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Frog virus 3 ORF 53R, a putative myristoylated membrane protein, is essential for virus replication *in vitro*

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ABSTRACT

Although previous work identified 12 complementation groups with possible roles in virus assembly, currently only one frog virus 3 protein, the major capsid protein (MCP), has been linked with virion formation. To identify other proteins required for assembly, we used an antisense morpholino oligonucleotide to target 53R, a putative myristoylated membrane protein, and showed that treatment resulted in marked reductions in 53R levels and a 60% drop in virus titers. Immunofluorescence assays confirmed knock down and showed that 53R was found primarily within viral assembly sites, whereas transmission electron microscopy detected fewer mature virions and, in some cells, dense granular bodies that may represent unencapsidated DNA-protein complexes. Treatment with a myristoylation inhibitor (2-hydroxymyristic acid) resulted in an 80% reduction in viral titers. Collectively, these data indicate that 53R is an essential viral protein that is required for replication *in vitro* and suggest it plays a critical role in virion formation.

Introduction

Viruses within the family *Iridoviridae* possess large double-stranded DNA genomes enclosed within icosahedral nucleocapsids (Chinchar et al., 2005). The family is divided into five genera, two that infect invertebrates (*Iridovirus* and *Chloriridovirus*) and three that infect cold-blooded vertebrates (*Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*). Whereas viruses within the *Megalocytivirus* and *Lymphocystivirus* genera infect only fish, ranaviruses cause systemic disease in a wide range of agriculturally and ecologically important amphibians, reptiles, and fish (Hyatt et al., 2000; Chinchar, 2002; Williams et al., 2005; Mendelson et al., 2006; Chinchar et al., 2009). Members of the genus *Ranavirus* are responsible for die-offs of farm raised frogs in China, Thailand, and North and South America, salamanders in western North America, and multiple fish species worldwide (Nakajima et al., 1998; Green et al., 2002; Whittington et al., 1996; Ariel et al., 1999).

Frog virus 3 (FV3) is the type species of the genus *Ranavirus* and the best characterized member of the family. Study of FV3 has illuminated many, if not most, of the key elements of iridovirus replication including the temporal regulation of viral gene expression, nuclear and cytoplasmic phases of infection, and transcriptional and translational control mechanisms (reviewed in Chinchar et al., 2009). Although sequence analysis of the FV3 genome identified 98 ORFs, the functions of only about a third of these genes, based on biochemical studies or BLAST analysis, are known or inferred (Tan et al., 2004; Eaton et al., 2007). The remaining genes fall into two categories: the majority shows homology to other iridovirus genes, but not to others in the database, whereas a smaller number are unique to the viruses encoding them. We postulate that these genes may play specific roles in viral metabolism and/or virion assembly or allow a virus to replicate in a specific host by inhibiting innate and acquired immunity.

Until recently, attempts at elucidating FV3 gene function have relied on classical biochemical and genetic approaches. For example, Chinchar and Granoff (1986) characterized 28 temperature sensitive mutants and ordered them into 19 complementation groups and four phenotypic classes. Mutants within classes II–IV played various roles

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in viral gene expression, whereas 16 class I mutants appeared to play a role primarily in virion assembly. However, expansion of these studies has been hindered by the difficulty of using site-directed mutagenesis or gene knockout via homologous recombination to generate specific viral mutants. To circumvent these problems, we and others examined the function of ranavirus gene products using either antisense morpholino oligonucleotides (asMOs) or siRNAs to knock down the expression of specific viral gene products and infer gene function by changes in phenotype (Sample et al., 2007; Xie et al., 2005; Dang et al., 2008). Specifically, we have used asMOs to knockdown the expression of the FV3 major capsid protein (MCP) and a viral homolog of the largest subunit of RNA polymerase II (vPol-IIα). Those studies confirmed that the MCP was required for virion assembly, whereas the vPol-IIα was essential for the expression of late gene products (Sample et al., 2007). We also showed that a highly abundant 18 kDa immediate early protein was nonessential for replication of FV3 in FHM cells *in vitro* (Sample et al., 2007). Here we seek to elucidate the function of a putative viral myristoylated membrane protein, 53R, using an asMO-mediated approach.

FV3 virions share structural features with other nuclear cytoplasmic large DNA viruses (NCLDVs) that possess icosahedral symmetry such as African swine fever virus (ASFV) and the phycodnaviruses that infect algae (Iyer et al., 2006; Wilson et al., 2009; Tulman et al., 2009). FV3 virions are ~150 nm in diameter and composed of four major structural components that, from inside-out, include a central DNA–protein core, an internal lipid membrane, an icosahedral capsid, and, in the case of virus released by budding from the plasma membrane, a viral envelope (Williams et al., 2005; Chinchar et al., 2009). ASFV and FV3 virion assembly occurs in morphologically distinct regions within the cytoplasm (i.e., viral factories/assembly sites, respectively) that contain both viral DNA and protein (Chinchar et al., 1984a,b; Wilson et al., 2009). In the ASFV system, cellular membranes, likely derived from the endoplasmic reticulum, are required for the formation of infectious virions and serve as the source of the internal lipid membrane that lies between the DNA core and the outer capsid shell (Cobbold et al., 1996; Rouiller et al., 1998). Moreover, there is evidence that an ASFV-encoded protein (p54) mediates the recruitment and utilization of these membranes (Rodriguez et al., 2004).

Myristoylated viral proteins have been shown to be required for the assembly of many viruses including human immunodeficiency virus 1 (HIV-1), arenaviruses, ASFV, vaccinia virus, and others (Göttlinger et al., 1989; Bryant and Ratner, 1990; Martin et al., 1997, 1999; Capul et al., 2007; Andrés et al., 2002a). These proteins associate with cellular membranes through hydrophobic myristoyl groups that allow them to embed within lipid bilayers (Maurer-Stroh et al., 2002). Myristoylation is dependent upon a conserved amino acid motif at the N-terminus of the protein, NH_2-M-G-X-X-X-(S/T/A) (Farazi et al., 2001). In the presence of this sequence, N-myristoyltransferase catalyzes the addition of myristate, a 14-carbon saturated fatty acid, to the penultimate G residue. In the case of ASFV, a precursor protein with a myristoylation motif, pp220, associates with cellular membranes and is required for virion assembly.

