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Murine cytomegalovirus M72 promotes acute virus replication *in vivo* and is a substrate of the TRiC/CCT complex

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ABSTRACT

Betaherpesvirus dUTPase homologs are core herpesvirus proteins, but little is known about their role during infection. Human cytomegalovirus (HCMV) UL72 and murine cytomegalovirus (MCMV) M72 have been designated dUTPase homologs, and previous studies indicate UL72 is dispensable for replication and enzymatically inactive. Here, we report the initial characterization of MCMV M72. M72 does not possess dUTPase activity, and is expressed as a leaky-late gene product with multiple protein isoforms. Importantly, M72 augments MCMV replication *in vitro* and during the early stage of acute infection *in vivo*. We identify and confirm interaction of M72 with the eukaryotic chaperonin tailless complex protein -1 (TCP-1) ring complex (TRiC) or chaperonin containing tailless complex polypeptide 1 (CCT). Accumulating biochemical evidence indicates M72 forms homo-oligomers and is a substrate of TRiC/CCT. Taken together, we provide the first evidence of M72's contribution to viral pathogenesis, and identify a novel interaction with the TRiC/CCT complex.

1. Introduction

Human cytomegalovirus (HCMV), a betaherpesvirus, is the major infectious cause of birth defects in developed countries (Griffiths, 2012). It has the potential to cause permanent neurological damage including microcephaly, cognitive impairment and sensorineural hearing loss in CMV-infected newborns (Boppana et al., 2013). Owing to the high cost of caring for children with congenital CMV infection, it has been prioritized as a candidate for vaccine development in the United States (Stratton et al., 2000). Immunocompromised individuals, including transplant recipients and HIV-infected persons, represent another susceptible population where HCMV represents a major cause of morbidity and mortality. In this group, problems arise due to acute infection or reactivation and include retinitis, hepatitis, and pneumonitis (Plosa et al., 2012; Ramanan and Razonable, 2013).

The narrow host range of HCMV makes it challenging to study the role of viral genes in the context of natural host. Hence, murine CMV (MCMV) infections of mice are used as a tractable animal model for studies of CMV pathogenesis. Sequence and functional homologs of viral genes among the CMVs of different hosts facilitate studies of specific pathogenic mechanisms (Barry et al., 2006; Schleiss, 2006). MCMV has a wide variety of genes that contribute to its ability to infect and evade the host responses. This leads to the establishment of an intricate life-long host pathogen relation, characteristic of all herpesviruses. The gamut of MCMV genes includes a subset of core genes that are evolutionarily conserved across herpesviruses. Most of these genes encode proteins required for replication and virus structure. However, many core genes remain relatively uncharacterized, and little is known about their contribution to infection. The MCMV gene, M72, designated as a 2′ deoxyuridine 5′triphosphate pyrophosphatase (dUTPase) homolog, is one such example of a core gene with no identified function (Mocarski Jr, 2007).

Cellular dUTPases are ubiquitous enzymes that convert dUTP to dUMP and pyrophosphate (PPi) to control cellular nucleotide pools and prevent misincorporation of uracil into cellular DNA. There are several examples of viral dUTPases among retroviruses and DNA viruses (Baldo and McClure, 1999). The dUTPase-encoding gene in non-primate lentiviruses is essential for replication in non-dividing cells (Hizi and Herzig, 2015). Similarly, a feline immunodeficiency virus (FIV) dUTPase mutant displays a reduced viral burden *in vivo*, suggesting a contribution to infection in the natural host (Lerner et al., 1995). Among herpesviruses, a dUTPase-encoding gene is present in all three

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herpesvirus family subdivisions, and the alpha- and gamma-herpesviruses dUTPases are functional enzymes (Davison and Stow, 2005). The varicella zoster virus (VZV) and simian varicella virus (SVV) dUTPases, encoded by ORF8 of each virus, contribute to virus replication in cell culture (Ross et al., 1997; Ward et al., 2009). Additionally, a herpes simplex virus (HSV) 1 dUTPase, UL50, null mutant is less virulent compared to wild type virus and exhibits decreased neurovirulence (Pyles et al., 1992).

