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Comparative mechanistic studies of de novo RNA synthesis by flavivirus RNA-dependent RNA polymerases

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Abstract

Flavivirus protein NS5 harbors the RNA-dependent RNA polymerase (RdRp) activity. In contrast to the RdRps of hepaci- and pestiviruses, which belong to the same family of *Flaviviridae*, NS5 carries two activities, a methyltransferase (MTase) and a RdRp. RdRp domains of Dengue virus (DV) and West Nile virus (WNV) NS5 were purified in high yield relative to full-length NS5 and showed full RdRp activity. Steady-state enzymatic parameters were determined on homopolymeric template poly(rC). The presence of the MTase domain does not affect the RdRp activity. Flavivirus RdRp domains might bear more than one GTP binding site displaying positive cooperativity. The kinetics of RNA synthesis by four *Flaviviridae* RdRps were compared. In comparison to Hepatitis C RdRp, DV and WNV as well as Bovine Viral Diarrhea virus RdRps show less rate limitation by early steps of short-product formation. This suggests that they display a higher conformational flexibility upon the transition from initiation to elongation.

Keywords: Dengue virus; Hepatitis C virus; Flavivirus; *Flaviviridae*; RdRP; Pestivirus; Polymerase; RNA synthesis; West Nile virus

Introduction

The *Flaviviridae* virus family comprises three genera of single-stranded positive-sense RNA viruses: hepaciviruses (Hepatitis C virus (HCV)), pestiviruses (e.g., Bovine Viral Diarrhea virus (BVDV)) and flaviviruses (e.g., Dengue virus (DV) and West Nile virus (WNV)). The genus flavivirus contains at least 70 mosquito-borne or tick-borne viruses. DV threatens up to 2.5 billion people in 100 endemic countries (Mackenzie et al., 2004). Between 50 and 100 million cases of dengue fever occur annually with 500,000 cases of the severe disease form dengue hemorrhagic fever, and 22,000 deaths mainly among children (Guzman and Kouri, 2002). Dengue has been classified a priority by the World Health Organization (WHO). It ranks as the most important mosquito-borne viral disease in the world (http://www.who.int/csr/disease/dengue). WNV was first isolated in Africa in 1937 and showed an extensive distribution throughout the world except in the Americas (Mackenzie et al., 2004). In 1999, a first WNV outbreak occurred in New York City. By the end of 2003, WNV activity had been identified in 46 states of the United States. In 2005 (until October, 18), WNV epidemics resulted in 2,316 reported cases of WN disease including 913 meningitis or encephalitis cases and 66 deaths (http://www.cdc.gov/ncidod/dvbid/westnile/). Kunjin virus (KV) is an Australian subtype of WNV (Scherret et al., 2001). In contrast to other WNV strains (Lanciotti et al., 2002), KV infections do not cause neuroinvasive disease in humans (Hall et al., 2002).

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Currently, there is no specific treatment available for DV or WNV infections. Different approaches towards efficient vaccines are under development (Hall and Khromykh, 2004; Hall et al., 2003; Pugachev et al., 2003). Apart from the vaccine approach and vector control measurements, antibody-based (Roehrig et al., 2001) and antiviral drug therapies might be considered relevant for future control of DV and WNV infections.

Viral RNA-dependent RNA polymerases (RdRps) represent targets of choice for anti-viral chemotherapy. They are essential to viral replication and present a unique evolutionary fate of being the only replicative RdRps preserved from the RNA world (Leipe et al., 1999). *Flaviviridae* RdRp duplicates the single-stranded flavivirus RNA genome in a single, continuous polymerization event. The RdRp enters at the 3′-end of the genome and is able to copy the whole RNA molecule in a primer-independent fashion (Bressanelli et al., 2002; Kao et al., 2001), referred to as *de novo* RNA synthesis. Viral RdRps belong to the SCOP (Structural Classification Of Proteins (Murzin et al., 1995)) superfamily of DNA/RNA polymerases. Crystal structures of various viral RdRps have been reported, among them that of two *Flaviviridae*, HCV and BVDV (Ago et al., 1999; Bressanelli et al., 1999, 2002; Choi et al., 2004; Lesburg et al., 1999). Their overall shape resembles a right hand with three subdomains: the "fingers", the central catalytic "palm" subdomain containing at least two strictly conserved acidic residues responsible for binding two catalytic Mg^{2+} ions, and the "thumb". The crystal structure of BVDV RdRp has shown interesting structural differences relative to that of HCV RdRp namely in a specific thumb region (named "β-hairpin" or "flap" for HCV RdRp and "β-thumb" region for BVDV RdRp) which is believed to play a role in the initiation of de novo RNA synthesis. Thus, although viruses of the *Flaviviridae* family have similar genome organization and replication mechanisms, these similarities do not necessarily translate into homologies at the molecular level.

