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Nonstructural proteins involved in genome packaging and replication of rotaviruses and other members of the Reoviridae

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Abstract

Rotaviruses, members of family Reoviridae, are a major cause of acute gastroenteritis of infants and young children. The rotavirus genome consists of 11 segments of double-stranded (ds)RNA and the virion is an icosahedron composed of multiple layers of protein. The virion core is formed by a layer of VP2 and contains multiple copies of the RNA-dependent RNA polymerase VP1 and the mRNA-capping enzyme VP3. Double-layered particles (DLPs), representing cores surrounded by a layer of VP6, direct the synthesis of viral mRNAs. Rotavirus core- and DLP-like replication intermediates (RIs) catalyze the synthesis of dsRNA from viral template mRNAs coincidentally with the packaging of the mRNAs into the pre-capsid structures of RIs. In addition to structural proteins, the nonstructural proteins NSP2 and NSP5 are components of RIs with replicase activity. NSP2 self assembles into octameric structures that have affinity for ssRNA and NTPase and helix-destabilizing activites. Its interaction with nucleotides induces a conformational shift in the octamer to a more condensed form. Phosphate residues generated by the NTPase activity are believed to be transferred from NSP2 to NSP5, leading to the hyperphosphorylation of the latter protein. It is suspected that the transfer of the phosphate group to NSP5 allows NSP2 to return to its noncondensed state and, thus, to accept another NTP molecule. The NSP5-mediated cycling of NSP2 from condensed to noncondensed combined with its RNA binding and helix-destabilizing activities are consistent with NSP2 functioning as a molecular motor to facilitate the packaging of template mRNAs into the pre-capsid structures of RIs. Similarities with the bluetongue virus protein NS2 and the reovirus proteins σNS and μ2 suggest that they may be functional homologs of rotavirus NSP2 and NSP5.

Keywords: Icosahedrons; Replication intermediates; Cryoelectron

1. Introduction

While the various genera of the Reoviridae differ markedly in their host range and in the types of diseases they cause, several features of the structure and replication pathway are common to all the viruses of this family. Most notably, the genome of all the members of the Reoviridae consists of 10–12 segments of dsRNA, with the plus-sense RNA of each segment indistinguishable from viral mRNA. The mature virions of this family are nonenveloped icosahedrons, formed by multiple concentric layers of protein. The core of each virion has $T = 1$ symmetry and contains not only one copy of each the genome segments, but also multiple copies of two enzymes, an RNA-dependent RNA polymerase and an mRNA-capping enzyme. Double-layered particles (DLPs) are formed by a second layer of protein, arranged in a $T = 13$ lattice, which surrounds the $T = 1$ core. Viral transcription is carried out by the RNA polymerase activity of DLPs through a process that is conservative. The nascent transcripts are extruded from the DLPs through channels present at the five-fold axes. Although the viral mRNAs contain 5′-methylated cap structures, they lack the poly(A) tails characteristic of most cellular mRNAs. Each viral mRNA encodes a single protein, or in a few cases, for two proteins in two different reading frames (Estes, 2001; Roy, 2001; Nibert and Schiff, 2001).

The viral mRNAs made by viruses of the Reoviridae direct the synthesis of protein and serve as templates for the synthesis of minus-sense RNA to form the dsRNA genome segments. Studies of the protein and RNA components and RNA polymerase activity of subviral particles (SVPs) isolated from infected cells have indicated that RNA replication is coordinated with the packaging of viral mRNAs. Indeed, such studies have suggested that as viral mRNAs moves into

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replication intermediates (RIs), the mRNAs are simultaneously replicated by particle-associated replicase activity to yield packaged dsRNA. Although, the packaging signals in viral mRNAs have not been described, the hypothesis that mRNA packaging precedes dsRNA synthesis implies that it is the mRNAs which contain cis-acting packaging signals as opposed to the double-stranded products of replication. Because the viruses of the Reoviridae contain equimolar numbers of genome segments, signals also must be present in the viral mRNAs that allow for gene-specific packaging (assortment) such that RIs can be assembled that contain one each of the full complement of viral mRNAs. Large electron-dense viral inclusion bodies (VIBs), representing concentrations of viral protein and RNA, form in the cytoplasm of infected cells soon after infection. The VIBs are believed to represent sites at which RIs are formed and at which RNA packaging and replication occur.

The protein components of RIs include not only the structural proteins that make up cores and DLPs, but also nonstructural proteins. Studies of the nonstructural proteins have shown that they have activities which are crucial for the formation of VIBs and for the packaging and replication of the viral genome. Herein, we review the current knowledge of the structure and function of the nonstructural proteins of the rotaviruses involved in genome packaging and replication and attempt to identify possible homologs of these proteins for other segmented dsRNA viruses.

