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Characterization of the NSP6 protein product of rotavirus gene 11

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Abstract

The 12 kDa non-structural protein 6 (NSP6) is the least studied of the rotavirus proteins. In an attempt to further characterize this protein mono-specific antisera was generated using purified protein expressed in *E. coli*. Pulse/chase radio-labeling of virus infected cells was used to show that it is expressed at a steady but low rate throughout the virus replication cycle. In contrast to the other rotavirus non-structural proteins, NSP6 was found to have a high rate of turnover, being completely degraded within 2 h of synthesis. NSP6 tagged with GFP was used to probe the intracellular distribution of the protein, perinuclear aggregates were observed in the cytoplasm of transfected cells. Following virus infection of these transfected cells the aggregates were seen to redistribute to the viroplasms. Consistent with its localization to the site of viral genome replication and packaging, NSP6 was found to be a sequence independent nucleic acid binding protein, with similar affinities for ssRNA and dsRNA.
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Keywords: Rotavirus; Non-structural protein; RNA binding protein

1. Introduction

Rotaviruses are members of the *Reoviridae* family and have been shown to be the predominant cause of acute viral gastroenteritis in children and young animals (Kapikian et al., 2001). The viral genome consists of 11 discrete segments of dsRNA which encode 6 structural proteins (VP1–VP6) and 6 non-structural proteins (NSP1–NSP6) (Prasad et al., 1988). The virus particle is composed of three concentric shells which form an icosahedron approximately 100 nm in diameter (Prasad et al., 1988). The genome segments are all monocistronic with the exception of the smallest (gene 11) which encodes two non-structural proteins NSP5 and NSP6 in overlapping reading frames (Estes, 2001).

Virus replication occurs in the cytoplasm and at 2–3 h post-infection cytoplasmic occlusion bodies termed viroplasms are first detected. These have been shown to be the sites of synthesis and replication of dsRNA, packaging of viral dsRNA into newly synthesized cores and the steps of viral morphogenesis that result in the formation of double-shelled pre-virions (Altenburg et al., 1980; Esparza et al., 1980; Patton and Gallegos, 1990; Patton et al., 1997; Wentz et al., 1996). Four (VP1, VP2, VP3 and VP6) of the viral structural proteins and several of the non-structural proteins including NSP5 and NSP6 have been shown to localize to viroplasms in infected cells (Gonzalez et al., 2000; Mattion et al., 1991; Petrie et al., 1982, 1984; Welch et al., 1989).

The precise role(s) of the two proteins encoded by gene 11 has only been partially characterized. The larger of the two, NSP5, is expressed from the long open reading frame (ORF) that initiates at the 5′ proximal AUG and has been shown to be a dimeric, O-glycosylated protein existing in several isoforms differing in their level of phosphorylation (Afrikanova et al., 1996; Gonzalez and Burrone, 1991; Poncet et al., 1997). In common with most of the other proteins found in viroplasms, NSP5 is a RNA binding protein but differs from the other non-structural proteins in exhibiting both ssRNA and dsRNA binding activity (Vende et al., 2002). NSP5 and a second rotavirus non-structural protein NSP2 have been shown to be essential for viroplasm formation (Campagna et al., 2005; Lopez et al., 2005; Silvestri et al., 2004; Vascotto et al., 2004). NSP5 and NSP2 have also been shown to form viroplasm-like structures when expressed from transfected cDNA copies of the viral genes and when NSP5 was expressed with an amino-terminal tag in the absence of other viral proteins suggesting that it may have a structural role in the formation of viroplasms (Fabbretti et al., 1999; Mohan et al., 2003). Similar transfection studies using cDNA copies of viral

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genes have also been used to show that NSP2 influences the phosphorylation status of NSP5 (Afrikanova et al., 1998). Characterization of NSP6 is much more limited, it is encoded in a +1 alternate reading frame in gene 11 that lies entirely within the NSP5 ORF and in most viral isolates the protein is a 92 amino acid in length. It has been proposed to localize to viroplasms from immunofluorescence experiments performed using sera which recognized both NSP5 and NSP6. NSP6 has been demonstrated to interact with NSP5 by using co-immunoprecipitation from cells co-transfected with the NSP5 and NSP6 ORF's and yeast two hybrid assays; however, this interaction could not be detected in virus infected cells (Torres-Vega et al., 2000). The C-terminal region of NSP5 has been shown to interact with NSP6, this region has also been implicated in both dimerization of NSP5 and its hyper-phosphorylation (Torres-Vega et al., 2000). Sequence analysis of gene 11 from different virus isolates has revealed that not all appear to actually encode a 92 amino acid NSP6 protein. Thus, two human virus isolates Mc323 (Kojima et al., 1996) and 512-C (Wu et al., 1998) do not possess the NSP6 start codon, while the Alabama strain of lapine virus (Gorziglia et al., 1989) and the porcine OSU strain (Gonzalez and Burrone, 1989) both appear to have truncated open reading frames for NSP6. This, together with its seemingly low level of expression in virus infected cells, has led to the suggestion that NSP6 may play a non-essential regulatory role within a rotavirus infection (Lopez et al., 2005; Mattion et al., 1991; Torres-Vega et al., 2000).