Many herpesvirus dUTPases have functions independent of their dUTPase activity. Kaposi's sarcoma-associated herpesvirus (KSHV) and murid herpesvirus-68 (MHV-68) ORF54 down-regulate a cell-surface ligand, NKp44L (Madrid and Ganem, 2012), and degrade IFN receptor 1 protein (Leang et al., 2011), respectively. EBV dUTPase, BLLF3, activates NF-κB (Ariza et al., 2009) and induces the secretion of pro-inflammatory cytokines in a TLR2-dependent manner (Ariza et al., 2013). Thus, there are functions associated with alpha- and gammaherpesvirus designated dUTPases independent of their catalytic activity.

Little is known about the betaherpesvirus dUTPase homologs. Preliminary characterization of the HCMV designated dUTPase homolog, UL72, revealed it to be catalytically inactive and dispensable for replication in cell culture (Caposio et al., 2004). Here, we report that the MCMV designated dUTPase homolog, M72, is also non-functional as a dUTPase enzyme, augments virus replication in some cell types and contributes to viral pathogenesis in the acute phase of replication in the natural host. We also find that the M72 protein is expressed from early times post infection, shows a complex expression profile that includes multiple shorter isoforms, and is a substrate of the eukaryotic chaperonin tailless complex protein -1 (TCP-1) ring complex (TRiC)/chaperonin containing tailless complex polypeptide 1 (CCT). Together, this initial characterization of M72 reveals new insight into betaherpesvirus dUTPases homologs and highlights the contribution of M72 to viral pathogenesis.

2. Materials and methods

2.1. Plasmids and transfections

M72 expression constructs were generated by PCR amplification of nucleotides 104,289 to 103,084 using MCMV K181 bacterial artificial chromosome (BAC) pARK25 (Accession No. AM886412.1) (Redwood et al., 2005), as template. Amplicons were cloned into the *EcoRI* and *XbaI* sites of p3XFLAG-CMV10 or p3XFLAG-CMV14 expression constructs (Sigma-Aldrich) or the *EcoRI* and *KpnI* sites of pEGFP-C1 (Clontech). Transfections were performed using GenJet transfection reagent (SignaGen Laboratories) according to manufacturer instructions. HA-CCT expression constructs (Kim et al., 2014) were a kind gift from Dr. Kyong-Tai Kim (Pohang University of Science and Technology, Republic of Korea). FLAG-tagged MHV68 ORF54 and ORF54$^{H80A/D85N}$ expression constructs (Leang et al., 2011) were a kind gift from Drs. Ting-Ting Wu and Ren Sun (University of California at Los Angeles).

2.2. Cells and reagents

STO (CRL-1503), HEK293Ts (ATCC CRL-3216), SVEC4–10 endothelial cells (ATCC CRL-2181) and RAW264.7 murine macrophages (ATCC TIB-71) were propagated in Dulbecco's modified Eagle's medium (DMEM – Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (FCS, Life Technologies, Inc.) and 1% penicillin-streptomycin-glutamine (PSG, Life Technologies, Inc.). NIH3T3 murine fibroblasts (ATCC CRL-1658) were propagated in DMEM containing 10% heat-inactivated bovine calf serum (BCS, Life Technologies) and 1% PSG. Bone marrow-derived macrophage (BMDM) cultures were generated as previously described (Kaiser et al., 2013). Briefly, pooled bone marrow cells from flushed tibias and femurs were harvested into Dulbecco's PBS, placed in culture for at least 18 h in DMEM containing 10% FBS, and then differentiated for 5–7 days in DMEM containing 20% FBS and 20% L929-conditioned medium. Phosphonoformic acid (PFA) was from Sigma-Aldrich.

