Characterization of rotavirus guanylyltransferase activity associated with polypeptide VP3

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Rotaviruses transcribe mRNA containing a 7mGpppGmp cap at the 5' end in vitro. Guanylyltransferase activity associated with the viral particle was detected by SDS–PAGE due to the formation of a nucleotide–enzyme complex when the virus was incubated with [γ-32P]GTP. Using purified viral particles it was shown that only the core polypeptide VP3 exhibits the ability to form a complex with the nucleotide. The reaction is specific for GTP or dGTP when Mg2+ is used as a cofactor. The reaction also depends on the incubation temperature and the pH, as described for other guanylyltransferases. The GMP–VP3 complex transfers the GMP to pyrophosphate, synthesizing GTP or GDP, resulting in the formation of a GpppG cap. These properties of the complex allowed the core polypeptide VP3 to be identified as the rotavirus guanylyltransferase.

Introduction

Rotaviruses, a genus of the Reoviridae family, have been associated with acute diarrhoea in a variety of animals including humans (Wyatt et al., 1978; Estes et al., 1983). The viral particle is composed of a double-shelled capsid surrounding the viral core. Recent observations have shown that the surface of the outer capsid contains mainly VP7 and projections consisting of dimers of VP4; the inner capsid is composed of VP6 molecules (Novo & Esparza, 1981; Prasad et al., 1988, 1990). The viral core contains the genome which consists of 11 dsRNA segments encoding at least 12 polypeptides (McCrae & McCorquodale, 1982; Suzuki et al., 1984; Mason et al., 1983). Each of the double-stranded viral RNA segments contains conserved sequences at both ends of the molecule and a cap at the 5' end of the plus strand (Imai et al., 1983a, b). The origin of the cap in the double-stranded segment seems to be due to the replication mechanism of rotaviruses, which utilizes as template viral mRNA which already contains a 7mGpppGmp cap (Acs et al., 1971; Spencer & Garcia, 1984; Patton, 1987).

Rotavirus genomes may be transcribed in vitro after activation of the viral particle by either incubation with low EDTA concentrations or a heat shock (Cohen et al., 1979; Spencer & Arias, 1981). The activated virus particles can synthesize RNA containing a capped end from each of the 11 genes. These transcripts are identical to those identified in infected cells and can be translated in vitro into viral polypeptides (Mason et al., 1980; Stacy-Phipps & Patton, 1987; Sandino et al., 1988).

Several reports have suggested that the virus particle contains three enzyme activities directly related to transcription: an RNA polymerase, a guanylyltransferase and an ATPase that may also be involved in replication of the viral genome (Spencer & Garcia, 1984; Flores et al., 1982; Gallegos & Patton, 1989). The assignment of viral structural polypeptides to these activities is not yet clear. It has been proposed that each of the activities belongs to one of the four polypeptides of the single-shelled particle, which are associated with the core or inner capsid (Bican et al., 1982).

The rotavirus inner core contains three polypeptides, VP1, VP2 and VP3. VP1 has been suggested to be the RNA polymerase, based on analysis of the sequence of gene 1, encoding VP1 (Cohen et al., 1989), whereas VP3 seems to be associated with transcription (A. M. Sandino, unpublished results). Recently, VP3 has been shown to have GTP-binding activity similar to the properties of guanylyltransferases, and it has also been suggested that it is required during dsRNA synthesis (Gallegos & Patton, 1989; Fukuhara et al., 1989). The polypeptides of the outer capsid are not related to transcription or replication in vitro or in vivo (Fukuhara et al., 1988; Sandino et al., 1986; Gallegos & Patton, 1989).