Like pp220 of ASFV, FV3 ORF 53R encodes a protein with an N-terminal myristoylation sequence that possibly plays an essential role in virion assembly (Tan et al., 2004). 53R is a highly conserved, virion-associated protein that is present in all five genera within the family *Iridoviridae* (Eaton et al., 2007; Zhao et al., 2008). Because of their putative myristoylation sequences, we postulate that FV3 ORF 53R and pp220 perform similar roles in virion assembly. To explore that possibility, we inhibited 53R protein expression using asMOs and determined the effect of knock down on virus replication and assembly. We showed that knockdown of ORF 53R had no effect on overall viral gene expression, but resulted in a decrease in virion formation and a reduction in the formation of mature virions. These results indicate that 53R, perhaps in a manner analogous to pp220, plays a key role in FV3 virion biogenesis.

Results

Knock down of ORF 53R expression

To determine the role of 53R, a putative 54.7-kDa myristoylated membrane protein, in FV3 biogenesis, we blocked its expression using asMOs and ascertained the effect of knock down on viral gene expression and virion formation. FHM cells were pretreated for 24 hours with an asMO targeted to the message for 53R. In addition, FHM cells were pretreated with a nonsilencing control asMO or one targeting the MCP as negative and positive controls, respectively. FHM cells were subsequently infected with FV3, and protein synthesis monitored by radiolabeling infected cells from 7 to 9 hours p.i. and analyzing host and viral protein synthesis by SDS–PAGE. As shown in Fig. 1, FV3 infection resulted in a marked inhibition of host cell protein synthesis (compare lane 1, mock-infected cells to lane 2, FV3-infected cells) and the appearance of at least 15 novel bands that likely represent FV3-specific proteins. Treatment of infected cells with either a nontargeting control MO or an inhibitor of myristoylation (see below) had no apparent effect on viral protein synthesis. However, as seen earlier, treatment with an asMO targeting the MCP resulted in a marked reduction in the synthesis of the MCP, without adversely affecting the synthesis of other viral proteins. In contrast, treatment with an asMO targeting 53R had no effect on the overall expression of viral proteins, and no reduction in a band of the expected size (~54 kDa) was observed. While this result could reflect the inability of this asMO to effectively knock down 53R synthesis, it could also be due to the presence of limiting amounts of 53R and/or the comigration of 53R with the more abundant MCP.

To resolve this issue, we obtained a plasmid expressing the 53R gene of *Rana gyrlio* virus (RGV), a ranavirus that is likely a strain of FV3 (Zhang et al., 2001; Zhao et al., 2008), and used this recombinant protein to develop polyclonal rabbit anti-53R serum. Using rabbit polyclonal anti-53R serum we demonstrated (Fig. 2) that 53R was not

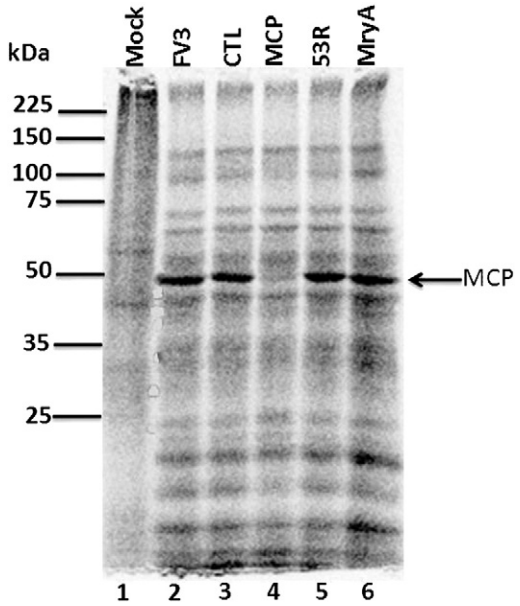

Fig. 1. SDS–PAGE analysis of asMO-treated, FV3-infected FHM cells. Mock-infected (lane 1) and virus-infected FHM cells (lanes 2–6) were exposed to the indicated asMOs (lanes 3–5) or treated with 1 mM 2-hydroxymyrisitic acid (lane 6) and protein synthesis monitored 7–9 hours p.i. Radiolabeled proteins were separated on a 10% SDS–PAGE and visualized by phosphorimaging. The position of the MCP is indicated by an arrow. Molecular weight markers (kDa) are shown to the left of the gel image. Lane headings here and elsewhere refer to untreated, FV3-infected FHM cells (FV3), or FV3-infected cells pretreated with a nontargeting control MO (CTL), an asMO targeted to the major capsid protein (MCP), an asMO targeted against 53R (53R), or 2-hydroxymyristic acid (MyrA).

