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Characterization of Frog Virus 3 knockout mutants lacking putative virulence genes

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ABSTRACT

To identify ranavirus virulence genes, we engineered Frog Virus 3 (FV3) knockout (KO) mutants defective for a putative viral caspase activation and recruitment domain-containing (CARD) protein (Δ64R-FV3) and a β-hydroxysteroid dehydrogenase homolog (Δ52L-FV3). Compared to wild type (WT) FV3, infection of *Xenopus* tadpoles with Δ64R- or Δ52L-FV3 resulted in significantly lower levels of mortality and viral replication. We further characterized these and two earlier KO mutants lacking the immediate-early18 kDa protein (FV3-Δ18K) or the truncated viral homolog of eIF-2α (FV3-ΔvIF-2α). All KO mutants replicated as well as WT-FV3 in non-amphibian cell lines, whereas in *Xenopus* A6 kidney cells replication of ΔvCARD-, ΔvβHSD- and ΔvIF-2α-FV3 was markedly reduced. Furthermore, Δ64R- and ΔvIF-2α-FV3 were more sensitive to interferon than WT and Δ18-FV3. Notably, Δ64R-, Δ18K- and ΔvIF-2α- but not Δ52L-FV3 triggered more apoptosis than WT FV3. These data suggest that vCARD (64R) and vβ-HSD (52L) genes contribute to viral pathogenesis.

Introduction

Ranaviruses such as Frog Virus 3 (FV3) are emerging pathogens that cause severe morbidity and mortality among fish, amphibians and reptiles worldwide (Chinchar et al., 2009; Duffus et al., 2015). The increase in prevalence and the expansion of host range suggests that ranaviruses are successful in overcoming host immune defenses. Although the general outlines of the FV3 replication cycle are known and 19 iridovirus genomes have been sequenced (Chinchar et al., 2009; Jancovich et al., 2015, 2010; Tan et al., 2004; Williams et al., 2005), the precise functions of most viral genes are still unknown. Previously a number of temperature-sensitive mutants were isolated and have proven useful in identifying genes essential for virus replication (Chinchar and Granoff, 1986; Goorha and Dixit, 1984; Goorha et al., 1981). In addition, transient knock down of viral gene function using antisense morpholino oligonucleotides (Sample et al., 2007) or siRNA (Whitley et al., 2011) have also elucidated the function of several viral genes. However, the random nature of temperature sensitive mutants and the inability to readily perform knock down *in vivo*, limits the usefulness of these approaches, especially if one wishes to target virulence genes. As an improvement on these approaches, we and others have recently used homologous recombination to directly knock out specific ranavirus genes and assess their roles in virus replication and virulence (Chen et al., 2011; Jancovich and Jacobs, 2011).

Among putative ranavirus immune evasion genes, the viral homolog of the cellular translation factor eIF-2α (vIF-2α) has received attention as an antagonist of protein kinase R (PKR) (Beattie et al., 1995; Rothenburg et al., 2011). Several ranaviruses including Epizootic Haematopoietic Necrosis Virus (EHNV; (Essbauer et al., 2001)); *Ambystoma tigrinum* Virus (ATV, (Jancovich and Jacobs, 2011)); and *Rana catesteiana* Virus Z (RCV-Z, (Rothenburg et al., 2011)) encode full-length vIF-2α genes. Furthermore, in both ATV and RCV-Z, vIF-2α was postulated to act as a pseudo-substrate and block PKR-mediated translational inhibition and cell death (Rothenburg et al., 2011; Jancovich and Jacobs, 2011). In addition, vIF-2α may also play a role in the degradation of PKR following ATV infection (Jancovich and Jacobs, 2011). In contrast to the above ranaviruses, the FV3 vIF-2α gene is truncated and lacks the N-terminal PKR binding domain and the central helicase domains (Chen et al., 2011). Thus, the precise functional role of the FV3 vIF-2 remains in question.

