Short
Communication

Functional differences between precursor and mature forms of the RNA-dependent RNA polymerase from rabbit hemorrhagic disease virus

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The genome region encoding the RNA-dependent RNA polymerase 3CD-like precursor from rabbit hemorrhagic disease virus (RHDV) (isolate AST/89) was cloned and expressed in *Escherichia coli* using polyhistidine fusion-based vectors. The full-length recombinant 3CD-like precursor polypeptide could not be purified as a consequence of its autoproteolytic processing. A Cys→Gly substitution of the 3C-like catalytic cysteine (C1212) impeded the cleavage and allowed the purification of the precursor at high yields using a polyhistidine fusion expression vector. Equimolar amounts of purified recombinant precursor (C1212G mutant) and mature 3D-like polymerase showed significant activity differences in genome-linked protein (VPg) uridylylation and RNA polymerization using *in vitro* assays. The data indicated that the precursor was more active than the mature polymerase in catalysing RHDV VPg uridylylation, whereas the latter enzyme form had higher activity than its precursor in RNA polymerization *in vitro* assays using a heteropolymeric RNA template.

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Rabbit hemorrhagic disease virus is the type species of the genus *Lagovirus* (Pringle, 1998) within the family *Caliciviridae*. Rabbit hemorrhagic disease virus (RHDV) is the causative agent of a lethal pathology in rabbits (Meyers *et al.*, 1991; Ohlinger *et al.*, 1990; Parra & Prieto, 1990). The viral genome is a positive-sense, single-stranded, polyadenylated RNA molecule of approximately 7.4 kb, with a virus-encoded protein (VPg) covalently attached to its 5' end. Relevant differences have been found concerning genetic organization (Green *et al.*, 2000) and ORF1 polyprotein processing (Belliot *et al.*, 2003; Liu *et al.*, 1999; Martín Alonso *et al.*, 1996; Meyers *et al.*, 2000; Sosnovtseva *et al.*, 1999) among members of the family *Caliciviridae*. One of the major differences is related to the structure and function of the RNA-dependent RNA polymerase (RdRp). The first report on a calicivirus RdRp showed that the RHDV '3D^{Pol}-like' (3D) mature product was enzymically active (López Vázquez *et al.*, 1998), whereas in feline calicivirus, the '3CD^{Pro-Pol}-like' (3CD) precursor was shown to be the active RdRp (Wei *et al.*, 2001) and no processing of this polypeptide into 3C and 3D seemed to occur in this viral system (Green *et al.*, 2002; Sosnovtseva *et al.*, 1999). More recently, it has been shown in Norwalk virus (NV) isolate MD145-12 that both the precursor 3CD and the mature 3D have similar RdRp activities (Belliot *et al.*, 2005) and that the Pro-Pol precursor is able to nucleotidylate VPg (Belliot *et al.*, 2008).

In RHDV, both the 3CD precursor and 3D mature polypeptides have been shown to co-exist within the cell using either RHDV-infected cells (Konig *et al.*, 1998) or transient expression experiments (Meyers *et al.*, 2000). The recombinant mature RdRp (3D) from RHDV has been functionally (López Vázquez *et al.*, 1998, 2000, 2001) and structurally (Ng *et al.*, 2002) characterized. In this work, we describe the cloning, expression in *Escherichia coli*, purification and functional characterization of the RHDV 3CD recombinant polypeptide using VPg uridylylation and RNA polymerization in *in vitro* assays.

The RHDV (isolate AST/89) 3CD coding region was amplified by PCR using the specific primers 3C5'B (5'-GGATCCCCTGGGTTTCATGAGACAC-3') and 3CD3' (5'-GGATCCTCACTCCATAACATTACAAACTC-3'). The 3C5'B primer created a *Bam*HI recognition sequence (underlined) and caused a Leu→Ser point mutation at residue 2 of the recombinant RHDV 3CD coding region. Primer 3CD3' included nine residues added to the specific RHDV sequences, which created an in-frame translational stop codon after the 3CD Glu C-terminal residue followed by a *Bam*HI recognition sequence. The resulting 1.97 kb *Bam*HI cassette, corresponding to nt 3334–5310 of the Spanish AST/98 RHDV isolate (GenBank accession no. Z49271) was then cloned in the correct orientation into vector pQE30 (Qiagen) yielding expression vector pQE-3CD (Fig. 1a). *E. coli* BL21 cells transfected with vector pQE-3CD and induced with IPTG did not overproduce the

