Virology Reports 5 (2015) 10–18

Contents lists available at ScienceDirect

Virology Reports

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

Mutations within ICP4 acquired during *in vitro* attenuation do not alter virulence of recombinant Marek's disease viruses *in vivo*

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ARTICLE INFO

Article history:
Received 14 June 2014
Received in revised form 17 November 2014
Accepted 23 November 2014
Available online 3 December 2014

Keywords:
Marek's disease virus
Virulence
Attenuation
Vaccine
Horizontal transmission
ICP4
BAC

ABSTRACT

Marek's disease (MD) is a T-cell lymphoma of chickens caused by the oncogenic Marek's disease virus (MDV). MD is primarily controlled by live-attenuated vaccines generated by repeated *in vitro* serial passage. Previous efforts to characterize attenuated MDVs identified numerous mutations, particularly a convergence of high-frequency mutations around amino acids 60–63 within ICP4 (RS1), therefore, ICP4 was considered a candidate gene deserving further characterization. Recombinant MDVs were generated containing a single Q63H mutation or double Q63H + S1630P mutations. Despite the repetitive nature of mutations within ICP4, neither recombinant virus decreased virulence, although one mutant reduced *in vivo* replication and failed to transmit horizontally. Our results indicate that these mutations are insufficient to reduce disease incidence in infected birds, and suggest that variants in ICP4 do not directly alter virulence, but rather may enhance MDV replication rates *in vitro*, offering an explanation for the widespread occurrence of ICP4 mutations in a variety of attenuated herpesviruses.

1. Introduction

Gallid herpesvirus 2, also commonly known as Marek's disease virus (MDV), is an oncogenic alphaherpesvirus of chickens. Afflicted birds display symptoms of Marek's disease (MD) including depression,

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http://dx.doi.org/10.1016/j.virep.2014.11.002

cachexia, and paralysis due to viral-induced T-cell lymphomas that ultimately result in death. The primary mode of MD control has been *via* the use of vaccines, which has significantly reduced losses due to MD since the 1970s (Purchase and Okazaki, 1971). Despite the widespread use of vaccinal control of MD, virulent strains of MDV able to overcome vaccines have emerged, leading to the need for periodic introduction of new and more effective MD vaccines approximately every 10 years (Witter, 1997). Currently the most protective vaccine commercially available, CVI988/Rispens, has been in use in the United States since the 1990s. Concern regarding the potential for MD outbreaks in Rispens-vaccinated flocks has highlighted the importance for vaccine development (Gimeno, 2008). One common method for vaccine production has been the use of live attenuated viruses generated *via* repeated *in vitro* serial passage. Several MD vaccines, such as HPRSS-16 and Rispens, were generated by *in vitro* serial passage and provide testament to the utility of this process in vaccine development (Churchill et al., 1969; Rispens et al., 1972).

To better understand this process and identify candidate genes involved in attenuation, previously we serially passed and sequenced the complete genome of four attenuated MDV replicates (three Md5BAC-derived viruses and one Md5 strain) to identify candidate mutations and genes involved in attenuation (Hildebrandt et al., 2014). Several candidate mutations present within genes in the unique long (UL) or unique short (US) regions of the viral genome were characterized *via* recombinant viruses. Among the five point mutations tested, one recombinant virus revealed a single nucleotide mutation in UL5 (helicase–primase subunit) able to reduce disease incidence by 90% or more. Due to the additional complexity involved in mutating genes present within the two long and short repeat regions (TRL/IRL and TRS/IRS) in MDV, these candidate mutations were not initially tested. Therefore, the purpose of this study was the characterization of a top candidate gene identified within the repeat regions of the MDV genome.

ICP4, encoded by RS1, is an immediate early transcriptional regulator in herpesviruses located within the TRS/IRS region of MDV and a gene commonly mutated in all of the attenuated replicates. All four passed viruses had mutations within ICP4, with three viruses containing high frequency (80–100%) nonsynonymous mutations within amino acids 60–63 in ICP4. Three other high frequency (40% or higher) nonsynonymous mutations in ICP4 were also observed, including one at amino acid position 1630 (85% frequency). Due to these numerous high frequency, parallel mutations within the attenuated replicates, ICP4 was considered a candidate gene for attenuation. Using Red-mediated recombineering, we generated two recombinant viruses to examine how these mutations in ICP4 impacted virulence of MDV.

2. Results

2.1. In vivo trials

Disease incidences resulting from challenge with either Mut ICP4-1 (single ICP4 Q63H SNV) or Mut ICP4-2 (double Q63H and S1630P SNVs) were 100% and 95%, respectively. Compared to the parental virus that showed 84% MD incidence there was no significant difference between MD incidence of the parental Md5B40BAC-c1 and either recombinant ICP4 viruses (Fischer's exact test $p = 0.5320$), therefore, these

Table 1
Mutational primers for generation of recombinant ICP4 viruses.

Primer name	Sequence
ΔICP4-f	TTGTCTAAATTGTTATGAGGTTTGGGGACAATATTTATTGACTAGTCTtagggataacagggtaatcgattt
ΔICP4-r	CCACCATCTATTTGCGCCCCTCCTAAACCCTAAAAATGGACAACCCGCCTAGACTAGTCAATAAATATTGTCCCCAAACC TCATAACAATTTAGACAAgccagtgttacaaccaattaacc
Mut ICP4 Q63H-f	GCTAGCCGGACAATCGGGTACATACCGCCCCCACTCCAGTTACCATGGACA**t**CGCTCGCTTTCCAGCGGCCC tagggataacagggtaatcgattt
Mut ICP4 Q63H-r	GGCTATGGGCAGCGGGGACGGGGCCGCTGGAAAGCGAGCG**a**TGTCCATGGTAACTGGAGTGGGGGCGGT ATGgccagtgttacaaccaattaacc
Mut ICP4 S1630P-f	ACCCGATCAGCTGTTTCGAGGTCCTGGGCGTCCCCGACGC**c**CCACTTCATCATCCCAGTCTtagggataacagggtaatcgattt
Mut ICP4 S1630P-r	ATGGGAGATTTATCAGACGCAGACTGGGATGATGAAGTGG**g**GCGTCGGGGACGCCCAGGACgccagtgttacaaccaattaacc

Nucleotides in uppercase indicate the region of primer homologous to MDV for integration into the MDV genome while nucleotides in lowercase are regions complementary to the kanamycin cassette. The single lowercase nucleotide in bold and underlined indicates the point mutation altered for generation of recombinant viruses.