2. Rotavirus

Rotavirus, one of the best-characterized viruses of the Reoviridae, is the major causative agent of infantile gastroenteritis (Kapikian, 2001). The virion is a large triple-layered icosahedron that contains a genome of eleven segments of dsRNA that encode six structural (VP1, VP2, VP3, VP4, VP6, VP7) and six nonstructural (NSP1-6) proteins (Estes, 2001). Cryoelectron microscopy has revealed that the innermost layer of the virion is made up of the core lattice protein VP2, arranged with $T = 1$ symmetry (Lawton et al., 1997b). Positioned at the inner surface at each of the twelve vertices of the core is an enzyme complex consisting of one copy each of the viral RNA polymerase, VP1 (Valenzuela et al., 1991) and the capping enzyme, VP3 (Chen et al., 1999; Lui et al., 1992; Lawton et al., 1997b). Anchored to each core enzyme complex is believed to be one of the dsRNA genome segments organized in a highly condensed form (Gouet et al., 1999; Pesavento et al., 2001). The $T = 13$ middle layer of the virion is made up of VP6, a major structural protein that interacts with the core protein VP2 and the outer capsid proteins, VP4 and VP7 (Prasad et al., 1988). DLPs, consisting of cores surrounded by VP6, produce transcripts which are extruded from the particles through channels at the icosahedral five-fold axes (Cohen, 1977; Lawton et al., 1997a).

Characterization of rotavirus intracellular SVPs has indicated that three species of RIs are formed in the infected cell: (i) the pre-core RI, (ii) the core RI, and (iii) the double-layered RI. The pre-core RI contains the structural proteins, VP1 and VP3, while the core RI, in addition, contains the structural protein VP2 and the nonstructural proteins NSP2 and NSP5. The double-layered RI is indistinguishable from the core RI except that the former also contains VP6. Pulse-chase analyses have provided evidence that the core RI and double-layered RI are formed by the sequential addition of VP2 and VP6 to the pre-core RI (Gallegos and Patton, 1989; Patton and Gallegos, 1988).

Only the core and the double-layered RIs have associated replicase activity able to catalyze the synthesis of dsRNA in vitro (Gallegos and Patton, 1989). Treatment with ssRNA-specific nuclease yields RIs that are no longer able to synthesize dsRNA. This observation indicates that the mRNA templates are not fully packaged within cores prior to replication. In contrast, the dsRNA product of RIs is resistant to degradation by the dsRNA-specific RNase, RNase V1, and therefore fully packaged (Patton and Gallegos, 1988). Taken together, these results suggest that packaging and RNA replication are concurrent processes with mRNA templates moving into cores as they are replicated by the viral replicase (Patton and Gallegos, 1990).

The presence of NSP2 and NSP5 in RIs suggests that these proteins have a role in the replication and/or packaging of rotavirus RNA. However, unlike the structural core proteins (VP1, VP2, VP3) (reviewed in Patton et al., 2002), the nature of the contribution of NSP2 and NSP5 to one or both of these processes is not apparent. Template-dependent cell-free replication systems containing only the core structural proteins, either virion-derived or recombinant, have been developed that support the synthesis of dsRNA from exogenous mRNA. Although promoting RNA replication, these systems do not support packaging of the dsRNA product into cores (Chen et al., 1994; Patton et al., 1997; Zeng et al., 1996). Hence, while sufficient to catalyze the synthesis of minus-strand RNA, the core proteins alone appear to lack the necessary activities required to promote packaging of dsRNA. To date, the only cell-free system to be described that supports RNA packaging along with replication is one that contains both structural and nonstructural proteins (Patton, 1986). In this system, which uses intracellular SVPs as a source of complexes with replicase activity, the dsRNA product is made by elongation of pre-existing nascent minus-strand RNAs associated with the particles. As the minus-strands are elongated, the dsRNA product is simultaneously packaged into cores contained in the SVP preparation. Based on the correlation between the presence of nonstructural proteins and the ability of cell-free systems to package RNA, it is possible to infer that some of the nonstructural proteins are required for this process.

Additional evidence that the nonstructural proteins have a role in genome packaging comes from studies of *tsE*(*1400*), a mutant of simian SA11 rotavirus with a temperature-sensitive lesion mapping to the gene for NSP2 (Ramig, 1982; Ramig and Petrie, 1984). At the nonpermis-

sive temperature (39 °C), despite the production of viral proteins, *tsE*-infected cells are defective in the production of viral ssRNA and dsRNA. Most remarkable, however, is the observation that virus particles formed in *tsE*-infected cells at the nonpermissive temperature are mostly empty (Chen et al., 1990; Ramig and Petrie, 1984). This finding indicates that successful packaging of viral RNA into progeny virions requires functional NSP2.

3. NSP2

NSP2 (35 Kda) is a highly conserved basic protein that is expressed at high levels in the infected cell where it localizes to VIBs (Petrie et al., 1984). Sedimentation analysis of NSP2, either derived from infected cell extracts or by expression from recombinant cDNAs, has shown that the protein exists not as a monomer but rather as a homo-multimer (Kattoura et al., 1994; Taraporewala et al., 1999). Determination of the solution structure of this multimer by equilibrium and velocity centrifugation has established that NSP2 self-assembles to form stable octamers (Schuck et al., 2001). This finding was confirmed when NSP2 was crystallized as an octamer with 4-2-2 symmetry (Jayaram et al., 2002). The structure of the NSP2 octamer was determined to a resolution 2.6 Å and revealed to have a doughnut-shaped structure with a 35 Å central hole formed by the head-to-head stacking of two tetramers (reviewed by Hariharan et al. Chapter 7 this volume).