In order to identify VP3 as the rotavirus polypeptide
with guanylyltransferase activity, an assay testing for the formation of complexes between GMP and the enzyme was used (Shuman & Hurwitz, 1981; Cleveland et al., 1986). Complex formation has become a specific assay for guanylyltransferase activity because it is the only enzyme known to bind a nucleotide covalently and transfer to a nucleotide acceptor containing a 5' diphosphate terminal (Shuman, 1982). In contrast to any other nucleotide-protein complex, the guanylyltransferase-GMP complex may transfer the GMP to an acceptor such as a nucleoside di- or triphosphate, an oligonucleotide containing a di- or triphosphate at the 5' end or to pyrophosphate, resulting in GTP synthesis (Cleveland et al., 1986). The purpose of the present communication is to characterize the properties of the rotavirus VP3 polypeptide as those of the viral guanylyltransferase initially identified by Fukuhara et al. (1989).

Methods

Virus purification. Human rotaviruses were obtained from stool samples of infants with acute diarrhoea and purified by flocc extraction and ultracentrifugation through a linear 25 to 35% sucrose gradient (Spencer & Arias, 1981). The electrophoretype of each isolate was characterized and viruses of the long electrophoretype (L) were used. The purity of the isolate was also determined by electron microscopy. Simian rotavirus SA-11 was obtained by infection of monolayers of MA-104 cell cultures and purified as previously described (Sandino et al., 1988). The RNA polymerase activity of each isolate was determined (Cohen et al., 1979).

Preparation of subviral particles. Single-shelled particles were obtained by EDTA treatment of double-shelled particles and cores were prepared by incubation with 0.5 mM-CaCl₂ as previously described (Sandino et al., 1986). The biological activity of both viral particles was determined by assaying their RNA polymerase activity (Spencer & Arias, 1981).

Protein determination. Viral protein concentration was determined as described previously (Bradford, 1976) using the Bio-Rad protein assay.

GMP binding assay. Purified viral particles were assayed for nucleotide binding in a 12 μl reaction mixture containing 50 mM-Tris-HCl pH 7.9, 15 mM-MnCl₂, 1 mM-MgCl₂, 1 μCi [³²P]GTP (specific activity 800 Ci/mmol) and 0.1 μg purified viral particles. After incubation for 20 min at 37 °C, EDTA was added to a final concentration of 10 mM. PAGE and processing for autoradiography were as described above.

Reversibility assay of the nucleotide-binding reaction. Virus-[³²P]GMP complex was prepared essentially as described above but the addition of EDTA was omitted. After the reaction mixture had been incubated at 37 °C for 20 min, the complex was washed three times by centrifugation with 3 volumes of 50 mM-Tris-HCl pH 7.9 and pelleted. The virus-[³²P]nucleotide complex was resuspended in 50 mM-Tris-HCl pH 7.9, 15 mM-MnCl₂ and increasing amounts of sodium pyrophosphate (5 to 25 mM) or GDP (3 to 15 mM) were added to the incubation mixture, which was kept at 37 °C for 30 min. The reaction mixture was then pelleted and the supernatant was subjected to polyethyleneimine (PEI)-cellulose thin-layer chromatography. Chromatography was done with 0.8 M-acetic acid, 0.9 M-LiCl. Plates were washed with ethanol, dried and exposed to an X-Omat Kodak film. The reversibility of the reaction was determined by the presence of a radioactive band that comigrated with GTP or GpppG.

Results

GMP binding to viral polypeptides

In order to determine which of the viral polypeptides was able to bind GMP from GTP, SA-11 double-shelled particles were incubated at 37 °C for 20 min in a reaction mixture containing 50 mM-Tris–HCl pH 7.9, 15 mM-MgCl₂ and increasing amounts of [³²P]GTP and then subjected to SDS–PAGE. As shown in Fig. 1, the viral particles contained only one polypeptide with the ability to form a complex with GTP that migrated in the position of VP3 or VP4. SA-11 single-shelled and core particles were able to bind the nucleotide in the same way as complete virions (data not shown), suggesting that VP4 is not the viral polypeptide involved in nucleotide binding. These results eliminated the possibility that the ability to form the complex could be associated with a component of the outer capsid, such as VP4 or VP7, or the inner capsid, which is composed of VP6 (Sandino et al., 1986; Liu et al., 1988). When a similar experiment was carried out using a human rotavirus isolate, a similar activity was observed. In the autoradiogram in Fig. 2 a similar viral polypeptide with the ability to bind GMP was observed migrating with an apparent Mr of 84K. The intensity of the bound radioactivity depended on the amount of virus added to the reaction and this suggested that nucleotide binding was limited by the availability of the polypeptide acceptor.

