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Regulation of human norovirus VPg nucleotidylylation by ProPol and nucleoside triphosphate binding by its amino terminal sequence *in vitro*

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ABSTRACT

The VPg protein of human Norovirus (hNoV) is a multi-functional protein essential for virus replication. The un-cleaved viral precursor protein, ProPol (NS5-6) was 100-fold more efficient in catalyzing VPg nucleotidylylation than the mature polymerase (Pol, NS6), suggesting a specific intracellular role for ProPol. Sequential and single-point alanine substitutions revealed that several positively charged amino acids in the N-terminal region of VPg regulate its nucleotidylylation by ProPol. We provide evidence that VPg directly binds NTPs, inhibition of binding inhibits nucleotidylylation, and NTP binding appears to involve the first 13 amino acids of the protein. Substitution of multiple positively charged amino acids within the first 12 amino acids of the N-terminal region inhibits nucleotidylylation without affecting binding. Substitution of only Lys20 abolishes nucleotidylylation, but not NTP binding. These studies indicate that positively charged amino acids in the first 20 amino acids of hNoV VPg regulate its nucleotidylylation though several potential mechanisms.

1. Introduction

The human Norovirus (hNoV) is a non-enveloped, single-stranded, non-segmented, positive-sense RNA virus belonging to the Caliciviridae family (Green, 2013; Green et al., 2010; Jiang et al., 1993). Infections with hNoV are the predominant cause of gastroenteritis worldwide, responsible for over 20 million cases per year in the United States, and account for 10–15% of severe cases of gastroenteritis in children aged less than 5 years old (Hall et al., 2011). In developing countries, hNoV is estimated to cause 218,000 deaths among children each year (Patel et al., 2008). Of the three genogroups of hNoV (GI, GII, GIV), GII hNoV has been the major cause of gastroenteritis outbreaks in the United States since 2001 (Siebenga et al., 2009).

The hNoV VPg is a 15.8 kDa multifunction protein covalently linked to the 5' end of hNoV genome (Belliot et al., 2008). Lack of VPg capping renders viruses non-infectious (Herbert et al., 1997). VPg has been proposed to serve as a protective cap for the hNoV genome against detection by the host immune system (Goodfellow, 2011). VPg is a recruiter of the cellular translation machinery, interacting with eukaryotic translation factors eIF3 and eIF4E (Chaudhry et al., 2006; Daughenbaugh et al., 2003; Goodfellow et al., 2005), a process that was recently linked to the C-terminal end of the VPg protein (Chung et al., 2014). A potential role in packaging of the newly synthesized viral genomes has also been hypothesized (Kaiser et al., 2006).

VPg is also the primer for the initiation of hNoV RNA synthesis when nucleotidylylated by the viral polymerase (Rohayem et al., 2006b). The mechanism of nucleotidylylation involves covalent attachment of a nucleoside monophosphate to the tyrosine at position 27 of hNoV by the viral polymerase independent of RNA (Belliot et al., 2008). While any one of the four NMPs can be linked to hNoV by its polymerase in the absence of an RNA template, GMP is preferred over UMP, which in turn, is preferred over CMP and AMP (Belliot et al., 2008). Evidence has been presented for the presence of an RNA sequence near the 3' end of the hNoV genome that has a potential to stimulate nucleotidylylation (Belliot et al., 2008).

The hNoV produces two equally active versions of its polymerase, the mature polymerase (NS6, Pol) and a protease-polymerase precursor protein (NS5-6, ProPol) that contains both protease and Pol activities (Green, 2013; Green et al., 2010). The relative intracellular functions and/or advantages of these two proteins are unknown and have been debated widely in the literature. During infections by the mouse norovirus, ProPol, as well as several other un-cleaved viral precursor proteins have been shown to be long-lived (Belliot et al., 2005; Green, 2013; Green et al., 2010). Both versions of the hNoV polymerase have been shown to catalyze Calicivirus VPg nucleotidylylation in qualitative assays in separate communications (Belliot et al., 2008; Fullerton et al., 2007; Machin et al., 2009, 2001; Rohayem et al.,

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2006b). Previous studies by our laboratory have examined the relative protease activity of ProPol and the mature hNoV protease (NS5, Pro) and found them to be essentially equivalent in activity in enzymatic assays (May et al., 2013, 2014; Viswanathan et al., 2013).