The NSP2 octamer possesses multiple activities. These include the ability to bind ssRNA nonspecifically and cooperatively, a characteristic that allows the protein to interact with RNA to form higher-order RNA protein complexes (Kattoura et al., 1992; Taraporewala et al., 1999). In addition, NSP2 octamers possess helix-destabilizing activity that can be observed upon incubation of the protein with RNA–RNA duplexes containing single-stranded overhangs. The helix-destabilizing activity (strand displacement activity) of NSP2 is Mg^{2+} and ATP-independent, and is unlike that of typical helicases (Taraporewala and Patton, 2001). NSP2 also has an associated Mg^{2+}-dependent NTPase activity that can hydrolyze any of the four NTPs to NDPs (Taraporewala et al., 1999). Assays performed in vitro with purified recombinant NSP2 and radiolabeled NTPs have indicated that the hydrolysis activity results in the phosphorylation of NSP2. Interestingly, NSP2 recovered from infected cells lacks any detectable phosphate groups. Hence, the phosphate groups generated by the NTPase activity in vivo may be rapidly transferred from NSP2 to another viral protein or removed by cellular phosphatases.

The addition of nucleotides and ssRNA to NSP2 stabilizes the octamer (Schuck et al., 2001). Specifically, sedimentation analysis has shown that the NSP2 octamer is less susceptible to Mg^{2+} induced disassembly into tetramers in the presence of nucleotides and ssRNA. Moreover, the binding of the nucleotides (but not RNA) to the octamer induces a conformational shift to a more compact form as seen by a small but significant increase in the rate of sedimentation (Schuck et al., 2001). Overall, the biochemical activities associated with NSP2 and the observation that the protein undergoes ligand-induced conformational change is consistent with a role for NSP2 in genome packaging.

Elucidation of the atomic structure of NSP2 has revealed that the probable sites involved in NTP binding and hydrolysis, and RNA binding are distinct and have little overlap (Jayaram et al., 2002). The NSP2 monomer has two structural domains, separated by a 25 Å deep cleft (Fig. 1). The C-terminal domain displays a prominent twisted

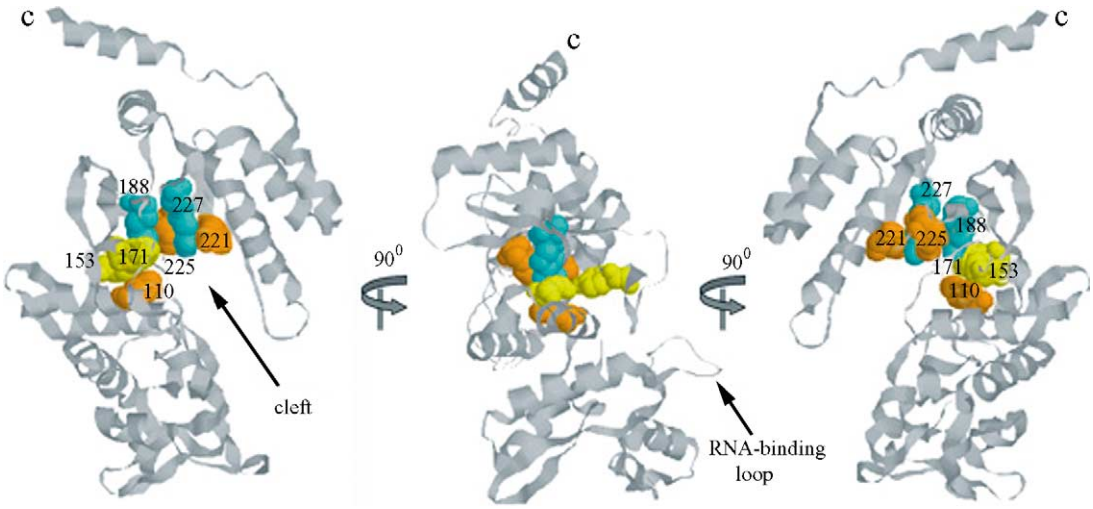

Fig. 1. Structure of the NSP2 monomer. Ribbon model of the x-ray structure of the monomeric unit of NSP2 at a resolution of 2.6 Å. The figure depicts the α-carbon backbone of the NSP2 monomer with the side-chains of the residues in the 25 Å-deep cleft that are involved in nucleotide-binding and Mg^{2+} coordination indicated in space-filling model. Among the residues implicated in nucleotide binding, the HIT motif residues (H110, H221, H225) are colored orange while the basic residues (K188, R227) are colored cyan. The residues involved in Mg^{2+} coordination (E153, Y171) are colored yellow. RNA binding is proposed to occur within a basic 24-residue loop (52–76) near the N-terminus of the monomer.