In Fig. 3 an autoradiogram of the time course of the reaction is shown. An increase in the radioactivity associated with VP3 up to 60 min of incubation was observed, suggesting that the acceptor capacity of VP3 in complete virions was not a condition limiting the reaction over short periods of incubation, such as 20 min. The results also suggested that during these incubation periods there was no detectable degradation of the nucleotide–VP3 complex.
Rotavirus guanylyltransferase

1 2 3 4 5 6

Fig. 1. PAGE of the covalent nucleotide-polypeptide complex. Complex formed by incubating purified double-shelled SA-11 particles (0.1 μg) in 50 mM-Tris-HCl pH 7.9 containing 15 mM-MgCl₂, with 2, 4, 6, 8 or 10 μCi [α-32P]GTP (lanes 2 to 6), for 20 min at 37 °C. The reactions were then processed as described in Methods. In lane 1, the electrophoretic mobility of simian rotavirus SA11 35S-labelled polypeptides is shown.

The pH optimum of the reaction had a narrow range close to pH 7.9 and pH values lower than 6.5 and higher than 8.5 inhibited complex formation (data not shown). An incubation temperature of between 30 and 45 °C was optimal for the binding of GTP to VP3 (data not shown).

Nucleotide specificity

One of the characteristics of the reactions involving nucleotides is their requirement for divalent cations as cofactors. Complex formation requires the presence of 15 mM-Mg²⁺ or 10 mM-Mn²⁺. In the range of concentrations studied using several [α-32P]nucleoside triphosphates. Of bind the nucleotide (data not shown).

Once the requirement for divalent cations had been determined, the specificity for complex formation was studied using several [α-32P]nucleoside triphosphates. Of all those used, only GTP and dGTP were able to form a complex with human rotavirus VP3 in the presence of Mg²⁺ (Fig. 4a). Complex formation was specific for VP3, even in the presence of other nucleoside triphosphates. The results were different when Mn²⁺ was used.

Fig. 2. Autoradiogram of PAGE of the covalent nucleotide-polypeptide complex formed with increasing amounts of human rotavirus isolate L. Increasing amounts of purified human rotavirus particles were incubated for 20 min at 37 °C in a 50 mM-Tris-HCl pH 7.9 containing 15 mM-MgCl₂ and 1 μCi [α-32P]GTP, and subjected to autoradiography. Lane 1, 0.1 μg; lane 2, 0.2 μg; lane 3, 0.4 μg; lane 4, 0.8 μg complete virus particles.

Fig. 3. Time course of the nucleotide-binding reaction. Human rotavirus isolate L (0.15 μg) was incubated in 50 mM-Tris–HCl pH 7.9 containing 15 mM MgCl₂ and 1 μCi[α-32P]GTP at 37 °C for 0, 5, 10, 20, 45, 60 or 75 min (lanes 1 to 7). The samples were processed as described.
Fig. 4. Specificity of the nucleotide-binding reaction for nucleosides and guanosine-2',3'-dialdehyde 5'-triphosphate (GTP-IO₄). (a) Human rotavirus L was incubated with different labelled nucleoside triphosphates (lane 2, GTP; lane 3, dCTP; lane 4, dGTP; lane 5, dATP; lane 6, UTP; lane 7, CTP), or in their absence (lane 1). (b) Human rotavirus L was incubated with increasing amounts of [α-³²P]GTP-IO₄. Lane 1, nucleotide-binding reaction in the absence of human rotavirus L; lane 2, nucleotide-binding reaction in the presence of human rotavirus L and [α-³²P]GTP as a control; lanes 3 to 6, human rotavirus L incubated with 5, 10, 15 or 20 μCi GTP-IO₄, as cofactor; in this case, all the nucleotides were able to form a complex with VP3 (data not shown).