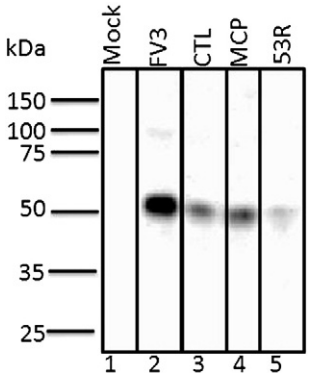

Fig. 2. Western blot analysis of asMO-treated, FV3-infected FHM cells. Mock-infected (lane 1) and FV3-infected FHM cells (lanes 2–5) were untreated (lane 2) or pretreated for 24 hours with a nontargeting control MO (CTL; lane 3), or asMOs targeted against the MCP (lane 4) or 53R (lane 5). At 9 hours p.i., lysates were prepared, and proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-53R serum for 2 hours followed by incubation with HRP-conjugated goat anti-rabbit antibody. Proteins were visualized using a chemiluminescent substrate. Molecular weight markers are shown to the left of the figure.

present in mock-infected FHM cells, but was clearly detected in FV3 lysates as a single band that comigrated at ~50 kDa along with the MCP (compare Figs. 1 and 2, lane 2). Consistent with an earlier study, there was a slight decrease in 53R levels when an asMO targeting the MCP or a nontargeting control MO were used. This reduction was likely due to the transfection reagent since we previously observed a similar global decrease in viral protein synthesis when EndoPorter was used alone (Sample et al., 2007). However, in contrast to this modest level of inhibition, prior treatment with an asMO targeting 53R markedly reduced 53R levels (Fig. 2, lane 5). Furthermore, the observed co-migration of MCP and 53R suggests that our earlier inability to detect 53R knock down by 1D SDS-PAGE was due to the presence of abundant levels of MCP that obscured detection of 53R. In addition, the observation that MCP expression is nearly completely blocked by asMO knock down (Fig. 1, lane 4) suggests that if MCP and 53R are present within the same region of the gel, MCP represents the bulk of the material, whereas 53R is a minor component.

To confirm this result and determine the intracellular location of 53R, we used anti-53R serum to demonstrate asMO-mediated knock down by indirect immunofluorescence assay (IFA). FHM cells were either mock-infected or infected with FV3 and at 24 hours p.i., were fixed, sequentially incubated with polyclonal anti-53R serum and FITC-conjugated goat anti-rabbit serum, and stained with Hoechst 33258. As expected, 53R-specific fluorescence was not detected in mock-infected cells (Fig. 3, A) whereas marked fluorescence was seen within viral assembly sites in both untreated cells (data not shown) and cells treated with a nontargeting control MO (Fig. 3D). Consistent with the Western blot findings, treatment with an asMO targeting 53R resulted in the almost complete absence of 53R staining within FV3-infected cells (Fig. 3G). In addition, we observed that mock-infected cells stained with Hoechst displayed well-formed nuclei, but that following virus infection, nuclei show marked alterations in appearance (Fig.3, compare B and E). Furthermore, small Hoechst-stained bodies appear adjacent to the nuclei and likely represent viral assembly sites. As shown in Fig. 3H, assembly sites persist after treatment with the anti-53R asMO indicating that wild-type levels of 53R are not needed for assembly site formation. Collectively, the results shown in Figs. 2 and 3 confirm that treatment with an asMO targeted against 53R resulted in a marked reduction in its synthesis.

In a previous study, we noted that a greater than 80% reduction in the synthesis of the 18K immediate early protein had no effect on the expression of other viral genes or the yield of infectious virus and indicated that 18K was nonessential for replication *in vitro* (Sample et al., 2007). To determine if 53R was essential for virus replication, we analyzed viral yields in cells treated with the anti-53R asMO. As shown in Fig. 4, treatment of FV3-infected FHMs with anti-53R asMO reduced viral titers by more than 60% and TEM analysis indicated a corresponding reduction in mature virions (Fig. 5). Most anti-53R asMO-treated, FV3-infected cells displayed viral assembly sites, but showed marked reductions in the number of mature virions (Fig. 5, compare A and B). However, aggregates, possibly representing viral protein–DNA complexes, could be observed in some 53R asMO-treated cells (Fig. 5C and D). The appearance of these structures was possibly due to a block in virion assembly due to limiting amounts of 53R protein. Collectively, these findings showed that like the MCP, 53R was essential for virus replication *in vitro*.

Role of myristoylation in FV3 virion morphogenesis

The above studies demonstrate that 53R is required for FV3 replication and virion assembly. However, these studies do not formally prove that 53R is myristoylated or that myristoylation is important in viral replication. To address these questions, we first asked what effect inhibition of myristoylation had on FV3 replication. Replicate FHM cells were treated with 1 mM 2-hydroxymyristic acid (myrA), an inhibitor of myristoylation, 1 hour before and continuously throughout infection, and viral protein synthesis was monitored by radiolabeling with [^{35}S]methionine from 7 to 9 hours p.i. SDS–PAGE analysis showed no difference between treated and untreated cells indicating that the inhibitor did not affect primary viral gene expression (Fig. 1, lane 6). Although myrA treatment had no effect on viral protein synthesis, we asked what effect myrA treatment had on virus replication by monitoring viral yields in control and myrA-treated cells. Consistent with other viral systems in which myristoylation was shown to be important for virus replication, treatment of FV3-infected cells with 1 mM myrA resulted in an 80% reduction in viral yields (Fig. 4). Furthermore, TEM analysis of myrA-treated FV3-infected cells showed a marked reduction in virion formation and the appearance of numerous empty virions (Fig. 6B–D).

To determine if myrA affected the intracellular localization of 53R, virus-infected cells treated with myrA were examined by IFA following incubation with anti-53R antibody and staining with Hoechst 33258 (Fig. 3J, K, and L). As seen previously, FV3 infection affected nuclear morphology and resulted in the appearance of lightly staining perinuclear bodies that likely represent viral assembly sites (Fig. 3H). However, even in the presence of myrA, assembly sites were stained by an antibody targeting 53R (Fig. 3J), suggesting that the lack of myristoylation did not grossly affect the intracellular localization of 53R.

