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Nucleotidylylation of the VPg protein of a human norovirus by its proteinase-polymerase precursor protein

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Abstract

Caliciviruses have a positive strand RNA genome covalently-linked at the 5'-end to a small protein, VPg. This study examined the biochemical modification of VPg by the ProPol form of the polymerase of human norovirus strain MD145 (GII.4). Recombinant norovirus VPg was shown to be nucleotidylylated in the presence of Mn^{2+} by MD145 ProPol. Phosphodiesterase I treatment of the nucleotidylylated VPg released the incorporated UMP, which was consistent with linkage of RNA to VPg via a phosphodiester bond. Mutagenesis analysis of VPg identified Tyrosine 27 as the target amino acid for this linkage, and suggested that VPg conformation was important for the reaction. Nucleotidylylation was inefficient in the presence of Mg^{2+}; however the addition of full- and subgenomic-length MD145 RNA transcripts led to a marked enhancement of the nucleotidylylation efficiency in the presence of this divalent cation. Furthermore, evidence was found for the presence of an RNA element near the 3'-end of the polyadenylated genome that enhanced the efficiency of nucleotidylylation in the presence of Mg^{2+}.
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Keywords: Norovirus; VPg; Polymerase; Nucleotidylylation

Introduction

Noroviruses (NoV), a major cause of acute gastroenteritis (Green et al., 2001), are members of the family *Caliciviridae*. The genome is composed of an approximately 7.5 kb single-stranded positive-sense RNA molecule that is covalently-linked at the 5'-end to a VPg protein, and is polyadenylated at its 3'-end. The genome is organized into three open reading frames (ORFs): ORF1, ORF2 and ORF3. ORF1 encodes the 200 kDa precursor of the nonstructural proteins, and ORF2 and ORF3 encode the major (VP1) and minor (VP2) structural proteins, respectively (Jiang et al., 1993; Lambden et al., 1993). The 200 kDa polyprotein of human norovirus strain MD145 (belonging to genogroup II.4) is cleaved by the viral proteinase (Pro) to release both precursors and fully processed viral proteins with the following gene order: NS1-2 (N-terminal protein): NS3 (NTPase): NS4 (p20): NS5 (VPg): NS6 (Pro): NS7 (RNA-dependent RNA polymerase) (Belliot et al., 2003; Sosnovtsev et al., 2006). The norovirus RNA-dependent RNA polymerase (RdRp) as well as RdRps from several calicivirus genomes have been expressed in bacteria as a mature form (designated Pol) or a ProPol RdRp precursor, purified, and characterized biochemically (Belliot et al., 2005; Fukushi et al., 2004; Ng et al., 2004; Rohayem et al., 2006a; Wei et al., 2001). Polymerase activity was demonstrated by both forms of norovirus RdRp (Belliot et al., 2005). The initiation of RNA

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synthesis by the calicivirus RdRp on an RNA template has been reported as primer-dependent in some studies and primer-independent in others (Belliot et al., 2005; Fukushi et al., 2004; Lopez Vazquez et al., 2001; Rohayem et al., 2006a; Wei et al., 2001). Various mechanisms by which initiation might occur in the absence of an added primer have been proposed. Moreover, a recent study has shown evidence that the uridylylated VPg protein may serve as a primer for the initiation of norovirus RNA synthesis on polyadenylated RNA templates (Rohayem et al., 2006b).

Studies of the role of the calicivirus VPg in replication and the nature of its linkage to the viral RNA have focused on feline calicivirus (FCV, genus *Vesivirus*) (Dunham et al., 1998; Herbert et al., 1997; Mitra et al., 2004), Norwalk virus and other human norovirus strains (genus *Norovirus*) (Rohayem et al., 2006b), and rabbit hemorrhagic disease virus (RHDV, genus *Lagovirus*) (Machin et al., 2001). Early work showed that genomic RNA purified from FCV and other vesivirus virions was not infectious after digestion of the VPg by proteinase K (Herbert et al., 1997), and it was proposed that the VPg was required for translation (Daughenbaugh et al., 2003; Goodfellow et al., 2005). Recent reports provided further evidence for the involvement of the calicivirus VPg in translation. Human and murine norovirus recombinant VPg proteins were shown to interact directly with the eukaryotic initiation factor eIF3 in a yeast two-hybrid system (Daughenbaugh et al., 2003), and the VPg proteins of both FCV and noroviruses were shown to interact with the translation factor eIF4E (Goodfellow et al., 2005). Moreover, certain translation initiation factor inhibitors, such as 4E-BP1, specific for eIF4E, could markedly reduce translation efficiency *in vitro* for some caliciviruses (Chaudhry et al., 2006).

