Virology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Virology

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

Viral precursor protein P3 and its processed products perform discrete and essential functions in the poliovirus RNA replication complex

Allyn Spear [1], Sushma A. Ogram, B. Joan Morasco, Lucia Eisner Smerage, James B. Flanegan *

Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL, USA

ARTICLE INFO

Article history:
Received 23 May 2015
Returned to author for revisions
12 June 2015
Accepted 28 July 2015

Key words:
Poliovirus (PV)
Reciprocal complementation
Poliovirus P3
Poliovirus 3CD
3D polymerase
VPg
5′Cloverleaf (5′CL)
3C-RNA binding activity
RNA replication complex
(−) Strand RNA

ABSTRACT

The differential use of protein precursors and their products is a key strategy used during poliovirus replication. To characterize the role of protein precursors during replication, we examined the complementation profiles of mutants that inhibited 3D polymerase or 3C-RNA binding activity. We showed that 3D entered the replication complex in the form of its precursor, P3 (or 3CD), and was cleaved to release active 3D polymerase. Furthermore, our results showed that P3 is the preferred precursor that binds to the 5′CL. Using reciprocal complementation assays, we showed that one molecule of P3 binds the 5′CL and that a second molecule of P3 provides 3D. In addition, we showed that a second molecule of P3 served as the VPg provider. These results support a model in which P3 binds to the 5′CL and recruits additional molecules of P3, which are cleaved to release either 3D or VPg to initiate RNA replication.

Introduction

Poliovirus (PV) is a prototypical human enterovirus, which is a member of the small, single-strand (ss), positive sense RNA virus family *Picornaviridae*. The PV genome, like that of other picornaviruses, is covalently linked to a viral protein, VPg, at the 5′ terminus. The PV genome also contains a conserved RNA structure in its 5′ terminal sequence, the 5′cloverleaf (5′CL), which has been shown to bind both cellular poly(C) binding protein (PCBP) as well as a viral precursor protein, 3CD (Andino et al., 1990b; 1993; Gamarnik and Andino, 1997; Parsley et al., 1997). Translation of the viral genome utilizes an internal ribosomal entry site (IRES) to drive synthesis of a single large open reading frame, which is subsequently processed by the viral proteases 2A, 3C and 3CD (reviewed in Palmenberg, 1990). Initial polyprotein processing occurs co-translationally at the boundary between the structural (P1) and non-structural (P23) proteins by 2A. All subsequent cleavage events are mediated by 3C and 3CD (Wimmer et al., 1993). Previous studies by Lawson and Semler (1992) showed that the predominant pathway for cleavage of the P23 polyprotein was the membrane-associated pathway starting with the cleavage at the junction of 2A–2B (Fig. 1). This generates 2A and the precursor protein, 2BC-P3, which is then rapidly processed to form 2BC and P3. P3 is processed to 3CD and 3AB. Cleavage of 3AB generates 3A and the viral protein primer, VPg (3B). 3CD is processed to yield 3C and the active form of the polymerase, 3D. VPg is uridylylated by the viral polymerase, 3D, to form VPgpUpU which is used as the primer for the initiation of both (−) and (+) strand synthesis.

Given the minimal coding capacity of these small RNA viruses, the differential use of polyprotein precursors and their products is a key strategy employed by PV to perform the many diverse functions required during viral replication using limited sequence space. An extension of this is the evolution of multiple activities within a single protein or protein precursor. The PV precursor protein, 3CD, exemplifies both of these concepts in that it performs multiple functions as a precursor, and these activities are functionally distinct from its processed products, 3C and 3D. As a precursor, 3CD exhibits no polymerase activity, however its processed product 3D, is the PV RNA-dependent RNA polymerase (RdRp) (Flanegan and Baltimore, 1979;Flanegan and Van Dyke,

* Corresponding author.
E-mail addresses: allyn.spear@ars.usda.gov (A. Spear), flanegan@ufl.edu (B.J. Flanegan).
[1] Present address: USDA-ARS, UWM School of Freshwater Sciences, 600 E Greenfield Ave, Milwaukee, WI 53204, USA.

http://dx.doi.org/10.1016/j.virol.2015.07.018
0042-6822/

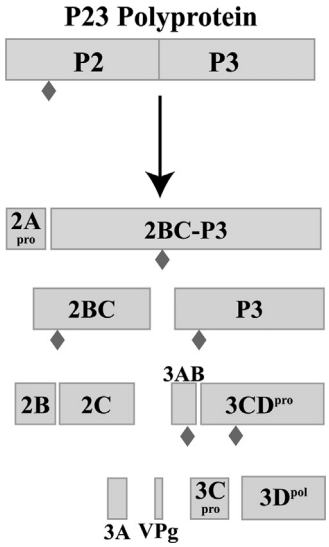

Fig. 1. Diagram of the poliovirus P23 polyprotein processing cascade showing the precursor and processed viral proteins. The P23 polyprotein is cleaved by the viral protease 3C/3CD at the cleavage sites shown as filled diamonds (◆).

