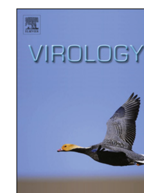
Virology 484 (2015) 80–85

Contents lists available at ScienceDirect

Virology

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



Sequence specificity for uridylylation of the viral peptide linked to the genome (VPg) of enteroviruses

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A R T I C L E I N F O

Article history:
Received 22 April 2015
Returned to author for revisions
17 May 2015
Accepted 22 May 2015
Available online 11 June 2015

Keywords:
Peptide priming
Nucleotide transfer reaction
RNA polymerase
Coxsackie virus
EV-71
Poliovirus
PCP-consensus sequence
Metal ion dependent phosphotransfer

A B S T R A C T

Enteroviruses (EV) uridylylate a peptide, VPg, as the first step in their replication. VPgpUpU, found free in infected cells, serves as the primer for RNA elongation. The abilities of four polymerases ($3D^{pol}$), from EV-species A–C, to uridylylate VPgs that varied by up to 60% of their residues were compared. Each $3D^{pol}$ was able to uridylylate all five VPgs using polyA RNA as template, while showing specificity for its own genome encoded peptide. All $3D^{pol}$ uridylylated a consensus VPg representing the physical chemical properties of 31 different VPgs. Thus the residues required for uridylylation and the enzymatic mechanism must be similar in diverse EV. As VPg-binding sites differ in co-crystal structures, the reaction is probably done by a second $3D^{pol}$ molecule. The conservation of polymerase residues whose mutation reduces uridylylation but not RNA elongation is compared.

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Introduction

The human enteroviruses (EV), which include the polioviruses (PV), coxsackie viruses (CVA, CVB) and many other pathogens, cause febrile rash, respiratory illness, and neurologic disease (Eyckmans et al., 2014; Pallansch et al., 2013). Although incidence of PV paralysis has been reduced by >99% globally through routine immunization and mass vaccination campaigns, there continue to be cases in areas where vaccine campaigns have been inhibited by social unrest (Moturi et al., 2014). Non-polio EV, such as EV A71 (Chan et al., 2011; Wang et al., 2014; Yu et al., 2014; Zheng et al., 2014) and EV 68(Stephenson, 2014) (Jacobson et al., 2012; Tokarz et al., 2012) can spread rapidly among children. These can cause severe respiratory illness and a range of neurological diseases, from aseptic meningitis to encephalitis and paralysis (Kreuter et al., 2011; Pallansch et al., 2013; Tao et al., 2014). Infections with other EV, such as CVB3, may contribute to diabetes (Salvatoni et al., 2013; Yeung et al., 2011) and heart disease(Chapman and Kim, 2008; Cooper, 2009).

There are currently no drugs approved for the treatment of the many different enterovirus infections (Abzug, 2014). As EV are omnipresent in the intestinal tract of humans and animals, there is little way to prevent occasional infections. Their antigenic diversity (Acevedo et al., 2014; Blomqvist et al., 2008) makes it difficult to develop vaccines to protect against the many different enterovirus pathogens. To aid in developing more widespread treatments for EV infections (Campagnola et al., 2011), it is important to identify common properties of the viral proteins involved in replication.

Early studies of poliovirus replication revealed that the 5′ end of the RNA was covalently bound to a small peptide, called VPg (for viral protein linked to the genome), which was essential for PV

Abbreviations: 3B, etc., Enteroviruses express one long polyprotein. This is cleaved into three fragments that are further cleaved to yield precursor and mature viral proteins. The third fragment is cleaved to form 3AB (3B is VPg), 3BC, 3CD (where 3C is a protease, and 3CD accelerates the uridylylation assay using *cre* RNA as template), and $3D^{pol}$ (the RNA polymerase); CV, coxsackievirus; DENV, Dengue virus; FMDV, foot and mouth disease virus; EV, enterovirus; FCV, feline calicivirus; IEP, isoelectric point; MNV, murine norovirus; RV, rhinovirus; PAGE, polyacrylamide gel electrophoresis; PCP, physical chemical properties; PCP-consensus, consensus sequence based on conservation of PCPs in each column of a multiple sequence alignment; pU, Uridylylated (i.e., VPgpU,VPgpUpU); PV, poliovirus; VPg, viral peptide linked to the genome; VPgpU, uridylylated VPg