Abbreviations: ANOVA, One-Way Analysis of Variance; BHK-21, baby hamster kidney-21 cells; βHSD, ORF 52L, β-hydroxysteroid dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FV3, Frog Virus 3; IE, immediate-early; i.p., intraperitoneal injection; MOI, multiplicity of infection; PFU, plaque forming units; p.i., post-infection; qPCR, quantitative real-time PCR; RV, Ranavirus; vCARD, ORF 64R, Caspase Activation and Recruitment Domain-containing protein; vIF-2α, ORF 26R, viral homolog of eukaryotic translation initiation factor-2 alpha; 18K, ORF 82R, FV3 18 kDa immediate early protein

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In addition to vIF-2α, a Caspase Activation and Recruitment Domain (CARD)-containing gene (vCARD) has also garnered attention as a putative immune-evasion protein. Typically CARD motifs modulate interactions among CARD-containing cellular proteins (Kawai and Akira, 2009, 2010). Cellular signaling molecules containing CARD domains include pro-apoptotic proteins, pro-inflammatory molecules and proteins participating in the cellular interferon responses, *e.g.*, RIG-I and MAVS (Besch et al., 2009; Meylan et al., 2005). Ranavirus vCARD is postulated to interact with one or more of these signaling molecules and to either block apoptosis or impair interferon (IFN) induction.

Lastly, a ranavirus homolog of β-hydroxysteroid dehydrogenase (vβHSD) has been identified as another possible immune evasion protein. βHSD homologs are present within poxviruses and have been shown to play a role in dampening host immune responses by elevating the levels of steroid hormones (Moore and Smith, 1992; Sroller et al., 1998). Whether the putative ranavirus homolog of βHSD functions in the same way remains to be determined.

We have recently developed a robust, dual-selection system (consisting of the puromycin-resistance gene fused to the gene for enhanced green fluorescent protein, PuroR/GFP) and successfully generated two FV3 KO mutants in which the genes encoding the 18K immediate-early protein and vIF-2α were replaced with PuroR/GFP (Chen et al., 2011). Here we use this technique to target two putative ranavirus virulence genes, vCARD (ORF 64R) and β–HSD (ORF 52L). In the first part of this report, we describe the generation and characterization of these two KO mutants, whereas in the latter sections we examine these two mutants along with two previously isolated ones lacking vIF-2α and the 18 kDa immediate early protein. The latter studies examined the ability of the KO mutants to replicate in *Xenopus* A6 cells, induce and respond to IFN, and trigger apoptosis. Marked differences between these four KO mutants and WT virus following infection of A6 cells suggest that the four targeted genes play critical roles in virus replication in *Xenopus* cells and modulate interferon and apoptotic responses.

Results

Generation and isolation of FV3 knockout mutants.

To generate FV3 knockout (KO) mutants, a recombination cassette was constructed in which the puromycin resistance gene fused to the EGFP gene by a five amino acid linker was placed under the control of the strong FV3 *18K* promoter (18Kprom-Puro-EGFP). The cassette was transfected into FV3-infected BHK cells and introduced into the FV3 genome at regions encoding vβHSD (ORF 52L;57,481–58,548) and vCARD (ORF 64R; 75,529–75,816) using homologous recombination (Fig. 1A). The resulting KO mutants (Δ52L- and Δ64R-FV3; Table 1) were initially selected for growth in the presence of puromycin and subsequently plaques purified until all plaques were GFP-positive (Fig. 1B).

Correct insertion of the marker cassette and replacement of the targeted gene were verified by PCR using primers flanking the gene of interest. Compared to WT FV3, both KO mutants produced PCR products consistent with the size of the Puro-GFP cassette and flanking regions (3.5 kb; Fig. 2 lanes 3 and 6), whereas PCR analysis of WT virus generated products of 2.6 kbp for *52L* gene and 2.0 kbp for *64R* gene (Fig. 2A, lanes 2 and 5, respectively). Furthermore, we did not detect amplicons indicative of WT gene products following analysis of either KO mutant confirming that KO mutants were not contaminated with WT virus. To further confirm that insertion of the Puro/EGFP cassette replaced the gene of interest, we performed PCR assays on each putative KO clone using primers that amplified within the two targeted genes (*52L* or *64R*) as well as within GFP and vDNA Pol II. No *52L*-specific product was amplified from Δ52L-FV3, and no 64R product was detected in Δ64R-FV3 (Fig. 2B). As expected, both KO mutants contained the inserted GFP gene as well as the viral DNA polymerase gene. Lastly, the presence of correct inserts was subsequently verified by cloning and sequencing each of the amplified fragments. Together, the above results confirmed the absence of WT contamination in the two newly generated KO mutants and the loss of the targeted genes.