anti-parallel β-sheet flanked by α-helices, which exhibits a HIT (histidine triad)-like fold that is commonly found among nucleotidyl hydrolases. Based on structural similarity with other HIT-proteins, in particular protein kinase C interacting protein (PKCI), a prototypical member of the HIT family of nucleotidyl hydrolases, the NTP-binding site has been proposed to be located within this cleft. In NSP2, the three histidine residues (the histidine triad, His 221, His

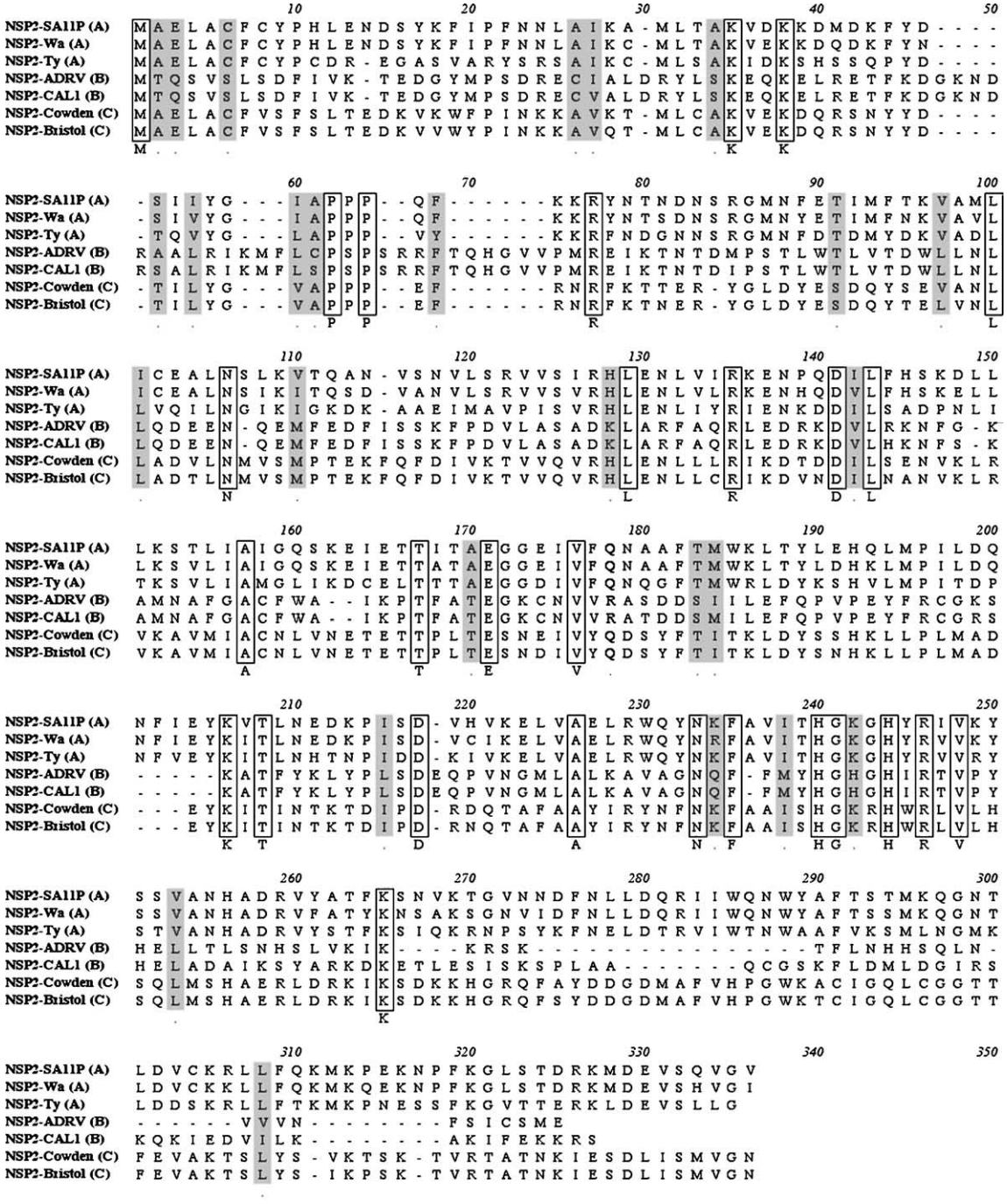

Fig. 2. Alignment of amino acid sequences of NSP2 derived from group A, group B and group C rotaviruses. Sequences of NSP2 derived from group A rotaviruses, simian strain, SA11-P (Genbank Accession number: AAA47298), human strain, Wa (Genbank Accession number: AAA47301), avian turkey strain, Ty (Genbank Accession number: AAA47300); group B rotaviruses: human strains, ADRV (Genbank Accession number: AAA47328) and CAL-1 (Genbank Accession number: AAF72868); and group C rotaviruses; bovine strain, Cowden (Genbank Accession number: CAA46742), and human strain, Bristol (Genbank Accession number: AJ132205), were aligned with the ClustalW program(Thompson et al., 1994, Nucleic Acids Res., 22, 4673–4680) of MacVector 6.5 (Oxford Molecular). Amino acids that are shaded and that are boxed indicate sequence similarity and identity, respectively. His 110 (residue 128 in the alignment), His 221 (residue 240) and His 225 (residue 244) have been proposed to make up the putative HIT motif involved in NTP binding and hydrolysis.