Another method for the study of polypeptides that interact with nucleotides is the use of [α-³²P]nucleoside-2',3'-dialdehyde 5'-triphosphates. These analogues form stable complexes with any nucleotide-binding polypeptide via their reactive aldehyde groups. The complex can be identified by its mobility in SDS-PAGE and comparison with the respective polypeptide (Chang et al., 1981). In Fig. 4(b) the binding of guanosine-2',3'-dialdehyde 5'-triphosphate to SA-11 rotavirus polypeptides is shown. Increasing amounts of nucleotide analogue were incubated with purified virions in 50 mM-HEPES buffer pH 7.9 in the presence of 15 mM-Mg²⁺ for 30 min at 37 °C. The results showed that the only rotavirus polypeptide to which the nucleotide bound to form a stable complex was VP3; the nature of this interaction may be different from that with GTP.

Effect of pyrophosphate, phosphate and DTT on complex formation

Other guanylyltransferases identified in different viruses and eukaryotic cells have been shown to be strongly stimulated by the addition of reagents that protect disulphide bonds (Monroy et al., 1978). As seen in Fig. 5, the addition of between 1 and 4 mM-DTT was inhibitory.

The effects of phosphate and pyrophosphate were also studied, based on the observation that pyrophosphate is able to release the GMP residue from the nucleotide–polypeptide complex of the guanylyltransferase leading to GTP formation (Shuman, 1982). In Fig. 5 the effect of the addition of pyrophosphate on complex formation was studied and concentrations as low as 0.1 mM-sodium pyrophosphate were seen to have a strong inhibitory effect. Phosphate (pH 7.9) also had an inhibitory effect but at 1000-fold greater concentrations; complexes were still detected in the presence of 50 mM-phosphate. This suggests that pyrophosphate may be acting as a nucleotide acceptor by inducing degradation of the complex, so driving the equilibrium of the reaction towards the formation of nucleoside triphosphate.
Effect of enzymes and 5'-acceptor molecules on complex stability. Nucleotide–polypeptide complexes were incubated with 50 mm-Tris-HCl pH 7.9 and human rotavirus L (lane 2), SA11 mRNA (lane 3), human rotavirus L mRNA (lane 4), DNA (lane 5), proteinase K (lane 6) and pancreatic RNAse (lane 7). Lane 1 is the same as lane 2 but the sample lacked human rotavirus L.

Effect of RNA, DNA and proteases

In order to identify the nature and some properties of the VP3–GMP complex, purified virus which had been incubated previously with [α-32P]GTP was reisolated by ultracentrifugation and then incubated with SA-11 mRNA or human rotavirus mRNA or DNA. The SA-11 or human L rotavirus mRNA was made in vitro in the absence of S-adenosylmethionine to avoid 5' cap formation on the mRNA (Spencer & Garcia, 1984). The results shown in Fig. 6 indicate that mRNAs of both SA-11 (lane 3) and human L (lane 4) rotaviruses were able to remove the GMP label from the virus, similar to the effect of pyrophosphate. The addition of linear or circular DNA had no effect and the radioactivity remained associated with VP3 (lane 5). After treatment of the prelabelled virus with an excess of pancreatic RNase (lane 7), the nucleotide–VP3 complex was resistant to degradation, whereas incubation with proteinase K completely degraded the labelled nucleotide–VP3 complex (lane 6).