This study has been aimed at achieving a more thorough analysis of this somewhat neglected viral protein in terms of its pattern of synthesis, intracellular distribution, stability and its nucleic acid binding properties.

2. Materials and methods

2.1. Cells, viruses and transient transfections

The BSC-1 line of African green monkey kidney cells and the Compton UK (Uktc) strain of bovine rotavirus (G serotype 6) were both grown as previously described (McCrae and Faulkner-Valle, 1981).

Transient transfections of BSC-1 cells (80% confluency) in 12-well dishes used 1.6 μg DNA and 2 μl Lipofectamine 2000 (Invitrogen). At 8 h post-transfection the DNA mix was removed and the cells overlaid with medium (GMEM) containing 1% FCS and incubated for a further 40 h. In those experiments requiring transfection and infection, the cells were infected at an m.o.i. of 3, 48 h post-transfection and then fixed 8 h post-infection.

2.2. Production of mono-specific sera

Mono-specific anti-NSP5 sera was generated by Harlan Sera Labs (Bicester, UK). A rotavirus sero-negative guinea pig was injected i.m. with 25 g of purified His-NSP5 in PBS in the presence of Freud's complete adjuvant. The animal was sacrificed at day 30 and a final bleed taken. Mono-specific anti-NSP6 was generated by ISL Ltd. A rotavirus sero-negative New Zealand White-Barrier Rabbit was injected i.m. with 200 g of purified NSP6 in PBS in the presence of Freud's complete adjuvant. The animal was boosted with the same dose 14 days later and five 5 ml bleeds were taken at 14-day intervals.

2.3. Immunofluorescence microscopy and antibodies

Expression of green fluorescent protein (GFP) was monitored by confocal microscopy. Transfected cells were washed once with phosphate-buffered saline (PBS) and then fixed in 100% methanol at −20 °C for 20 min and washed twice with PBS. Transfected cells that were subsequently infected were fixed using the same procedure at 8 h post-infection. Intracellular distribution of NSP5 was monitored by indirect immunofluorescence. Fixed cells were blocked in 1% bovine serum albumin (BSA) in PBS for 1 h, washed three times in PBS and then incubated with guinea pig anti-NSP5 serum (1:100) in PBS 1% BSA for 1 h. Cells were washed three times with PBS and then incubated with (1:100) Alexafluor 594 conjugated goat anti-guinea pig serum (Molecular probes) and mounted for confocal microscopy using Vector Shield mounting media (Vector Laboratories).

2.4. Oligonucleotide primers for PCR

The primers used for the amplification and addition of a 6× histidine tag for the NSP5 ORF were 5′-GCGCATATGCACCACCACCACCACCACATGTCTCTCAGTATTGACGTGAC-3′ and 5′-CTGCAGGTGACCTCTCAGGTCAGACCTACA-3′.

The primers used the amplification and addition of a 6× histidine tag for the NSP6 ORF were 5′-GCGCATATGCACCACCACCACCACCACATGAATCATCTTCAACAGCGTCA-3′ and 5′-CTGCAGGATGAATCTAGGTTCGCATTCACT-3′.

The primers used for amplification of the NSP6 ORF for insertion into the phrGFP-N1 vector were 5′-CGCGAGCTCATGAATCATCTTCAACAGCGT-3′ and 5′-GCGAAGCTTTCACTTTAATTCCTTTATTAA-3′.

The primers used for inserting rotavirus UKtc gene 7 into the PCIneo vector were 5′-GGAATTCCTCGAGGGCTTTTAAAGCGTCTCAGTCG-3′ and 5′-GCGTCTAGACTGCAGGTCACATAAGCGCTTTCTATTCTTG-3′.

The primers used for inserting the actin gene into the PCIneo vector were 5′-GCAGGCTAGCATGGATGATGATATCGCCGCGCT-3′ and 5′-GCGGCTCGAGCTAGAAGCATTTGCGGTGGA-3′.

2.5. Plasmid constructions

The GST tag region of pET42b vector (Novagen) was removed by digestion with the restriction enzymes *Nde*I and *Pst*I. The coding regions of both NSP5 and NSP6 were then amplified with specific primers containing an *Nde*I restriction site followed by six histidine residues at the 5′ terminus and a *Pst*I restriction site at the 3′ terminus of each ORF. The PCR products were cleaved with these restriction enzymes and ligated into the prepared pET42b vector.

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