225 and His 110) and a cluster of basic residues (Arg 227, Lys 188), located at the base of the cleft, are probably involved in NTP binding and hydrolysis (Fig. 1). The cofactor for the NTPase activity, Mg^{2+}, is believed to be coordinated by the side-chain carboxyl- and hydroxyl-oxygen atoms of Glu 153 and Tyr 171, respectively. His 225, the putative catalytic residue for NTP hydrolysis, is strictly conserved in NSP2 of group A, B and C rotaviruses, as is His 221. His 110 is conserved among group A and C viruses but not in group B, where it is replaced by a similarly charged lysine (Fig. 2). The conservation of residues that make up the HIT motif in NSP2 and other basic residues in the cleft where NTP binding occurs suggest that the NTPase activity is associated with NSP2 encoded by all rotavirus strains.

The N-terminal domain of NSP2 is largely α-helical and exhibits no structural similarity to any known protein structure. Between the two sub-domains within the N-terminal domain lies a 24-residue highly basic loop (Fig. 1), which lines four prominent grooves that form diagonally across the two-fold axes of the outer surface of octamer. Based on the relative concentration of charged residues on the surface of the octamer, these grooves were proposed to be the sites where ssRNA binds (Jayaram et al., 2002). In this model, the RNA would wrap around the octamer and hence, any conformational change induced by ligand binding would affect the RNA–protein interaction.

The octamer is the functional form of NSP2 and the form required for productive virus replication as shown by the structural and biochemical analysis of NSP2 of *tsE*(*1400*) (Taraporewala et al., 2002). Loss of the ability of NSP2 to form stable octamers at the nonpermissive temperature interferes with all the known biochemical activities of the protein. How the octamer functions to facilitate packaging is yet unclear.

4. NSP5

NSP5, the larger of the two proteins encoded by segment 11, is a highly acidic protein, rich in serine and threonine residues, that forms dimers (Poncet et al., 1997). In the infected cell, NSP5 undergoes O-linked glycosylation (Gonzalez and Burrone, 1991) and exists in both hypo- and hyper-phosphorylated forms (Welch et al., 1989). Phosphorylation of the protein at serine residues results in isoforms ranging in size from 26 to 32–34 kDa (Welch et al., 1989; Afrikanova et al., 1996; Poncet et al., 1997; Blackhall et al., 1997). Protein-protein cross-linking of infected cell lysates has shown that NSP5 forms complexes with VP1 and NSP2 (Kattoura et al., 1992; Afrikanova et al., 1998). The interaction of NSP5 with NSP2 has been confirmed using yeast two-hybrid assays (Poncet et al., 1997). Unlike NSP2, which has a poor affinity for dsRNA, NSP5 efficiently binds both ss and dsRNA nonspecifically (Vende et al., 2002). Like VP2, the protein accumulates in VIBs in the infected cell (Petrie et al., 1984). By transient expression of viral proteins in uninfected cells, Fabbretti et al. (1999) were able to provide strong evidence that NSP5 and NSP2 are necessary and sufficient for the formation of VIBs. Such inclusions were not detected if either protein was expressed alone in uninfected cells.

The molecular basis of the phosphorylation of NSP5 has been the subject of many studies (Afrikanova et al., 1996; Poncet et al., 1997; Blackhall et al., 1998; Afrikanova et al., 1998; Torres-Vega et al., 2000; Eichwald et al., 2002). Despite these efforts, the source of the kinase activity responsible for the hyperphosphorylation of NSP5 has not been fully resolved. Several studies have suggested that NSP5 possesses a low level of autokinase activity. However, this activity has been shown to account for only a small fraction of the total phosphorylation that NSP5 undergoes in infected cells, thereby suggesting that other viral proteins and/or cellular proteins, e.g., phosphatases and protein kinases, also affect the phosphorylation of NSP5. Poncet et al. (1997) have provided evidence indicating a linkage between hyperphosphorylation of NSP5 and localization of the protein in VIBs. Because VIB formation is dependent upon the interaction of NSP2 and NSP5, these investigators proposed that NSP2 plays a role in the hyperphosphorylation of NSP5. This proposal is in fact supported by the results of two recent studies. One of these studies, carried out by Afrikanova et al. (1998), showed that the transient expression of NSP2 and NSP5 in uninfected cells results in the hyper-phosphorylation of NSP5, to an extent comparable to that seen for the protein in infected cells. Such was not the case when NSP5 was expressed by itself. The second study by Vende et al. (2002) showed that incubation of purified recombinant NSP2 with NSP5 in an in vitro phosphorylation assay causes an enhancement in the extent of NSP5 phosphorylation.