Formation of GTP and GpppG from the VP3–GMP complex

Nucleotide-labelled virus was incubated with pyrophosphate to study whether the nucleotide could be removed from the virus and released as GTP under these conditions.
conditions, a unique property of all known guanylyltransferases (Shuman & Hurwitz, 1981). In Fig. 7(a) the results of this experiment are shown. \([\alpha^{-32}P]GTP\)-labelled virus was then purified by centrifugation until no soluble GTP substrate could be detected, and further incubated in a final volume of 20 \(\mu\)l with different concentrations of sodium pyrophosphate in the presence of 15 mM-MgCl\(_2\) for 20 min at 37 °C. The reaction mixture was centrifuged and 5 \(\mu\)l aliquots of supernatant were subjected to thin-layer chromatography on PEI-cellulose plates.

A mixture of unlabelled GTP, GDP and GMP was added to the supernatant spots as internal markers and their positions after chromatography are indicated in Figure 7(a). The results indicate that the addition of 15 to 20 mM-pyrophosphate allowed the detection of the formation of GTP from the nucleotide–VP3 complex present in the purified particle. According to the position of the nucleotide markers, the only product formed under these conditions was GTP. In Fig. 7(b) a similar experiment to the one described above was performed but GDP was used as the acceptor (lanes 1 to 3). In this case, the bound GMP was transferred from VP3 to form a product that migrated in the same position as GpppG; this product was made only when GDP was present in the reaction mixture (lane 5). The amount of product obtained was dependent on the concentration of GDP used (Fig. 7b). In order to identify the product as a GpppG cap, GMP-bound virus was incubated with 15 mM-GDP, as described above, and centrifuged. The supernatant containing the cap was adjusted to pH 8-5 with 1 M-Tris and incubated for 60 min at 37 °C in the presence of 0-5 \(\mu\)g bacterial alkaline phosphatase (BAP) (10 mg/ml). The reaction mixture was phenol-extracted, washed with chloroform and seeded on the plate together with nucleotide and GpppG markers. Control experiments were done in which the virus left after incubation with PP, or GDP was analysed by SDS–PAGE; in this case no radioactivity remained associated with a band migrating in the position of VP3 (data not shown). In Fig. 7(b), lane 4 the radioactive product obtained was shown to be resistant to BAP hydrolysis and the mobility of the product was identical to that of the GpppG cap marker. This result suggests that the complex formed between GTP and rotavirus VP3 became unstable when incubated with an acceptor, such as an RNA with a 5' phosphate or pyrophosphate, and produced GTP from the covalently bound GMP, as has been described for guanylyltransferases (Shuman, 1982).

**Discussion**

The presence of a 5' cap structure in the plus strand of the rotavirus genome RNA has been previously reported (Imai et al., 1983a). The origin of this structure may be due to the presence of cellular or virus-encoded guanylyltransferases which introduce the m\(^7\)GpppG\(^{3'\text{cap}}\)Gp at the 5' end of each of the 11 mRNAs during viral transcription in infected cells (McCrae & McCorquodale, 1982). During rotavirus transcription *in vitro* it has been shown that the 'activated' virus is able to make transcripts from each of the genes and, when S-adenosylmethionine is added to the reaction mixture, the synthesized products contain a 5' end cap structure identical to that found in the viral genome (Spencer & Garcia, 1984). The purpose of this work was to characterize the viral polypeptide with guanylyltransferase activity initially described by Fukuhara et al. (1989).