The largest non-structural protein NS5 of flaviviruses harbors the RdRp activity. Signature-sequence analysis suggests that NS5 is made of two domains separated by an ill-defined inter-domain region. The three-dimensional structure of a N-terminal domain of 30 kDa of DV has been determined (Egloff et al., 2002). It bears a (nucleoside-2′-O-)-methyltransferase (MTase) activity involved in the formation of the type-1 cap of genomic RNA, unique to flaviviruses in contrast to pestiviruses and hepaciviruses. Accordingly, RdRps of HCV and BVDV, named NS5B, do not contain a MTase domain. The DV MTase domain ($NS5MTase_{DV}$) domain is able to bind GTP as well as GTP analogues, such as ribavirin 5′-triphosphate, a broad-spectrum inhibitor of viral replication (Benarroch et al., 2004). A C-terminal domain of ca. 70 kDa harbors the RdRp, as judged by the presence of signature-sequence motifs A to F (Koonin, 1991; O'Reilly and Kao, 1998). The crystal structure of the RdRp domain is not known. Two nuclear localization sequences (bNLS from amino acid 320 to 369 and a/bNLS from 370 to 405) are thought to map in the inter-domain region (Brooks et al., 2002).

Full-length NS5 of DV ($NS5_{DV}$) and WNV ($NS5_{WNV}$) have been obtained from *E. coli* expression systems (Ackermann and Padmanabhan, 2001; Nomaguchi et al., 2004; Tan et al., 1996) and baculovirus/insect cell expression systems (Guyatt et al., 2001; Steffens et al., 1999). The number of enzymatic studies of flavivirus RdRps is still limited and reports contradictory results. $NS5_{DV}$ and $NS5_{WNV}$ were shown to be active on specific subgenomic RNA templates of positive and negative polarity (Ackermann and Padmanabhan, 2001; Nomaguchi et al., 2004), whereas in the same study, enzymatic activity on unspecific heteropolymeric templates was virtually undetectable. In contrast, another report showed that recombinant $NS5_{KV}$ was active on an unspecific heteropolymeric RNA (Guyatt et al., 2001). Recombinant $NS5_{WNV}$ present in insect cell extracts was reported to be active on homopolymeric RNA templates (poly(rC)) but exclusively in the presence of an oligo $(rG)_{18}$ primer (Steffens et al., 1999). On subgenomic RNA templates, $NS5_{DV}$ and $NS5_{WNV}$ were able to initiate polymerization de novo generating a template-size product (Ackermann and Padmanabhan, 2001; Nomaguchi et al., 2004). Products twice the template size were also observed originating presumably from a copy-back mechanism. In the copy-back mechanism, templates adopt a hairpin conformation due to the existence of short complementary sequence stretches in the 3′ region. The 3′-end hybridizes internally and is then elongated. Copy-back elongation is considered unique to in vitro experimental conditions whereas de novo initiation is thought to be the mechanism used in infected cells during *Flaviviridae* virus replication (Bressanelli et al., 2002; Kao et al., 2001). For RNA synthesis by $NS5_{DV}$ on a negative-sense subgenomic template, the ratio of de novo versus copy-back products increases when the temperature during initiation is lowered. Consequently, a model was proposed where the enzyme adopts a closed conformation around the 3′ of the template in the initiation complex and then opens up for elongation (Ackermann and Padmanabhan, 2001). De novo RNA synthesis requires unusually high GTP concentrations in comparison to

Fig. 1. Structure-based sequence alignment of RdRp domains of NS5 of DV (DV_2_NGC) and WNV (strain IS-98-ST1 (WNV_IS98ST1) and Kunjin subtype, strain MRM61C (WNV_Kunjin)) and single-domain RdRps NS5B of HCV and BVDV. Residue numbering corresponds to full-length $NS5_{DV}$. Secondary-structure prediction for $NS5Pol_{DV}$ done by PredictProtein (Rost, 1996) is shown above the alignment. Secondary structural elements of $NS5B_{HCV}$ and $NS5B_{BVDV}$ (Choi et al., 2004; Lesburg et al., 1999) are shown below. The sequence of $NS5B_{BVDV}$ starts with the first residue considered to belong to the RdRp core (Choi et al., 2004). RdRp subdomains (fingers—blue, palm—green, thumb—orange) are indicated by colored lines above the alignment. Motifs A to F are shown by red bars below the alignment. Strictly conserved residues within *Flaviviridae* family RdRps are given in white on a red background. Two NLSs (bNLS and a/bNLS) present only in flavivirus RdRps are delineated by red rectangles in the predicted secondary structure line. Alignment was generated by comparison of the two structures using Swiss-Prot PDB viewer (http://www.expasy.org/spdbv/) and the flavivirus sequences were then aligned manually using Seaview (Galtier et al., 1996). Graphic visualization was generated by Espript (Gouet et al., 2003).