Discussion

The abovementioned results indicate that FV3 ORF 53R, a putative myristoylated membrane protein, was required for a productive FV3 infection *in vitro*. Herein we showed that blocking 53R expression using a gene-specific asMO resulted in a marked reduction in 53R protein expression and an ~60% drop in viral titers. The reduction in the level of mature virions suggests that 53R, in a manner analogous to some myristoylated viral proteins, is required for virion assembly. Moreover, asMO-mediated repression of 53R expression was gene specific and did not affect the synthesis of other viral proteins or block the formation of viral assembly sites. Furthermore, whereas TEM analysis showed that in most cells fewer mature virions were present, in a minority of cells dense granular bodies (DGB) were detected within viral assembly sites. DGBs may represent protein–DNA complexes that were not encapsidated, or viral structural proteins that aggregated and were not capable of forming mature capsids in the absence of normal levels of 53R. While the precise composition of DGBs is unknown, similar structures were observed following

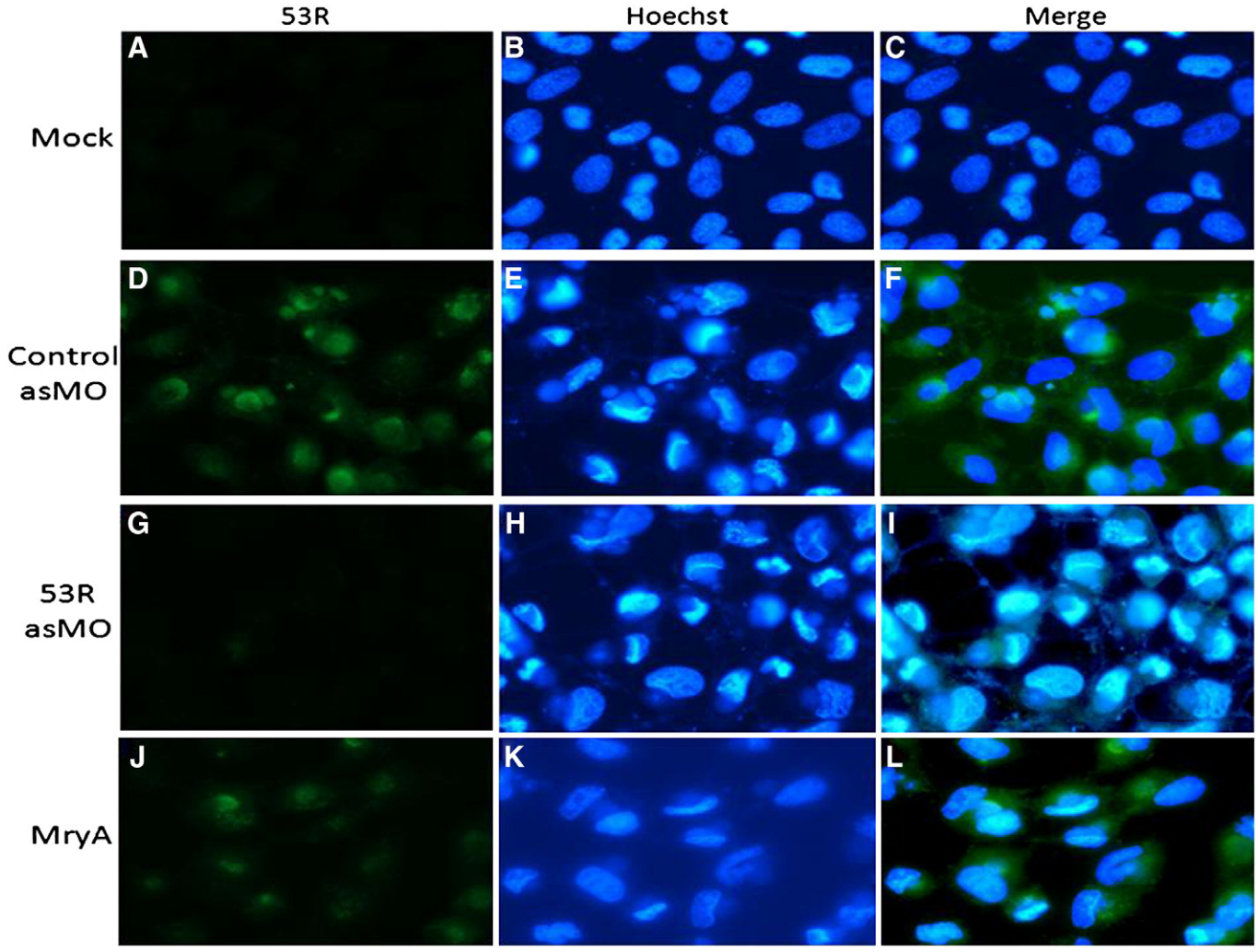

Fig. 3. Intracellular localization of FV3 53R by indirect immunofluoresence microscopy. FHM cells were mock-infected (A–C), or FV3 infected following treatment with a nontargeting control asMO (D–F), an anti-53R asMO (G–I), or myrA (J–L) as described in Materials and methods. Twenty-four hours after infection, the cells were fixed, permeabilized, and incubated with rabbit anti-53R serum and FITC-conjugated anti-rabbit antibody, followed by staining with Hoescht 33258.

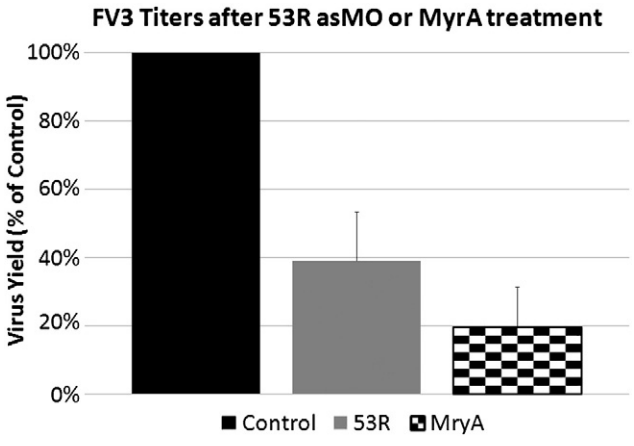

Fig. 4. FV3 yields following treatment with anti-53R asMO and myrA. FHM cells were treated with anti-53R asMO 24 h before FV3 infection or treated with 1 mM 2-hydroxymyristic acid 1 h before and continuously throughout FV3 infection. At 24 hours p.i., replication was stopped by freezing and viral yields determined by plaque assay. Shown are the averages and standard deviations of seven replicates from anti-53R-treated cultures and four replicates from myrA-treated cells.

infection of three Class I FV3 temperature-sensitive mutants incubated at nonpermissive temperatures (Chinchar and Granoff, 1986, RC Sample and VG Chinchar, unpublished observations) and in wt-infected cells shifted to 37 °C after initial growth at permissive temperatures (Tripier et al., 1977). Collectively, these results support the importance of 53R in virion assembly.