Previous studies on hNoV VPg have primarily been modelled on investigations with Poliovirus, due to similarities between the characteristics and functions of several genes of these two single-stranded positive-sense viruses. However, there are significant differences between VPg of these two virus families. The hNoV VPg is comprised of 133 amino acids, while the poliovirus VPg contains only 22 amino acids. While hNoV VPg is absolutely essential for hNoV translation initiation (Chaudhry et al., 2006), poliovirus VPg is not, due to the presence of IRES structures within its 5' UTR that recruit host cell ribosome machinery (Pelletier and Sonenberg, 1988; Rohll et al., 1994). Nucleotidylylation of hNoV VPg occurs in the absence of RNA while uridylylation of poliovirus VPg requires a cis-acting *cre* element within the RNA coding sequence of Poliovirus protein 2C (Lyons et al., 2001; Morasco et al., 2003; Murray and Barton, 2003; Paul et al., 2000; Rieder et al., 2000).

The VPg of Potyviruses, such as Potato Virus A (PVA), shares more similarity to the VPg of Caliciviruses with regard to size (189 amino acids *vs.* 133, respectively), a requirement for translation initiation, and nucleotidylylation in the absence of RNA (Puustinen and Makinen, 2004). The PVA VPg is also capable of directly binding nucleoside triphosphates, a process that was found to serve as a pre-requisite for its nucleotidylylation (Puustinen and Makinen, 2004), which has not been studied for Calicivirus VPg. A nucleotide-binding sequence containing several lysine residues (^{38}AYTKKGK^{44}) is present upstream of the PVA VPg nucleotidylylation site (either Y63 or Y119), that is required for both binding of UTP and successful nucleotidylylation (Puustinen and Makinen, 2004; Rantalainen et al., 2011). This amino acid sequence was also shown to be necessary for RNA and protein binding properties of PVA VPg (Puustinen and Makinen, 2004; Rantalainen et al., 2011). Of related interest, deletion of the first 3, 8, or 20 amino acids of hNoV VPg, which are also lysine-rich, progressively reduced its nucleotidylylation at Tyr27 (Belliot et al., 2008).

In this report, we investigate the role of specific amino acids in the lysine-rich region comprising the first 20 amino acids of hNoV VPg in the regulation of its nucleotidylylation. We provide the first evidence that hNoV VPg directly binds nucleoside triphosphates and that prevention of binding inhibits nucleotidylylation of the protein. We also demonstrate that the un-cleaved hNoV precursor protein, ProPol (NS5-6) (Green, 2013; Green et al., 2010), which possesses both the protease and RNA-dependent RNA polymerase functions of the mature protease (Pro, NS5) and polymerase (Pol, NS6), is vastly superior to Pol with respect to nucleotidylylation of hNoV VPg, suggesting a role for this precursor protein in virus replication.

2. Results

2.1. Nucleotidylylation of native hNoV VPg by hNoV ProPol is significantly greater than nucleotidylylation catalyzed by hNoV Pol

The primary purpose of these studies was to examine the influence of amino acids near the amino terminal end of the hNoV VPg on its nucleotidylylation by its polymerases. Essentially all previous studies of hNoV VPg nucleotidylylation utilized a His_6-tag on the amino terminal end of the protein. For our studies, it was deemed essential to remove amino terminal tags from hNoV VPg to eliminate any potential effects of additional amino acids on the reaction.

Initial studies quantitatively compared the hNoV VPg nucleotidylylation activities of both versions of the hNoV polymerase. Nucleotidylylation of amino terminal His_6-tagged hNoV VPg by hNoV Pol or hNoV ProPol was essentially equivalent (Fig. 1, Panel A, lanes 2 and 3), consistent with previous reports (Belliot et al., 2008).