Fig. 1. (a) Diagram depicting the 3CD coding sequence in the RHDV genome and simplified schemes of the pQE-30 derivative expression vectors. The numbers above the rectangles indicate amino acid residues from the RHDV ORF1 polypeptide N terminus. (b) Protein patterns observed after SDS-PAGE and Coomassie blue staining of samples at different stages of the purification of recombinant RHDV 3CD^{C1212G}. Lanes: 1, molecular mass marker (kDa); 2, crude homogenate of IPTG-induced *E. coli* BL21 cells transfected with recombinant vector pQE-3CD^{C1212G}; 3, soluble cell-free extract; 4, cell-free extract proteins precipitated using 25% ammonium sulphate; 5, eluate from a Vivapure Q Maxi spin column using buffer B containing 500 mM NaCl; 6, eluate from a Ni²⁺-chelating Sepharose Fast Flow column using buffer C containing 100 mM imidazole. (c) Western blot analysis of increasing amounts of purified recombinant 3CD^{C1212G} mutant (lanes 1–3: 0.4, 2 and 4 µg, respectively) using a specific anti-3D serum. Lane 4 contains 2 µg purified 3D (López Vázquez *et al.*, 1998).

expected product (data not shown), and Western blot analyses, using anti-3D-specific antibodies, showed that the 3CD protein fusion was cleaved into several smaller polypeptides, the most abundant one having the expected mass for mature 3D. In order to produce full-length 3CD, two separate strategies were used. In the first, we aimed to preserve 3C activity by mutating the P1 residue (E¹²⁵¹G) located at the 3C–3D boundary. This was carried out taking into consideration that previous studies (Wirblich *et al.*, 1995) have reported that this residue is critical for 3C cleavage and that only glutamic acid, glutamine and aspartic acid are tolerated at this position. The second point mutant was made by performing a C¹²¹²G mutation of the Cys residue of the 3C catalytic triad. This mutation has previously been shown to abolish 3C proteinase activity (Boniotto *et al.*, 1994). For construction of the C1212G or E1251G mutant, an RHDV cDNA (nt 3517–7130) cloned into pBluescript SK⁺ vector was used to perform *in vitro* site-directed mutagenesis using the specific mutagenic oligonucleotide primer 3CD^{C1212G} (5'-CCCACGGTGAC-GGTGGGCTGCCG-3') or 3CD^{E1250G} (5'-AAGGGAGTT-TATGGAACATCAAACCTTCTTC-3') by the Chameleon double-stranded site-directed mutagenesis method (Stratagene). The resulting mutant DNAs were digested with *Asp7181* and *BglII* restriction enzymes and subcloned

into *Asp7181/BglII* double-digested pQE-3CD vector, giving rise to pQE-3CD^{C1212G} and pQE-3CD^{E1251G} expression plasmids (Fig. 1a). Expression studies using pQE-3CD^{E1251G} indicated that no prominent bands of the expected size for the His–3CD fusion were observed after Coomassie blue staining of SDS-PAGE gels. In addition, immunodetection studies using anti-3D-specific antibodies showed that the E¹²⁵¹G mutation did not completely abolish the self-cleavage of the fusion protein. Nevertheless, some full-size 3CD precursor could be observed in contrast to the wild-type construct, in which 3CD was completely processed (data not shown). Mass spectrometry analysis of 3CD cleavage products indicated that the mutation did in fact abolish cleavage at the natural ET peptide bond, indicating that the observed products were the result of lower efficiency secondary cleavages at alternative susceptible bonds. In contrast, the pQE-3CD^{C1212G} expression vector directed the synthesis of a His–3CD fusion that was not self-processed (Fig. 1b, lane 2). The protein profile of pQE-3CD^{C1212G}-transformed IPTG-induced *E. coli* cultures using SDS-PAGE indicated that most of the over-produced 3CD in the crude cell extract was present in the soluble fraction (Fig. 1b, lane 3) and that this major protein band could be precipitated efficiently using ammonium sulphate (Fig. 1b, lane 4). The recombinant 3CD^{C1212G}

protein was then purified by combining anion-exchange chromatography using Vivapure Q columns (Fig. 1b, lane 5) and Ni^{2+} -chelating Sepharose chromatography yielding a recombinant 3CD^{C1212G} that was more than 98% homogeneous by SDS-PAGE (Fig. 1b, lane 6). To rule out the presence of trace amounts of contaminant mature 3D in the purified 3CD^{C1212G} preparation, Western blot analyses were performed using increasing amounts of the purified 3CD (Fig. 1c, lanes 1–3). Although the purified 3CD precursor migration observed in Fig. 1(c, lanes 1–3) differed slightly as a consequence of the increasing protein load, the lack of detection of additional smaller bands with the expected size for the mature 3D (Fig. 1c, lane 4) indicated that no detectable 3D was present and that the purified 3CD preparation contained only the RdRp precursor, which was not contaminated with the RHDV RdRp mature form.