N-myristoylation is an irreversible protein modification that occurs co-translationally in eukaryotes and their viruses and promotes weak and reversible protein–membrane and protein–protein interactions (Farazi et al., 2001). Although myristoylation is required for the replication of retroviruses (Göttlinger et al., 1989), poxviruses (Ravanello et al., 1993; Ravanello and Hruby, 1994a, 1994b), herpesviruses (Harper et al., 1993), arenaviruses (Perez et al., 2004), geminiviruses (Fondong et al., 2007), picornaviruses (Goodwin et al., 2009), and reoviruses (Chandran et al., 2002), viruses, with the likely exception of entomopoxvirus, do not encode myristoyltransferases, but rely on cellular enzymes. Myristoylation occurs on the penultimate G residue of proteins bearing the consensus sequence (NH_2-M-G-$(X)_3$-[S/T/A]-COOH) and may play a role in either virion assembly, virion entry or both. Several examples illustrate this point. In vaccinia virus, six myristoylated viral proteins have been identified and three (L1R, A16L, and E7R) have been characterized in detail (Martin et al., 1997). A16L and E7R are soluble proteins, whereas L1R is membrane associated. Analysis of conditional lethal mutants demonstrated that L1R is an integral component of the membranes of

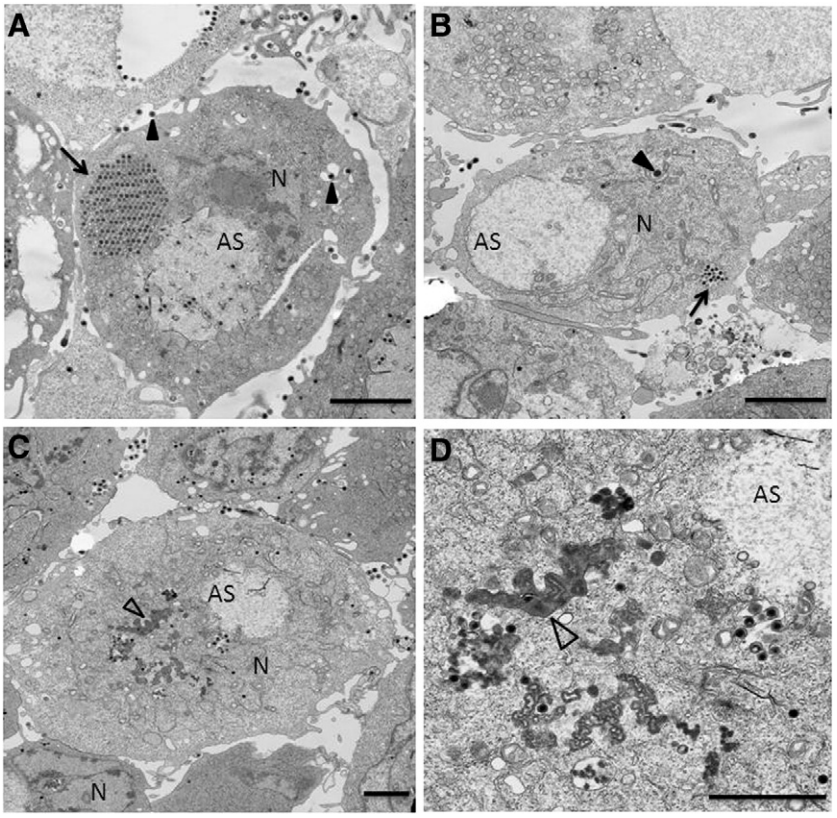

Fig. 5. Transmission electron microscopy of control and anti-53R asMO-treated, FV3-infected FHM cells. FHM cells were treated with CTL or anti-53R asMOs and 24 hours later infected with FV3 (MOI = 20 PFU/cell). At 9 hours p.i., cultures were fixed in 2% glutaraldehyde and processed for electron microscopy. (A) Control asMO-treated, FV3-infected FHM cells. (B–D) Anti-53R asMO-treated, FV3-infected FHM cells. Panel D is an enlargement of the cell shown in panel C. N, nucleus; AS, assembly site; closed arrowheads indicate mature virions; open arrowhead indicate dense granular bodies; arrow indicates a paracrystalline array. Scale bars represent 1.5 μm.

intracellular mature virions and is essential for virion morphogenesis (Ravanello and Hruby, 1994a,b). In nonenveloped, RNA-containing icosahedral viruses such as poliovirus and foot-and-mouth disease virus (family *Picornaviridae*) and reovirus (family *Reoviridae*) myristoylation is thought to be required both for virion assembly (Goodwin et al., 2009) and virus entry (Chandran et al., 2002; Belnap et al., 2000). Myristoylation