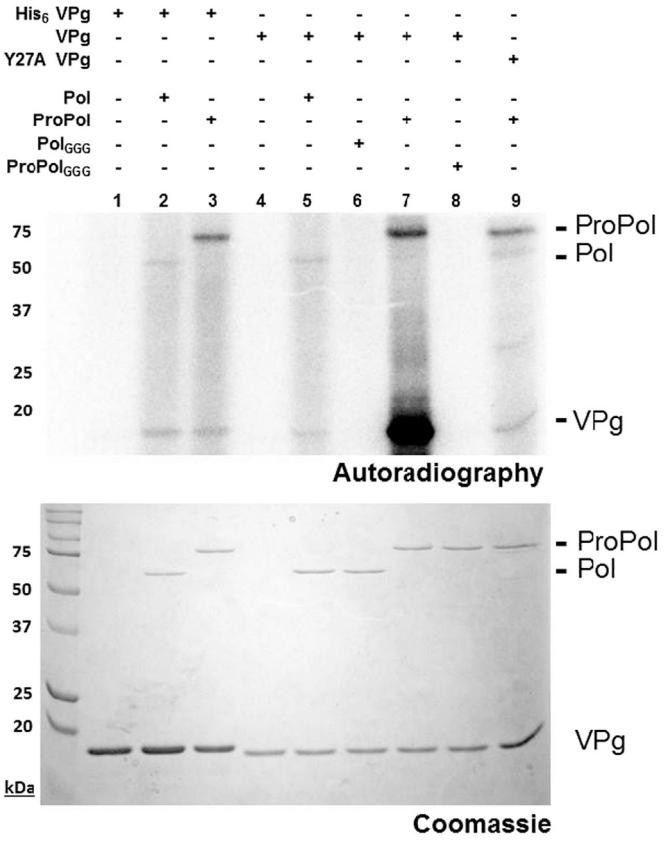

Fig. 1. Nucleotidylylation of His_6-labeled and native hNoV VPg. Reactions were conducted as described in the methods section using α-^{32}P GTP. Top Panel: autoradiography image: Bottom Panel: SDS PAGE gel in Panel A stained with Coomassie dye. Lanes 1–3, His_6-hNoV VPg; Lanes 4–9, native hNoV VPg. Lanes 2 and 5, Pol; Lanes 3, 7, and 9, ProPol. Reaction negative controls: Lanes 1 and 4, reactions lacking either of the hNoV polymerases. Lanes 6 and 8, reactions containing inactive hNoV polymerases (active site motifs mutated from ^{342}GDD^{344} to ^{342}GGG^{344} (Jablonski and Morrow, 1995; Vazques et al., 2000)). Lane 9, native hNoV VPg with Tyr27 substituted by Ala (primary site of nucleotidylylation (Belliot et al., 2008)); compare directly to Lane 7, hNoV ProPol catalyzed reaction using native hNoV VPg.

Nucleotidylylation reactions of His_6-tagged hNoV VPg and tag-less (native) hNoV VPg, when catalyzed by hNoV Pol, revealed no significant difference in the levels of nucleotidylylation (Fig. 1, Panel A, lanes 2 and 5). However, nucleotidylylation of native hNoV VPg by hNoV ProPol was markedly greater than that observed for His_6-tagged hNoV VPg (Fig. 1, Panel A, lanes 3 and 7).

In time course studies, hNoV ProPol was approximately 100 times more efficient at nucleotidylylation than hNoV Pol (Fig. 2, Panel A), although the *de novo* RNA synthesis activities of both polymerases were comparable (Fig. 2, Panel B). The overall levels of nucleotidylylation of native hNoV VPg catalyzed by hNoV Pol were comparable to those previously reported for His_6-tagged hNoV VPg (Belliot et al., 2008). As previously reported (Belliot et al., 2008), nucleotidylylation of hNoV VPg required an active hNoV polymerase, as substitutions of the ^{342}GDD^{344} motif in the active site with ^{342}GGG^{344}, which eliminates hNoV Pol activity (Vazques et al., 2000), inhibited hNoV VPg nucleotidylylation by either hNoV Pol or ProPol (Fig. 1, Panel A, lanes 6 and 8). The tyrosine at position 27 of hNoV VPg has been reported to be the preferred nucleotidylylation site (Belliot et al., 2008). Substitution of an alanine at position 27 in native VPg essentially abolished nucleotidylylation to a level consistent with previous reports (Fig. 1, Panel A, lane 9). Based on these data, all further studies on hNoV nucleotidylylation were carried out using native hNoV VPg and ProPol.