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http://dx.doi.org/10.1016/j.virol.2015.05.016
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Virus	VPg Sequence	GenBank Accession #
EV A71	GAYSGAPKQVLKKPALRTATVQ	AJD77327
EV103	GAYTGLPFNKPKVPTIRQAKVQ	FJ007373
SV6	GAYTGLPFNKPKVPTIRQAKVQ	AF326766
EV108	GAYTGLPFTKPKVPTIRQAKVQ	AF414372
BaEV	GAYSGMPQTKPKVPTIRQAKVQ	AF326750
EV4	GAYTGMPNQKPKVPTLRQAKVQ	AY302557
CVB1	GAYTGMPNQKPKVPTLRQAKVQ	M16560
CVB3	GAYTGVPNQKPRVPTLRQAKVQ	M88483
EV5	GAYTGMPNQKPKVPTLRQAKVQ	AF083069
CVB6	GAYTGMPNQKPKVPTLRQAKVQ	AF039205
EV11	GAYTGMPNQKPKVPTLRQAKVQ	X80059
EV6	GAYTGMPNQKPKVPTLRQARVQ	AY302558
EV3	GAYTGMPNQKPKVPTLRQAKVQ	AY302553
EV7	GAYTGIPNQKPKVPTLRQAKVQ	AY302559
EV87	GAYTGLPNQKPKVPTLRQAKVQ	AY843305
CVB1	GAYTGLPNQKPKVPTLRQAKVQ	AA084300
EV75	GAYTGMPNQKPKVPTLRQAKVQ	AY556070
EV30	GAYSGMPNQKSKVPTLRQAKVQ	AF162711
CVA20	GAYTGMPNQKPKVPTLRQAKVQ	AF499642
CVA18	GPYTGLPSKKPNIPTIRTAKVQ	BAE20393
CVA17	GAYTGLPNKKPNIPTIRTAKVQ	AF499639
EV102	GAYTGLPNKKPNVPTIRTAKVQ	EF555645
CVA13	GAYTGLPNKRPNVPTIRTAKVQ	AF499637
CVA21	GAYTGLPNKKPNVPTIRVAKVQ	AF546702
CVA11	GAYTGLPNKRPSVPTIRTAKVQ	AF499636
PV3	GAYTGLPNKRPNVPTIRTAKVQ	K01392
PV1	GAYTGLPNKKPNVPTIRTAKVQ	J02281/ V01149
PV2	GAYTGLPNKRPNVPTIRTAKVQ	M12197
CVA15	GAYTGLPNKRPNVPTIRTAKVQ	AF499638
CVA24	GAYTGLPNKKPSVPTVRTAKVQ	D90457
EV96	GAYTGLPSKKPNVPTIRAAKVQ	EF015886
EV99	GPYTGLPTRKPNVPTIRTAKVQ	EF555644
CVA1	GAYTGLPNAKPKVPTIRAAKVQ	AFQ55937
CVA22	GAYTGLPNVKPKVPTIRAAKVQ	AF499643
.	*:*:*:* *:::*::*:*:**	

*100% conserved positions; :similar

Fig. 1. Sequences of EV VPgs used to design the PCP-consensus VPg with their gene bank accession numbers. Only the unique sequences were used in calculating the consensus.

replication (Ambros and Baltimore, 1978; Lee et al., 1977). A uridylylated form of VPg, VPgpUpU, was shown to be present in the cytoplasm of infected cells (Crawford and Baltimore, 1983). Subsequently, it was shown that VPgpU could be formed in an in vitro reaction containing the polymerase ($3D^{pol}$) and a template RNA. The uridylylated peptides, VPgpU or VPgpUpU, prime viral RNA synthesis (Paul et al., 1998). VPg sequences are present in the genomes of all picornaviruses. Larger VPg proteins were also identified in caliciviruses and other families that were even more distinct from the picornaviruses (Goodfellow, 2011) but which may have arisen from combinations of picornavirus gene sequences during evolution of the eukaryotic cell (Koonin et al., 2008).