1979;Harris et al., 1992). The 3CD precursor also has the ability to bind to stem-loop 'd' of the 5′CL. While this ability is partially retained by its processed product 3C, the binding affinity of 3C for the 5′CL is 10-fold lower than that of 3CD (Andino et al., 1993). Although 3CD and 3C are proteases, their cleavage specificities and activity levels are different. This difference is particularly apparent in the processing of the viral capsid precursor (P1) as well as processing at the 3C–3D junction (Parsley et al., 1999). Despite these functional differences, there are very few structural differences between 3C and 3D individually and within the 3CD precursor as determined by x-ray crystallography (Gruez et al., 2008;Marcotte et al., 2007;Thompson and Peersen, 2004). A critical difference, however, is that the N-terminal glycine residue in 3D is buried in a pocket near the base of the fingers domain in the active 3D structure, but it is part of the 3C–3D linker in the inactive 3CD precursor. Studies on the structure of both PV and Coxsackievirus B3 (CVB3) 3D indicate that the buried N-terminal glycine residue is required to activate the picornaviral polymerase (Gruez et al., 2008;Thompson and Peersen, 2004).

Current models of PV replication complex formation invoke genomic circularization mediated by ribonucleoprotein (RNP) complexes formed at the 5′CL and the 3′NTR-poly(A) tail to promote initiation of (−) strand synthesis (Barton et al., 2001; Herold and Andino, 2001; Lyons et al., 2001;Ogram and Flanegan, 2011;Teterina et al., 2001). Viral precursor protein, 3CD, in the presence of PCBP or 3AB, was observed to form RNP complexes with the 5′CL (Andino et al., 1990b; 1993; Harris et al., 1994; Parsley et al., 1997; Xiang et al., 1995). In a previous study, a revertant that contained a second site suppressor mutation in the RNA binding domain of viral protein 3C was isolated from cells transfected with viral RNA with an insertion mutation in stem-loop 'd' (Andino et al., 1990b). This finding provided evidence for a direct interaction between 3CD and stem-loop 'd' of the 5′CL. Additional studies showed that mutations in stem-loop 'd' of the 5′CL, which disrupted 3CD binding, also inhibited PV RNA replication (Andino et al., 1993; Parsley et al., 1997; Xiang et al., 1995; Barton et al., 2001;Vogt and Andino, 2010). Furthermore, mutations in the 3C-RNA binding domain in 3CD disrupted the formation of the 5′CL-RNP complex and inhibited viral RNA replication (Andino et al., 1993;Hammerle et al., 1992;Harris et al., 1994;Blair et al., 1998).

The results of the above studies suggest that the diverse activities associated with 3CD, and presumably other precursor proteins like P3, are required for PV RNA replication. Consistent with this idea, previous studies showed that P3 was required to complement an RNA replication-defective mutation in the 3A region of protein 3AB (Towner et al., 1998). In addition, the results of another study showed that P3 was required for complementation of a VPg-linkage mutant (Liu et al., 2007). Taken together, these findings suggest that viral precursor proteins and their processed products play distinct and essential roles in the assembly of the poliovirus RNA replication complex. However, the precise molecular mechanisms which drive the requirement for the viral precursor proteins have not been delineated. To characterize these mechanisms, we utilized the HeLa S10 translation-replication system to examine the complementation profiles of viral transcript RNAs, which contain either a 3D polymerase mutation or a 3C-RNA binding mutation. These mutant RNAs were assayed for their ability to assemble functional replication complexes and initiate (−) strand synthesis. The results of the experiments with the 3D polymerase mutant showed that 3D is initially assembled into the replication complex in the form of its inactive precursor, P3 or 3CD, and that 3D itself cannot assemble into the complex. The precursor proteins are subsequently cleaved to release active 3D for VPg uridylylation and RNA replication. In the experiments with the 3C-RNA binding mutant, our results showed that P3, and not 3CD, is the preferred precursor that binds to the 5′CL. Furthermore, the results of reciprocal complementation assays demonstrated that the molecule of P3 that binds the 5′CL does not serve as the 3D polymerase provider. Instead, a second molecule of P3, which does not bind RNA, functions as the source of active 3D polymerase. Interestingly, our results also showed that a second molecule of P3 that does not bind RNA served as the source of VPg in the replication complex. These findings support a model in which P3 binds to the 5′CL and then recruits additional molecules of P3, which are then cleaved to release either active 3D or VPg for RNA replication. Taken together, our results illustrate that the viral precursor protein, P3, performs distinct and essential functions in the replication complex.

Results

Previous studies have identified several functions associated with the viral protein 3CD that are required for PV RNA replication. The initial goal of this study was to use a genetic complementation analysis to identify specific functions of 3CD that are required to form functional replication complexes to initiate (−) strand synthesis. Specific mutations that disrupted 3D polymerase activity, the processing of 3CD to 3C and 3D and the RNA binding activity of 3C (and 3CD) were constructed in a subgenomic RNA transcript, PV P23 RNA (Fig. 2B). P23 RNA encodes all of the viral replication proteins and serves as an efficient template to measure (−) strand synthesis. For the complementation analysis, protein expression RNAs that encoded P3, 3CD, 3C or 3D were constructed as described (see Materials and methods) (Fig. 2C). (−) Strand synthesis was measured in preinitiation replication complexes (PIRCs) isolated from HeLa S10 translation-RNA replication reactions containing one of the P23 RNA mutants and one of the viral protein expression RNAs. To confirm that equivalent levels of the viral proteins were synthesized in each reaction, translation of the viral transcript RNA was measured in the presence of [^{35}S]-methionine.

Characterization of 3D polymerase activity mutants

The first mutant, P23-3D(G327M) RNA, contained a mutation in the active site of the 3D, which abolishes all polymerase activity