Comparative functional analyses of the purified recombinant 3CD with respect to the previously characterized mature 3D (López Vázquez *et al.*, 1998) were first attempted by exploring their RdRp activities using a synthetic RNA template. For this purpose, we made a 2.1 kb RNA transcript from a PCR amplicon made using primers 5'-GTGAAATTATGGCGGCTATGTCGCG-3' and 5'-ACCGCGGCCGCTAATACGACTCACTATAGGGGTGCTCACTCTTGATAAGGTCC-3', the latter including a modified T7 promoter (*italic*) and a *NotI* restriction site (*underlined*). The amplicon was then transcribed *in vitro* using RiboMAX, a large-scale RNA production system from Promega, yielding a synthetic transcript including 2145 residues from the 3' end of the RHDV genome negative strand.

The RdRp assay protocol used was based on that described previously for RHDV recombinant 3D (López Vázquez *et al.*, 1998). Appropriate concentrations (70 nM to 1.08 µM) of purified 3D or 3CD^{C1212G} polymerase were added to 50 µl reactions containing 1 µg RNA transcript, 50 mM HEPES (pH 8.0), 10 µM each ATP, CTP and GTP, 5 µM UTP, 4 µM dithiothreitol, 3 mM magnesium acetate, RNaseOUT (Invitrogen) and 10 µCi (370 kBq) [α -³²P]UTP. The reaction mixtures were incubated at 30 °C for 60 min, extracted with phenol/chloroform and precipitated with ethanol in the presence of 0.3 M sodium acetate (pH 6.0) and 20 µg tRNA. The precipitates were dissolved in formaldehyde/agarose electrophoresis sample buffer. After electrophoresis, the gels were dried and radiographed. The radiograph of the dried gels indicated the presence of major RNA products (1× and 2× template size) whose abundance increased with the 3D or 3CD concentration (Fig. 2a), indicating relevant UMP incorporation rate differences between both types of RdRp. A quantitative analysis of the gels using an Instant Imager (Packard Instrument Company) (Fig. 2b) revealed that the mature RdRp (3D) was four to five times more active than its precursor (3CD). In both cases, the rate of UMP incorporation into RNA products showed a linear

Fig. 2. (a) Autoradiograph of an agarose/formaldehyde gel showing ³²P-labelled RNA products synthesized using increasing concentrations of purified RdRp (3D) or mutant RdRp precursor (3CD^{C1212G}), using a synthetic RNA template. Lanes: 1, 0.07 µM; 2, 0.13 µM; 3, 0.27 µM; 4, 0.54 µM; 5, 1.08 µM. Arrow heads indicated the electrophoretic mobility of RNA products of template (t) size or twice the template size (t 2×). (b) Quantitative analysis of UMP incorporated into the labelled RNA products shown in Fig. 2(a), using an Instant Imager.

dependence on the enzyme concentration from 67 nM to 0.54 µM (Fig. 2b).

We have described in a previous study that, in addition to its RdRp activity, RHDV 3D is able to catalyse VPg uridylylation (Machín *et al.*, 2001). In order to investigate the putative VPg uridylylation activity of the RHDV 3CD, the protocol used was based on that described previously (Machín *et al.*, 2001). Briefly, reaction mixtures (20 µl) contained 50 mM HEPES buffer (pH 7.5), 1 mM MnCl₂, 100 µM UTP, 2 µCi [α -³²P]UTP (800 Ci mmol⁻¹) and appropriate amounts (50 nM to 1 µM) of purified 3D or 3CD and VPg (0.14 nmol). Incubations were carried out at 30 °C and the reactions were stopped after 60 min by the addition of SDS-PAGE loading buffer and analysed using 15% polyacrylamide gels containing SDS, which were dried and autoradiographed once the runs were complete. The radiograph of the dried gels indicated that, under the conditions used, the 3CD precursor was clearly more active than the mature 3D (Fig. 3a) and that there was a linear dependence of the VPg uridylylation rate on the enzyme