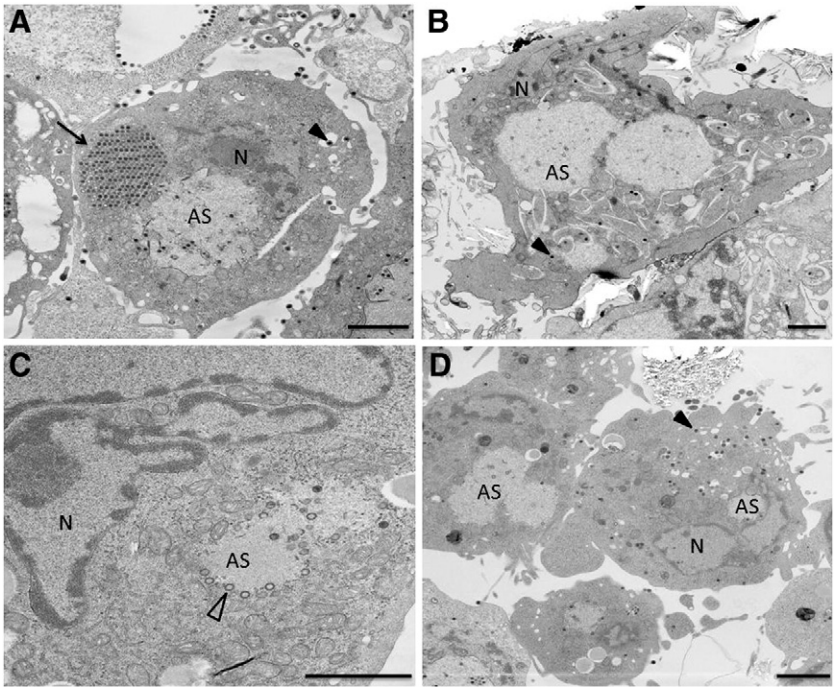

Fig. 6. Transmission electron microscopy of myrA-treated, FV3-infected cells. (A) Nontreated, FV3-infected FHM cells. (B–D) MyrA-treated (1 mM), FV3-infected FHM cells. At 9 hours p.i., cells were processed for transmission electron microscopy as indicated in Materials and methods. N, nucleus; AS, assembly site; arrow indicates a paracrystalline array; closed arrowheads indicate virions; open arrowheads indicate empty capsids. Scale bars represent 1.5 μm.

of the picornavirus capsid precursor protein P1 is thought to play a role in virion assembly by stabilizing the five protomeric subunits that form the pentameric vertices (Chow et al., 1987; Marc et al., 1990, 1991), Likewise, externalization of N-myristoylated VP4 exposes amphipathic sequences within VP1 that may mediate entry (Belnap et al., 2000).

Assembly mechanisms, more relevant to the study of FV3 than those cited above, are provided by the ASFV model. ASFV is a large, icosahedral dsDNA-containing virus that is phylogenetically related to poxviruses, iridoviruses, phycodnaviruses, mimiviruses, and ascoviruses (Iyer et al., 2006; Wilson et al., 2009). ASFV has been described as a "poxvirus in iridovirus clothing" in reference to its poxvirus-like genomic organization and iridovirus-like virion morphology (Tulman et al., 2009). Like FV3, ASFV formation takes place within perinuclear cytoplasmic viral factories or assembly sites (AS) that bear a striking resemblance to aggresomes. ASFV viral factories and aggresomes (and likely FV3 AS) are located close to the microtubule-organizing center, exclude cellular organelles, recruit chaperones and mitochondria, and are surrounded by the vimentin network (Heath et al., 2001; Murti and Goorha, 1989; Murti et al., 1985). ASFV recruits cellular membranes that are likely derived from the endoplasmic reticulum (ER) or Golgi to viral factories where virion formation takes place (Rodriguez et al., 2004). Viral assembly is thought to involve the deposition of capsid protein (p72) and pB438L on the convex surface of the membrane and pp220 and pp62 onto the concave surface (Andrés et al., 1997, 2002a,b; Suárez et al., 2010). pp220 is a myristoylated polyprotein that gives rise to four virion-associated cleavage products (p150, p37, p34, and p14), whereas pp62 is a polyprotein that is cleaved to generate p35 and p14. Together pp220 and pp62 and their cleavage products make up about one-third of virion mass and form the "core shell" that lies between the internal lipid membrane and the DNA core. Although pp220 is thought to interact with lipid membranes through its myristoyl group, pp220 is not needed for the formation of virus-like particles since in its absence p72 associates with membranes and leads to the formation of empty capsids (Andrés et al., 2002a). In addition to pp220, ASFV also encodes at least one other myristoylated protein, pE248R that is required for viral infectivity (Rodríguez et al., 2009). Like pp220, pE248R associates with the membrane fraction in infected cells, but it appears to function at the level of virion entry, rather than in virion assembly. pE248R belongs to a class of myristoylated membrane proteins related to the vaccinia virus L1R protein. These proteins contain disulfide bridges and, at least in the case of pE248R, are thought to be a final substrate for virus-encoded redox enzymes such as the flavin adenine dinucleotide-linked sulfhydryl oxidase encoded by pB119L (Rodríguez et al., 2006).

To identify possible homologs of FV3 53R, PSI-BLAST analysis was conducted using 53R as the query (Altschul et al., 1997). Aside from the expected identification of conserved iridovirus proteins, PSI-BLAST showed significant homology to at least two ascovirus proteins and the ASFV pE248R protein (*E* value 2e-37) but did not detect homology to pp220 or any of its cleavage products. Since inspection of the FV3 genomic sequence (Tan et al., 2004) identified no other ORFs containing the consensus myristoylation sequence, it is likely that 53R is the only candidate for myristoylation among the FV3 proteome. Whether 53R functions like pE248R or pp220 remains to be resolved. FV3 53R (522 amino acids) is more than twice the size of either vaccinia L1R (250 amino acids) or ASFV pE248R (248 amino acids) and sequence similarity among these three proteins is very low (10–11%). Currently, we do not know if FV3 53R functions at entry, like pE248R, during assembly, like pp220, or in both capacities. The observation that virion assembly is diminished in anti-53R asMO-treated cells suggests a role similar to pp220, whereas the PSI-BLAST result showing homology with pE248R supports a role in entry.