A wealth of data indicates that mutations throughout the 22 amino acid sequence of PV1-VPg reduce uridylylation in vitro and lower or eliminate the formation of infectious virus (Kuhn et al., 1988a, 1988b; Paul et al., 2003). However, there are many gene sequences known for EV VPgs, which differ at positions (when aligned with PV-VPg) that are known to affect uridylylation (Fig. 1). Deep sequencing of viral isolates may reveal even more diversity (Acevedo et al., 2014), introduced through the high mutation rate of viral $3D^{pol}$ (Gnadig et al., 2012). Indeed, VPg seems to be evolving at a very rapid rate, as the sequences of the four EV $3D^{pol}$ included here are much more conserved, ranging from 67–74% identity.

However, function eventually constrains sequence variability. To determine the minimum requirements for uridylylation, we analyzed the sequences, the underlying conservation of physico-chemical properties, and the structures of VPg and their binding sites on the polymerases in co-crystal structures. VPg, before uridylylation, in solution has a flexible, or even disordered structure (Schein et al., 2006a), which might also be stabilized by binding to cellular components or the polymerase. In contrast, chemically synthesized uridylylated PV-VPgpU has a very stable structure in solution (Schein et al., 2010). The NMR structure indicated that the positively charged residues directly coordinate with the UMP moiety of the modified tyrosine. Such a stable structure is probably needed for VPgpU to effectively prime RNA synthesis.

To determine the specificity endowed within the diversity of sequences of VPg, we chose four diverse EV polymerases and determined whether they could recognize VPgs that differed greatly in sequence from their own encoded peptide. We purified the $3D^{pol}$ of three important human pathogens, from EV-A71 (species A), CVB3 (species B), and CVA24 (species C, and closely related in sequence to PV-$3D^{pol}$) (Smura et al., 2014). Our results indicate that the diversity in the sequences of the VPg of species A–C correlates with their different binding sites for uridylylation on the $3D^{pol}$. The underlying physical chemical properties of the VPgs were captured in a single consensus sequence. All four of the polymerases tested could uridylylate this artificial sequence, while still showing preference for their own VPg. The ability of all to uridylylate a consensus peptide, coupled with evidence that VPg-based replication can be done in trans (Chen et al., 2013), suggests that there is indeed a common mechanism for VPg uridylylation. However, the specificity we show here, coupled with the different binding sites seen in co-crystal structures, supports a “2 molecule mechanism” (Sun et al., 2012), where the VPg can be located at different positions on one polymerase molecule, and uridylylated by a second polymerase molecule.

Results

Deriving a PCP-consensus VPg for EV species A–C

The sequences of 31 diverse EV were used to derive a PCP-consensus for VPg (Fig. 1). Only 9 residues (without inserting gaps) of the 22 are conserved across EV species A, B, and C. Seven of these residues are also conserved in analogous positions in diverse Rhinoviruses (RV, enterovirus species D; Table 1). The conservation of G1 and Q22 reflects the sequence needed for protease cleavage of the P3 domain of the polyprotein (Pathak et al., 2007). Despite the relatively low absolute identity, the physical chemical properties at each position are more conserved. For example, there is always a positively charged residue at positions 8–10 in the sequences, and arginine is always present at position 17. The absolute sequence number of the positively charged residues is not conserved (e.g., K9 is a Q, N, R or T in the different sequences). However, all 5 VPg sequences synthesized for this study have the same predicted IEP (10.9) and charge (+4) at pH 7.

The unique VPg sequences (from species A, B, and C) chosen for this study are compared in Table 1 with those of other picornavirus sequences and the uridylylation site of larger VPgs from other virus families. The IEP and net charges for the sequences of RV VPgs are somewhat lower. The sequences of the three genome encoded VPgs of the distantly related Foot and mouth disease virus (FMDV; genus *Aphthovirus*) are, like FMDV polymerase (see the alignments in Fig. S3), significantly different from those of the

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