Fig. 3. (a) Autoradiograph after SDS-PAGE analysis of VPg uridylylation reactions using increasing concentrations of purified RdRp (3D) or mutant RdRp precursor (3CD^{C1212G}) in the presence of [α -³²P]UTP and 0.14 nmol purified recombinant VPg. Lanes: 1, no RdRp or RdRp precursor added; 2, 0.05 μ M; 3, 0.1 μ M; 4, 0.25 μ M; 5, 0.5 μ M; 6, 1.0 μ M. (b) Quantitative analysis of UMP incorporated into VPg (in Fig. 3a) using increasing concentrations of purified RdRp (3D) or mutant RdRp precursor (3CD^{C1212G}), using an Instant Imager.

concentration, at least in the 0.25–1 μ M range (Fig. 3b). The [³²P]UMP incorporated into VPg was quantified using an Instant Imager, showing that the 3CD precursor was 8–13-fold more active than 3D in this kind of reaction (Fig. 3b). VPg nucleotidylylation in the presence of other NTPs indicated that GTP was preferred over UTP, CTP and ATP (not shown). Nevertheless, the differences in activity between 3CD and 3D for any particular NTP remained similar to the data shown for UTP in Fig. 3.

The comparative activity assays performed using both RHDV RdRps acting on a synthetic RNA template (Fig. 2) indicated that the mature form (3D) was fivefold more active than its precursor (3CD), in clear contrast with reports for NV, in which both enzyme forms were equally active (Belliot *et al.*, 2005). It should be noted that the 3D used in the present study had three additional amino acid residues (GSM) at its authentic N terminus (López Vázquez *et al.*, 1998), and that this fact has been argued by other authors to be the cause of its lower activity with respect to other calicivirus RdRps (Wei *et al.*, 2001). Nevertheless, if this proves to be a negative factor, it should

then be expected that a 3D enzyme with an authentic N terminus would be even more active than the one used in this work, thus amplifying the differences observed in the RdRp activity described here.

As calicivirus polymerases must also be able to act on VPg at some stage of their replication cycle, we extended our comparative studies to analyse putative functional differences using VPg uridylylation assays. Our data indicated that the 3CD precursor was up to 13-fold more active than 3D.

The data reported in this study were consistently reproducible in our hands and indicate a tendency towards functional specialization of the two polymerase forms in the RHDV system. The precursor 3CD seems to be more efficient than its 3D derivative during the initial steps of genome replication, which, in light of our current knowledge, should involve the addition of NTP residues to a VPg protein primer. Once the 3CD precursor is processed into 3D, this latter form of the enzyme develops an increased capacity to act on RNA rather than on protein primers. The conversion of 3CD into 3D would then contribute to an increase in the efficiency of late replication events, which require the extension of the growing RNA chains covalently linked to VPg. Several published reports (López Vázquez *et al.*, 1998, 2000; Wei *et al.*, 2001) have provided evidence that the calicivirus polymerases characterized so far show poor template specificity and that they can act equally well on non-viral RNAs *in vitro*. The increased VPg nucleotidylylation activity of 3CD could also be envisaged as part of the virus specificity mechanisms involved in ensuring that the viral RdRp acts primarily on viral genome replication and not on other cellular RNAs. Thus, in our studies, the 3CD enzyme form did not work very efficiently on ‘naked RNA’ and showed a clear preference for a protein primer (VPg) of viral origin. This activity would yield abundant VPg–RNA primers *in vivo* that could then be extended more efficiently by the mature 3D.

The results reported in this study indicate that, despite the evident similarities to related viral systems such as those of picornaviruses, the biological role of the 3CD precursor appears to be very different in members of the *Caliciviridae*, as the calicivirus 3CD is catalytically active (in contrast with the picornavirus 3CD), showing the same activities as the mature RdRp. In addition, critical discrepancies concerning 3CD activity can be found within this virus family in which members of two of the genera (*Lagovirus* and *Norovirus*) process the 3CD precursor into smaller functional products (Belliot *et al.*, 2003; Meyers *et al.*, 2000), whereas in the remaining two (*Vesivirus* and *Sapovirus*), 3CDs cannot be considered as precursors but as bifunctional enzymes, as no further processing of these polypeptides has been demonstrated (Oka *et al.*, 2005; Wei *et al.*, 2001). The biological role of the existence and functional specialization of RdRp forms within members of the family *Caliciviridae* should be investigated further.

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