At present, the composition of the FV3 core shell is unknown. Although IFA indicates that 53R is present within FV3 AS, we do not know its precise location. However, using rabbit anti-53R serum, we should be able to determine by immunoelectron microscopy whether 53R is associated with membranous structures, virion intermediates, or virions. Preliminary coimmunoprecipitation data suggest that 53R associated with at least one other viral protein, possibly the MCP. If that result is confirmed, it may suggest that 53R and MCP are closely associated, perhaps bound to the same membrane fragment. However, it would not distinguish between independent binding of MCP and 53R to the membrane, or whether prior binding of 53R is required for subsequent binding by the MCP.

A recent study by Zhao et al. (2008) showed that 53R preferentially associated with viral AS in cells infected with *Rana grylio* virus, an isolate that is likely a strain of FV3 (Zhang et al., 2001). Multiple alignment indicated that RGV 53R was >99% identical to FV3 53R and that among divergent rana-, megalocyti-, irido-, chlorirido-, and lymphocystiviruses regions of identity, including two conserved cysteines, were present. Treatment of RGV virions with NP-40 plus/minus dithiothreitol resulted in the appearance of 53R in the soluble fraction suggesting that 53R was membrane associated. However, it is not clear from these results whether 53R was associated with the internal lipid membrane or the external viral envelope.

As indicated above, among the 98 putative FV3 ORFs, only ORF 53R possesses an N-terminal myristoylation motif and is thus the only candidate for myristoylation. To formally demonstrate myristoylation of 53R, studies are in progress using ^{3}H-myristic acid to label, and polyclonal rabbit anti-53R serum to immunoprecipitate 53R and specifically detect the myristoylated protein. Moreover, we have obtained evidence consistent with this view by demonstrating that treatment with myrA markedly reduced FV3 replication and resulted in an almost complete inhibition of virion formation. The observed reduction in virus yields was not a consequence of the toxicity of the compound since growth of FHM cells in myrA for 48 hours had no observable effect on host cell viability (data not shown). In addition, viral protein synthesis was neither quantitatively nor qualitatively altered in myrA-treated cells (Fig. 1). Moreover, although the myristoyl group is likely required for membrane association, its presence was not required for targeting 53R to assembly sites since blocking myristoylation did not affect the subcellular location of 53R. Finally, as suggestive as these results are, it is possible that myristoylation affects some other process required for virus replication. Formal proof that myristoylation of 53R is required for viral infection will require the generation of a nonmyristoylatable mutant as was done previously with the p220 protein of ASFV (Andrés et al., 1997).

Taken together, our findings provide evidence that both myristoylation and a putative myristoylated membrane protein were required for a productive FV3 infection. These studies will help to further illuminate the ranavirus assembly process and better understand the interplay that occurs between host and virus during productive infections. Additional characterization of the 53R protein will be required in order to determine its exact role during FV3 infection. Coimmunoprecipitation experiments are in progress to identify viral or host proteins that may interact with 53R. In addition, immunoelectron microscopy will determine which cellular organelles, if any, interact with 53R. Successfully completed, these studies will indicate whether the assembly of iridovirus virions is similar to that of ASFV, or whether iridoviruses possess unique features that distinguish them from their closest relatives.

Materials and methods

Cells and virus

FV3 infections were performed in fathead minnow cells (FHM, ATCC No. CCL-42) grown in Dulbecco's modified Eagle's medium (DMEM) containing 4% fetal bovine serum (D4) at 26 °C in a humidified incubator in 95% air and 5% CO_2. FV3 (Granoff et al.,

1966; ATCC VR-569) was propagated on confluent monolayers of FHM cells grown in 150-cm^2 flasks and incubated in Eagle's minimum essential medium with Hank's salts (HMEM) supplemented with 4% fetal bovine serum (H4). To generate virus stocks, FHM cells were infected at an MOI = 0.01 PFU/cell and harvested ~5 days later when cytopathic effect was marked. Virions were released by three freeze–thaw cycles and clarified by low-speed centrifugation. Titers were determined by plaque assay on FHM monolayers under an overlay of 0.75% methylcellulose.

Antisense morpholino oligonucleotides

AsMOs and delivery agents were purchased from GeneTools (Philomath, OR) and were used according to the manufacturer's protocol. Three different morpholino oligonucleotides (MO) were utilized: a nontargeting negative control (CTL) (5′CCTCTTACCTCAGTTACAATTTATA3′), an asMO targeting the major capsid protein (MCP) (5′TGAACCAGTTACAGAAGA**CAT**TTCC3′) as a positive control, and an asMO targeting ORF 53R (53R) (5′TGTTTGATAGATTCCGCTGCTC**CAT**3′). As indicated by underlining and boldface type, both anti-MCP and anti-53R bound slightly upstream of, or precisely at, the AUG initiation codon. AsMOs were used at a final concentration of 10 μM and the delivery agent EndoPorter (EP) was used at final concentration of 6 μM.

FV3 protein synthesis

FHM cells were grown to ~80% confluency (~1.9×10^6 cells/well) in six-well tissue culture plates. On the day of assay, the media was removed by aspiration and replaced with 1 mL of D4. Subsequently, 6 μL of EndoPorter and 10 μL of a 1 mM stock of the indicated asMO were added, and the cultures incubated at 26 °C. Twenty-four hours after treatment, the cultures were infected with FV3 at a MOI = 20 PFU/cell. To detect viral proteins, replicate cultures were radiolabeled with methionine–cysteine free Eagle's minimum essential medium with Earle's salts (EMEM) containing 30 μCi/mL [^{35}S]methionine–cysteine (EasyTag Express Protein Labeling Mix, Perkin-Elmer) from 7 to 9 hours p.i. At 9 hours p.i., the radiolabeled medium was removed, and cell lysates were prepared by disrupting the cells in 120 μL direct sample buffer (125 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.02% 2-mercaptoethanol, 0.01% bromophenol blue). Radiolabeled proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels (Laemmli, 1970) and visualized by autoradiography using either Kodak XAR film or a phosphorimager (BioRad, Personal Molecular Imager).