The discovery that recombinant VPg expressed from RHDV could be uridylylated by the viral polymerase provided initial evidence that the calicivirus VPg might also serve a primer function during RNA replication, similar to that of the picornavirus VPg (Machin et al., 2001). As noted above, evidence for such activity has been reported recently for the norovirus VPg (Rohayem et al., 2006b). It is assumed that the first step in a VPg-mediated priming mechanism would be nucleotidylylation at a specific amino acid residue of the VPg. In RHDV, a tyrosine residue located at position 21 of the VPg was shown to be uridylylated *in vitro* by recombinant Pol in the presence of Mn^{2+} in a template-independent manner. Mutagenesis of the corresponding conserved tyrosine residue (position 24) in the FCV VPg was lethal for the growth and recovery of the virus (Mitra et al., 2004). These studies suggest that a conserved tyrosine residue is the site of nucleotidylylation for the caliciviruses, but this site has not been identified in the noroviruses.

In this study, we examined biochemical modifications of the VPg by the ProPol form of the MD145 RdRp in an effort to gain further insight into the role of the VPg in RNA replication for the noroviruses. One aim was to determine whether the norovirus VPg undergoes nucleotidylylation by the ProPol form of the viral polymerase and to map the site where this modification occurs. We showed that the VPg was nucleotidylylated by the ProPol form of the human norovirus RdRp in a template-independent manner in the presence of Mn^{2+}, and that the linkage between the nucleotide and the VPg was covalent. We identified Tyrosine 27 of the MD145 VPg as the target amino acid for this activity, and showed that the linkage was susceptible to treatment with phosphodiesterase. Efficient nucleotidylylation of the VPg could be achieved in the presence of Mg^{2+} cations, but only in the presence of selected polyadenylated viral RNA templates. In addition, evidence was found for an enhancing element in calicivirus RNA that might function similarly to the *cis*-acting replication element (CRE) of the picornaviruses.

Results

Synthesis and purification of recombinant VPg proteins

Recombinant VPg proteins were expressed either as N- or C-terminal 6× His-tagged fusions, or as proteins without a His-tag (summarized in Fig. 1B). The VPg proteins with an N-terminal His-tag were expressed in the pET system and purified on a nickel-nitrilotriacetic (NTA) column to greater than 85% homogeneity (Fig. 1C, lanes 1 to 7). The recombinant proteins rVPg-His, rΔ3VPg-His, rΔ8VPg-His and rΔ20VPg-His, in which the His-tag was engineered at the C-terminus, were purified to over 90% homogeneity (Fig. 5A, panel II). The SUMO-VPg recombinant fusion proteins (rHis-SUMO-VPg, rHis-SUMO-Y27A/VPg, rHis-SUMO-Y30A/VPg) were purified on a Ni-NTA column (as shown for rHis-SUMO-Y27A/VPg in Fig. 1C, lane 9) and then incubated with Ulp1 proteinase. The mixture was again passed through a Ni-NTA column to bind the cleaved SUMO-His-tag and the His-tag-free recombinant VPg protein was collected in the flow-through (as shown for rHis-SUMO-Y27A/VPg in Fig. 1C, lane 10). The efficiency varied, but at least 50% of the fusion protein was cleaved with Ulp1 (data not

Fig. 1. MD145 VPg DNA constructs generated for study. (A) The primary deduced amino acid sequence of the MD145 VPg is shown. The tyrosine residues at positions 27, 30, 41, 46, 53, 54, and 126 are shown in bold type, and the lysine residues in the N-terminal region are underlined. The N-terminus of the VPg encoded in truncated VPg constructs Δ3-, Δ8- and Δ20-VPg-His is indicated. (B) cDNA clones engineered for expression of the MD145 VPg and its mutated forms in bacteria. His-tag is represented by the black box in the diagram, and is located at the N- or C-terminus. The VPg constructs were designated according to the mutagenized tyrosine (e.g., pET-His-Y27A/VPg). Double tyrosine-mutated VPg constructs were designated similarly. VPg proteins with sequential deletions from the N-terminus were designated according to the first amino acid in the truncated VPg (e.g., pET-Δ3VPg-His). For the SUMO-VPg constructs, the SUMO peptide is indicated by a grey box. For the Ulp1 cleavage site, SUMO peptide and VPg amino acids are indicated by lower and lower cases, respectively. The resulting construct was named as indicated above. (C) SDS-PAGE analysis of 1 μg bacterially-expressed recombinant VPg proteins (lanes 1–7) and $Pro^{-}Pol$ (lane 8) that were engineered with an N-terminal His-tag. The mutated rY27A/VPg (Fig. 1C, lanes 9 and 10) was expressed without a His-tag (see Materials and methods). The rHis-SUMO-Y27A/VPg fusion protein (lane 9) and tag-free rY27A/VPg (lane 10) were resolved by SDS-PAGE. Ulp1 proteinase treatment is indicated above the gel. The migration of the molecular weight marker (Mark XII, Invitrogen) is indicated in kDa here and in the following figures.