The mechanism by which NSP2 could mediate the hyperphosphorylation of NSP5 is unknown. One possibility is that the interaction of NSP2 and NSP5 results in the activation of an inherent (auto) kinase activity of NSP5. Alternatively, the NTPase activity of NSP2 could generates phosphate moieties which are transferred from NSP2 to NSP5 (Taraporewala et al., 1999; Vende et al., 2002). The latter explanation is supported by the observation that phosphorylated NSP2 can be detected when transiently expressed in vivo but not in infected cells where both NPS2 and NSP5 are present (Taraporewala et al., 1999).

In addition to studies providing results that NSP2 plays an important role in NSP5 phosphorylation, other studies have provided results that cellular kinases and phosphatases contribute to the protein's phosphorylation (Blackhall et al., 1997; Eichwald et al., 2002). For example, Blackhall et al. (1998) have shown that the presence of staurosporine, a strong inhibitor of protein kinase C and a wide range of protein kinases, decreases the extent of NSP5 phosphorylation in the infected cell. These investigators also noted that the addition of the phosphatase inhibitor, okadaic acid, to

infected cells results in an increase in the phosphorylated isoforms of NSP5. From these data, it was concluded that NSP5 may cycle between hyper- and hypo-phosphorylated forms in infected cells via the action of cellular kinases and phosphatases. Notably, Eichwald et al. (2002) have also provided results raising the possibility that NSP5 phosphorylation is mediated by a cellular kinase, but that the activation of this kinase is dependent on NSP2. Mapping of the phospho-acceptors in NSP5 that are targets of the kinase activity indicate that they reside in the C-terminal region of the protein. Interestingly, this same region is involved in NSP5 dimerization, NSP6 interaction, and the formation of VIBs (Eichwald et al., 2002).

5. Importance of NSP2-NSP5 interaction

Why NSP5 exists in many phosphorylated forms is not known but the ability of the protein to cycle between the forms may be critical for the function of NSP2. Because NTP hydrolysis by NSP2 is correlated with increased phosphorylation of NSP5 (Vende et al., 2002), it may be proposed that the transfer of the phosphate groups from NSP2 to NSP5 may allow the NSP2 octamer to move from a transient phosphorylated state to a nonphosphorylated state. In this scenario, once dephosphorylated the NSP2 octamer is free to again bind a molecule of NTP. As described above, NTP binding by NSP2 induces a conformational shift in the octamer structure. NTP binding would be followed by NSP2-catalyzed NTP hydrolysis, autophosphorylation, and dephosphorylation coupled with NSP5 hyperphosphorylation. Though speculative, repeated cycling of the NSP2 octamer from the loose to the compact structure accompanied by NTP hydrolysis could provide the mechanical force necessary to overcome the entropy of packaging eleven viral mRNAs into core-like structures.

The hyperphosphorylation of NSP5 is dependent upon dimerization of the protein (Torres-Vega et al., 2000). Since NSP5 requires NSP2 to accumulate in VIBs and localization of NSP5 in VIBs is correlated with hyperphosphorylation of the protein, it is reasonable to assume that it is the dimeric form of NSP5 that interacts with the NSP2 octamer. The surface of the NSP2 octamer in the region where the two tetramer rings stack is highly basic (Jayaram et al., 2002) and, therefore, could serve as the site of interaction with the highly acidic NSP5 protein. A part of this region in the NSP2 octamer is also the presumed site for ssRNA binding (Jayaram et al., 2002). Since both, NSP2 and NSP5 bind ssRNA (Kattoura et al., 1992; Taraporewala et al., 1999; Vende et al., 2002), and can be co-immunoprecipitated only when infected cells are exposed to UV-light under which RNA–protein cross-links are formed, (Poncet et al., 1997; Afrikanova et al., 1998), it is possible that the NSP2 and NSP5 proteins can interact, such that RNA is juxtaposed between them.

In addition to binding ssRNA, NSP5 also binds dsRNA (Vende et al., 2002). Since rotavirus RNA replication is coordinated with packaging of the mRNA templates into RIs which are structurally similar to cores or DLPs, the VP2 and/or VP6 shells of protein would seemingly preclude the physical interaction of NSP5 and other nonstructural proteins with dsRNA contained in the core of the RIs. If not able to interact with the dsRNA genome, then what is the purpose of the dsRNA-binding activity of NSP5? It is conceivable that NSP5, via its dsRNA-binding activity, is responsible for guiding NSP2, which has helix-destabilizing activity but poor dsRNA-binding activity, to regions within the viral mRNA with secondary structures that need to be destabilized and unfolded for packaging to occur (Vende et al., 2002). The interference posed by higher order structures within plus-strand mRNAs during packaging was demonstrated by Qiao et al. (1995) using an in vitro packaging system for ϕ6, a bacteriophage with a genome of three segments of dsRNA. The fact that genome packaging for ϕ6 also involves movement of plus-strand mRNAs into $T = 1$ cores indicates that the mechanism of packaging used by this phage may be quite similar to that of the viruses of the Reoviridae.