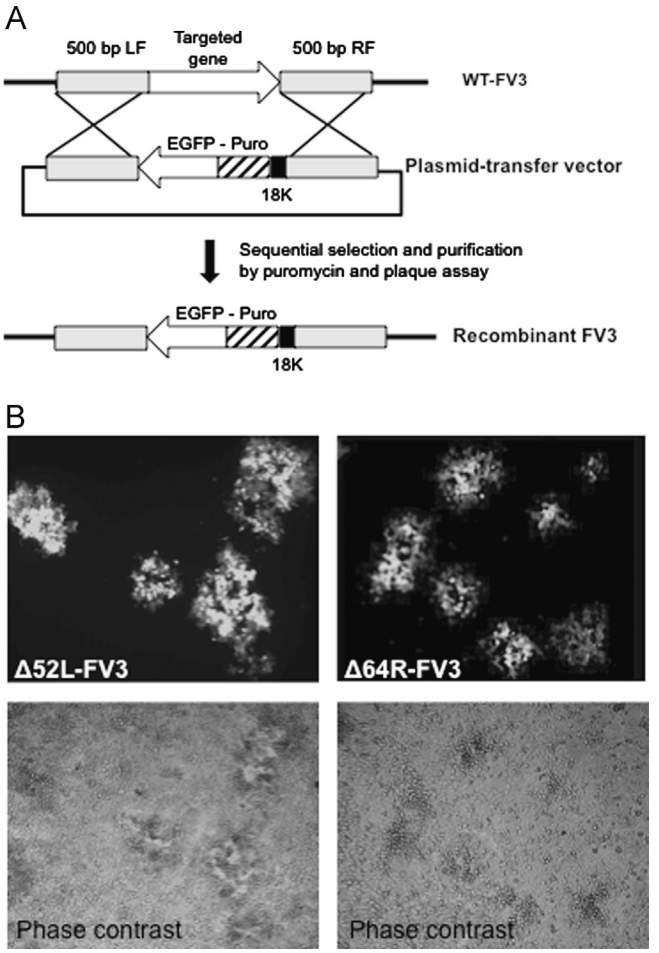

Fig. 1. Generation and selection of FV3 KO mutants. (A) Schematic of site-specific integration of the 18Kprom-Puro-EGFP cassette into the FV3 genome. Constructs consisting of the 18Kprom-Puro-EGFP cassette and regions (approximately 500 bp) flanking the targeted insertion sites (gray) were introduced into pBluescript SK(+). The recombination vectors (p18Kprom-Puro-EGFP) were transfected into WT FV3-infected BHK-21 cells and recombinant FV3 generated by homologous recombination. Sequential selection was performed based on virus replication in the presence of puromycin (50 μg/ml) and expression of EGFP were performed. (B) Fluorescence (top) and phase contrast (bottom) microscopy of BHK-21 cells infected with Δ52L-FV3 and Δ64R-FV3 after six consecutive rounds of selection. All the plaques produced by these recombinant viruses were EGFP-positive indicating that were not contaminated with WT virus.

Assessment of replication in BHK and FHM cells.

To determine whether deletion of the 52L or 64R genes or insertion of the GFP cassette affected growth in susceptible BHK-21 and FHM cells, we monitored viral replication and transmission by examining both single- and multiple-step growth curves (Fig. 3A and B, respectively). WT and KO mutants exhibited nearly identical replication kinetics following single-cycle infections of BHK-21 cells and multi-step infections of FHM cells. These results indicate that the 52L (vβHSD) or 64R (vCARD) genes were not