FV3 viral yields

To determine virus yields, 80% confluent FHM monolayers were treated with gene-specific asMOs as previously described and infected with FV3 at a MOI of 20 PFU/cell. Twenty-four hours after infection, viral growth was stopped by freezing the cultures, and virions, released by three cycles of freeze–thaw, were clarified by low-speed centrifugation. Virus-containing supernatants were serially diluted 10-fold, and 200 μL of each dilution was added to duplicate wells of confluent FHM monolayers grown on six-well plates. After a 1-hour absorption period, the cells were overlaid with 2 mL of DMEM containing 0.75% methyl cellulose. After 7 days, the overlay was removed, and plaques were visualized by staining with 1% crystal violet in 70% ethanol.

Transmission electron microscopy

Replicate cultures were treated with asMOs as previously described and infected at a MOI = 20 PFU/cell. At 9 hours p.i., the medium was removed and the cells were rinsed gently with 0.1 M sodium phosphate, pH 7.4. The cells were scraped into fresh buffer, pelleted, fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer, and stored at 4 °C until processed for electron microscopy. At that time, the samples were postfixed in 1% osmium tetroxide and stained with 2% uranyl acetate in deionized water. The samples were dehydrated in a stepwise fashion in ethanol, the ethanol was removed by treatment with 100% propylene oxide, and the samples were embedded in 100% Epon. Thin sections (0.1 μm) were floated onto 200-mesh copper hexagonal grids (Electron Microscopy Science, Fort Washington, PA), counterstained with lead citrate, and examined using a Leo 906 (Zeiss) transmission electron microscope.

Inhibition of myristoylation

FHM cells were grown to confluency (~2.4×10^6 cells/well) in six-well tissue culture plates. One hour before FV3 infection, the medium was removed by aspiration and replaced with D4 containing 1 mM 2-hydroxymyristic acid (myrA, Sigma Chemical, cat. no. H6771), a potent inhibitor of myristoylation (Nadler et al., 1993). Subsequently, both control and experimental samples were infected with FV3 at an MOI = 20 PFU/cell. After a 1-hour attachment period, the inoculum was removed and replaced with 2 mL D4 or D4 with 1 mM myrA. Replicate cultures were processed for SDS–PAGE analysis, plaque assay, and transmission electron microscopy as indicated above. To maintain inhibition, treated samples were continuously in the presence of myrA from 1 hour before infection until the termination of the experiment.

Prokaryotic expression, protein purification, and antibody preparation

A plasmid, pET32a/53R, expressing the 53R protein from *Rana gyrlio* virus was obtained from Zhao et al. (2008) and used to transform BL21 competent *Escherichia coli*. Transformed bacteria were treated with 0.8 mM IPTG for 4 hours at 37 °C to induce expression of recombinant 53R protein. Recombinant 53R, with an N-terminal polyhistidine tag, was harvested using MagneHis Ni-Particles (Invitrogen) according to the manufacturer's protocol. For the initial immunization, recombinant 53R (133 μg) was mixed with an equal volume of Freund's complete adjuvant and used to subcutaneously immunize a rabbit. Subsequent immunizations were repeated at 14-day intervals using 53R (66 μg) mixed with an equal volume Freund's incomplete adjuvant. Serum was collected biweekly to monitor seroconversion, and a week after the 3rd immunization, the animal was exsanguinated and serum was prepared. Antibody titers were determined by ELISA using purified recombinant 53R as antigen.

Western blot

FHM cells were infected with FV3 at an MOI = 20 PFU/cell. At 9 hours p.i., the cells were lysed in direct sample buffer and host and viral proteins, separated by electrophoresis on a 12% SDS-polyacrylamide gel, were transferred to a nitrocellulose membrane at 100 V for 1 hour at 4 °C. After blocking in Tris-buffered saline (50 mM Tris–HCl, pH 8.0, 150 mM NaCl) containing 0.01% Tween (TBST) and 10% milk at 4 °C, the membrane was incubated with anti-53R serum, diluted 1:1000 (vol./vol.) in TBST-10% milk, for 2 hours at 4 °C with agitation. Subsequently, the primary antibody was removed; the membrane was washed three times with TBST and incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (H + L) antibody (1:2000 dilution, Southern Biotechnology Associates, Inc., cat. no. 4050-05) for an additional 2 hours at 4 °C. After washing, immunoreactive bands were visualized with a SuperSignal West Pico chemiluminescent substrate kit (Pierce Biotechnology) using the ChemiDoc XRS system (BioRad). Mock-infected FHM cell lysates were used as negative control.

Indirect immunofluorescence assay (IFA)

FHM cells were grown to ~80% confluency on poly-L-lysine-treated microscope slides. Replicate cultures were either untreated or treated with a control asMO or one targeting 53R for 24 hours before infection. Cells were infected with FV3 at a MOI = 20 PFU/cell and a subset of samples incubated in D4 containing 1 mM myrA for 1 hour before, and continuously throughout, infection. At 24 hours p.i., the cells were briefly washed with PBS and fixed by submersion in 3.7% formaldehyde for 1 min. After fixation, the cells were permeabilized using 100% cold methanol and briefly washed with PBS. After blocking with 1% BSA in PBS, the cells were incubated with rabbit anti-53R serum (diluted 1:500, vol./vol., in PBS–1% BSA) at room temperature for 1 hour. After washing, cells were incubated with FITC-conjugated goat anti-rabbit serum (1:5000, Southern Biotechnology Associates) and stained with 1 μg/mL Hoechst 33258 for 15 min. After washing, glass cover slips were mounted using ProLong Gold antifade reagent (Invitrogen) and fluorescence was observed using a Nikon Eclipse 50i microscope.

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