6. NSP6

The smaller protein (11 kDa) encoded by an alternative open reading frame of segment 11 is NSP6 (Mattion et al., 1991). NSP6 localizes to VIBs (Mattion et al., 1991) and interacts with NSP5 (Torres-Vega et al., 2000; Gonzalez et al., 1998), properties that raise the possibility that NSP6 is involved in genome replication or packaging. The C-terminal region of NSP5 that interacts with NSP6 is also the region of NSP5 required for dimerization; therefore, NSP6 may influence the formation of NSP5 dimers (Torres-Vega et al., 2000). And since dimerization of NSP5 affects the hyper-phosphorylation of the protein, NSP6 may indirectly control the phosphorylation status of NSP5 (Torres-Vega et al., 2000). The low level of expression of NSP6 in rotavirus infected cells is consistent with a regulatory role for the protein. Some rotavirus group A strains, such as Mc323 and Alabama, and all group C strains do not encode an NSP6 (Torres-Vega et al., 2000). This means that NSP6 may not be required for rotavirus replication or that either a viral or a cellular protein in these strains functionally substitutes for NSP6.

7. Homologs of NSP2 and NSP5 for reovirus and bluetongue virus (BTV)

Rotavirus NSP2 and NSP5 share some interesting parallels with proteins encoded by the reovirus and bluetongue virus (BTV), members of the orthoreovirus and orbivirus genera, respectively, of the Reoviridae. Because of the

structural similarity of the capsids of these viruses and the similarities in their pathways of replication, it is reasonable to predict that rotavirus, reovirus, and BTV encode for proteins with homologous functions. However, identifying such homologs, particularly for the nonstructural proteins, is complicated by the lack of any detectable sequence homology between the proteins made by these viruses. Despite this limitation, analysis of the BTV protein NS2 (41 kDa) and the reovirus protein σNS (41 kDa) suggest that they may be the homologs of rotavirus NSP2. All of these proteins have been implicated in viral genome replication and/or packaging (Gillian and Nibert, 1998; Huismans and Joklik, 1976; Gomatos et al., 1980, 1981; Thomas et al., 1990).

Like rotavirus NSP2, BTV NS2 and reovirus σNS are expressed in high levels in the infected cell where they localize to VIBs (Brookes et al., 1993; Thomas et al., 1990, Becker et al., 2001). The proteins are found associated with viral mRNA during replication (Antczak and Joklik, 1992; Huismans et al., 1987; Helmberger-Jones and Patton, 1986) and, as with rotavirus NSP2, σNS was also found associated with particles that had replicase activity (Gomatos et al., 1980). The three proteins, rotavirus NSP2, BTV NS2 and reovirus σNS, share two common and distinctive features that may be critical for their role in genome replication and/or packaging. (I) Each protein forms homomultimers made up of ∼ 4–8 subunits (Gillian and Nibert, 1998; Uitenweerde et al., 1995). (II) The homomultimers preferentially bind ssRNA in a manner that results in the formation of large RNA–protein complexes (Uitenweerde et al., 1995; Theron and Nel, 1997; Gillian et al., 2000), most likely due to the binding of multiple multimeric units to a single RNA molecule. As a consequence of their saturative and high affinity binding to ssRNA, NSP2 and σNS have been reported to possess passive strand displacement activity, that unlike typical helicases, is Mg^{2+}- and ATP-independent (Gillian et al., 2000; Taraporewala et al., 2001). The strand displacement activity is predicted to confer on these proteins the ability to remove secondary structures in the mRNA templates that would, otherwise, impede RNA packaging into cores and subsequent mRNA to dsRNA replication. In some respects, NSP2 and σNS mimic functionally the single-stranded DNA-binding proteins (SSBs), a ubiquitous and highly conserved group of proteins which possess strand-displacement activity (Boehmer and Lehman, 1993; Monaghan et al., 1994; Tsurumi et al., 1998; Hosoda et al., 1974). The SSBs bind and keep the ssDNA at the replication fork unwound, and thereby, assist in the processivity of DNA replication (Kornberg and Baker, 1992). BTV NS2 also possesses strand displacement activity though the specific activity appears to be much less than that of rotavirus NSP2 (Taraporewala and Patton, unpublished data). One explanation for this observation is that BTV NS2 when not bound to ssRNA is more polydisperse than rotavirus NSP2 and therefore, is less stable as a multimeric unit (Taraporewala and Patton, unpublished data).

BTV NS2 and rotavirus NSP2 are involved in NTP binding and hydrolysis (Taraporewala et al., 1999; Horscroft and Roy, 2000; Taraporewala et al., 2001). Both proteins hydrolyze all of the four NTPs; however, there are fundamental differences in the nature of this activity. NS2 has a strong nucleotidyl phosphatase activity that cleaves all three phosphodiester bonds (α, β and γ) of the NTP, while NSP2 can cleave only the γ-phosphodiester bond (NTPase activity) (Taraporewala et al., 2001). Comparison of the primary amino acid sequences of NS2 from diverse orbivirus strains has shown no obvious conservation of histidine residues that could potentially represent an NTP-binding HIT-motif, similar to that of rotavirus NSP2 (Jayaram et al., 2002). Also, unlike NSP2, NTP hydrolysis catalyzed by NS2 does not result in autophosphorylation of the protein, though in infected cells, NS2 but not NSP2 can be detected as a phosphoprotein (Taraporewala et al., 2001, Theron et al., 1994; Devaney et al., 1988; Taraporewala et al., 1999). BTV NS2 is phosphorylated when expressed in insect cells (Thomas et al., 1990) and recombinant BTV NS2 is phosphorylated in vitro when incubated with uninfected cell lysates (Theron et al., 1994; Taraporewala et al., 2001). Hence, the phosphorylation of NS2 in vivo may be due to the action of cellular kinases.

