression of the viral life cycle.

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REFERENCES

15. Daniels R, et al. 2006. Simian virus 40 late proteins possess lytic properties
that render them capable of permeabilizing cellular membranes. J. Virol. 80:6575–6587.