2.3. Generation of recombinant viruses

BAC mutagenesis and diagnosis was performed by recombineering as previously described (Upton et al., 2010). Briefly, *E. coli* DH10B cells containing pARK25 and pSIM6 (Datta et al., 2006) were grown to O.D.600 of 0.4–0.6, recombination functions induced by incubation at 42 °C and cells made electrocompetent by multiple washes in ice cold water. The levansucrase (SacB) and kanamycin (Kan) genes were amplified from plasmid pTBE100 (Upton et al., 2010) with 60 nucleotide base pair overhangs corresponding to MCMV genomic sequences. PCR reactions were treated with *DpnI*, and amplicons gel purified then used to electroporate induced bacteria. Kanamycin-resistant, sucrose-sensitive clones were selected and assessed for insertion and genomic integrity by PCR and RFLP analysis. M72.SK (primers; SG005, 5′-*ACGG GACGCCTGCACAACGTCGGAAGGCGTCGCGACCTCGAGGAACAAAAG CAGCAGCAC*AATTCGAGCTCGGTACCCGG-3′ and SG006, 5′-*ATCACG ATCTTGTTGACGGTCGTATCCGGCACCGAAGCGGCGACCGACACCGGT ACGGAGGCC*ATCCCGGGAAAAGTGCCACC-3′: italic indicates viral sequence) deletes viral sequence between nucleotide 102,772 – 105,791 and M72Flag.SK (primers; FC001, 5′*GGTAAACGTAGTTTTCCTGAGTA CACTAGACAAGAGGTAATCTCTCTAGG*AATTCGAGCTCGGTACC CGG-3′ and FC002, 5′- *ACCGCGCGGAAGAGAGAATGCGAAGCGGTCG AGACTCGTCGAACGAGGGC*ATCCCGGGAAAAGTGCCACC) deletes the M72 stop codon (nucleotides 103,083 – 103,086) and introduces an additional 2.9Kb of sequence. Specific mutations were introduced by a second round of recombineering with individual amplicons generated by overlap extension PCR. M72StopS (primers; SG007, 5′-ATGAAGGA TCCCTTTAATATCTTCGAC**TAGT**ACGAACCTTCC-3′ and SG015, 5′-GGAAGGTTCGT**ACTA**GTCGAAGATATTAAAGGGATCCTTCAT 3′; bold indicates nucleotide substitution, underline indicates diagnostic restriction enzyme recognition site) and M72StopN (primers; SG009, 5′-ATGAAGGATCCCTTTAATA**GCTAGC**ACGACGACGAACCTTCC-3′ and SG016, 5′-GGAAGGTTCGTCGTCGT**GCTAGC**TATTAAAGGGATCCTT CAT-3′) contain an engineered stop codon and a *SpeI* (nt 104,124 – 104,127), or an *NheI* (nt 104,135-104,130) diagnostic restriction site, respectively. M72.3XFlag inserts three tandem FLAG epitopes at the C-terminal end of M72, and was constructed by recombineering with an amplicon generated by overlap extension PCR (primers; FC003, 5′-GGACGTGTAAGTGTGTGGATTGTTG-3′ and FC009, 5′-TGACCTAGA GAGATTACCTCTTGTCTAG using template pARK25; FC004, 5′- GATG GCCAAGATCATCTTCACGAC-3′ and FC010, 5′- CTAGACAAGAGGTAA TCTCTCTAGGTCACTACTTGTCATCGTCATCCTTGTAG-3′ template p3XFLAG-CMV-14-M72). Colonies were screened for kanamycin sensitivity and sucrose resistance, and positive clones confirmed by PCR and RFLP analysis. PCR amplification and diagnostic restriction digest of M72 region confirmed the incorporation of the mutagenesis in the M72 mutants. Infectious virus was reconstituted as previously described (Upton et al., 2010), amplified by growth in STO cells in the presence of 25 μg/ml 6-thioguanine (Sigma-Aldrich, St. Louis, MO), and plaque purified by limiting dilution. Parallel stocks were produced by infecting BALB/cJ mice with initial transfection supernatants of WT and M72Stop mutants (M72StopS and M72StopN). Infected salivary glands were harvested 14 days post infection (d.p.i.), sonicated, clarified and used to infect NIH3T3 fibroblasts. Viral stocks were generated, clarified, concentrated and titered by plaque assay as previously described on NIH3T3 fibroblasts (Upton et al., 2010). All viral stocks were confirmed to be GFP negative, indicating excision of the BAC. All M72 viral stocks were confirmed by sequencing of the recombineering junctions, introduced mutations, and surrounding regions.

2.4. Infections, in vitro growth, and determination of viral titers

Viral titers were determined by plaque assays performed on NIH3T3