Reovirus σNS has no detectable activity that hydrolyzes the phosphodiester bonds of NTPs nor is there any evidence that the protein undergoes phosphorylation (Gillian et al., 2000). However, the protein does interact with another viral protein, μ2, which has affinity for ssRNA and is the determinant of the NTPase activity associated with reovirus cores (Brentano et al., 1998; Noble and Nibert, 1997). σNS is also dependent on μ2 for the formation of VIBs in the cell (Becker et al., 2001; Mbisa et al., 2000), a phenomena paralleling the cooperative role noted for rotavirus NSP2 and NSP5 in the formation of VIBs. In contrast, BTV NS2 alone can trigger the formation of VIBs in the cell (Thomas et al., 1990). From these data, it can be predicted that functional domains are conserved among proteins encoded by the various viruses of the Reoviridae, but that these domains may be distributed differently among sets of viral proteins. As an illustration, reovirus σNS-μ2 in combination seem to have the same activities as rotavirus NS2-NSP5 and these pairs of proteins seem to function as homologs of BTV NS2.

8. P4, a potential NSP2 homolog of the segmented dsRNA phage ϕ6

Another possible homolog of NSP2 is the packaging protein P4 encoded by ϕ6. A well defined in vitro packaging and replication system has been reconstituted for this phage from purified recombinant proteins (Gottlieb et al., 1991). The successful development of a in vitro packaging system for ϕ6 and lack thereof for viruses of the Reoviridae may be partially attributed to the fact that the process of nucleocapsid assembly and RNA packaging for ϕ6 does not require

the participation of nonstructural proteins (Butcher et al., 1997). P4, an important component of the ϕ6 in vitro packaging system, is a structural 35 kDa protein with nonspecific NTPase activity (Gottlieb et al., 1992; Paatero et al., 1995). Plus-strand packaging in ϕ6 is dependent on the energy provided by NTP hydrolysis (Gottlieb et al., 1991).

P4 forms stable hexamers that are associated with the ϕ6 core at the five-fold axis of the P1 icosahedral shell (de Haas et al., 1999). The mismatch between the six-fold symmetry of the P4 hexamer and the five-fold symmetry of the P1 shell has been suggested to generate a vertex that functions in RNA packaging in a manner akin to the role that the portal vertex plays in DNA packaging of dsDNA phages (de Haas et al., 1999). The enzymatic similarity of the ϕ6 P4 and rotavirus NSP2 proteins allows the speculation that for rotavirus, packaging of viral mRNA into cores may be facilitated by a mismatch between the four-fold symmetry of the NSP2 octamer positioned at each of the five-fold vertices of the VP2 shell. Hypothetically, the threading of the viral mRNA templates through these portals would be followed by the synthesis of the minus-strand RNA by the viral polymerase, VP1, that is positioned at the inner surface of the VP2 shell at each of the vertices, thus, coordinating the events of packaging and minus-strand synthesis. For rotavirus, the complete packaging of all eleven plus-strand RNA templates is not required for dsRNA synthesis to commence. This is in contrast to ϕ6, where expansion of the cores following complete packaging of all three plus-strand RNA templates stimulates minus-strand synthesis by the polymerase (Zeng et al., 1996; Frilander et al., 1992, 1995; de Haas et al., 1999).

It is interesting to note that virion-derived core particles or core-like particles made with recombinant VP2 with or without VP1 and VP3, exhibit extreme insolubility and tend to aggregate (Zeng et al., 1996; Patton et al., 1997) similar to the behavior observed for VLPs made with the P1 protein of ϕ6 (Butcher et al., 1997). The solubility of the recombinant P1 particle increases when co-expressed with P4 protein (Butcher et al., 1997). This observation raises the question whether NSP2, which exhibits very high solubility as a recombinant protein or NSP5 which is highly acidic, could alter the physical and structural properties of the rotavirus core making it more amenable for genome packaging. Once packaging is accomplished, the nonstructural proteins must be displaced by VP6, such that further assembly of the virion can proceed (Patton and Gallegos, 1988).

Despite some lines of evidence that are consistent with the idea that NSP2 acts as homolog of the P4 protein, in fact, no data has yet been found that NSP2 interacts with any of the structural proteins of assembled core proteins. Absence of such data raises significant questions as to the possibility that NSP2 is indeed the functional equivalent P4. However, it is possible that it is not NSP2 alone that associates with the core structural proteins, but the interaction of NSP2 with NSP5 forms a complex that binds to cores in such a way that mimics the P4 protein.

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