Virology 405 (2010) 448–456

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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

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ARTICLE INFO

Article history:
Received 24 February 2010
Returned to author for revision
26 March 2010
Accepted 17 June 2010
Available online 14 July 2010

Keywords:
Frog virus 3
Iridovirus
Ranavirus
Virion assembly
Antisense morpholino oligonucleotides
Transmission electron microscopy
Immunofluorescence assay
Myristoylated viral protein
Viral membrane protein

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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0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.06.034

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

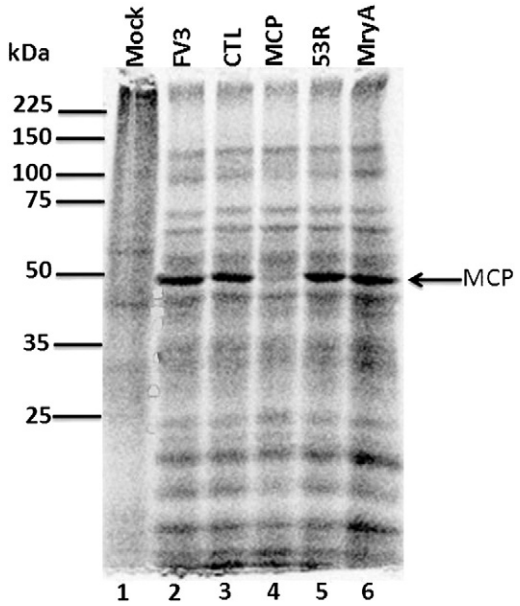

Fig. 1. SDS–PAGE analysis of asMO-treated, FV3-infected FHM cells. Mock-infected (lane 1) and virus-infected FHM cells (lanes 2–6) were exposed to the indicated asMOs (lanes 3–5) or treated with 1 mM 2-hydroxymyrisitic acid (lane 6) and protein synthesis monitored 7–9 hours p.i. Radiolabeled proteins 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).