In order to determine the presence of a viral guanylyltransferase activity, an assay that has been shown to be specific for such activity and which consists of the detection of the intermediate of the reaction, a covalent GMP–enzyme complex made from the GTP, was used (Cleveland et al., 1986; Shuman, 1982). The GMP–enzyme complex transfers the GMP residue to a diphosphate-terminated acceptor, which may be either a 5' diphosphate-terminated RNA molecule yielding a cap RNA or a 5' diphosphate nucleoside yielding a cap fragment or pyrophosphate, to produce GTP (Shuman & Hurwitz, 1981). Based on this information, the formation of the nucleotide–guanylyltransferase complex was investigated using purified rotavirus particles (SA-11 and human L). The results obtained revealed the presence of only one nucleotide–polypeptide complex comigrating with rotavirus SA-11 VP3 (Fig. 1). The use of subviral particles, such as the viral core, suggested that VP6 and the outer shell polypeptides are not involved; identical results were obtained using human rotavirus. Experiments using 2',3'-dialdehyde GTP showed that the only viral polypeptide able to interact with the nucleotide was VP3, although the nature of the complex formed may be different from that made with GTP.

The kinetics of the reaction showed saturation at 60 min under the present conditions, probably due to lack of access for GTP to VP3 located in the viral core (Fig. 3). The inhibitory effect of reagents such as DTT and 2-mercaptoethanol makes this activity similar to those identified in vaccinia virus or HeLa cells (Monroy et al., 1978; Shuman, 1982). The nucleotide–VP3 complex showed the characteristics of a covalent complex, based on its stability when boiled in the presence of SDS, its sensitivity to treatment with proteinase K and its resistance to pancreatic ribonuclease.

GMP–VP3 complex formation was dependent on the presence of divalent cations such as Mg\(^{2+}\) or Mn\(^{2+}\), as has been described previously for other guanylyltransferases (Shuman, 1982). The major difference was in the specificity of the nucleotides; whereas all other guanylyl-
Rotavirus guanylyltransferase activities are highly specific for GTP or dGTP in the presence of either MgCl₂ or MnCl₂, the rotavirus enzyme was able to form complexes with UTP, CTP, ATP, TTP, dCTP and dATP when Mn²⁺ was used as cofactor (data not shown). The loss of specificity of other viral enzymes that interact with nucleotides in the presence of Mn²⁺ has been reported (Spencer et al., 1980).

The rotavirus nucleotide–VP3 complex, as with other guanylyltransferases, was shown to be sensitive to the addition of phosphate and low concentrations of pyrophosphate. These characteristics permit investigation of the nature of the nucleotide–VP3 complex because the nucleotide–protein complex of guanylyltransferase transfers the GMP to an acceptor, such as pyrophosphate or an oligonucleotide having a di-phosphate 5'-terminated end, synthesizing GTP or a capped oligonucleotide respectively (Monroy et al., 1978). Transfer of the nucleotide to the pyrophosphate molecule was confirmed by the experiment shown in Fig. 7(a). The same conclusion was reached from the results of the experiment in which the complex was incubated with GDP; in this case a product was formed which migrates in the same position as GpppG (Fig. 7b). The detection of this cap was dependent on the amount of GDP added. Therefore, the complex formed with VP3 involves a GMP residue and, together with the above mentioned properties of this polypeptide, it corresponds to the viral guanylyltransferase. This conclusion is also supported by the detection of capped RNA when in vitro transcription is performed with the purified virus particle (Spencer & Garcia, 1984). Cap formation seems to be important because the addition of 5'-adenosylnitromethane stimulates the rate and yield of rotavirus mRNA synthesis, lowering the $K_m$ values for each of the substrate nucleoside triphosphates (Spencer & Garcia, 1984). Considering the results of in vitro transcription, and those of the present study, it seems that the capping activity present in rotavirus also includes a methyltransferase activity similar to that reported for reovirus (Cleveland et al., 1986).

During viral infection, cells transcribe viral cap mRNAs but, when replication begins, the cap structure is not added to the 5' end of minus-strand RNA (Imai et al., 1983a), although VP3 is a constituent of the replicase particle together with VP1 and some of the non-structural polypeptides (Gallegos & Patton, 1989).

This suggests that the guanylyltransferase activity is active only as a constituent of a multienzyme complex related to transcriptional activity. The polypeptide function may vary according to the proteins interacting with it. Furthermore, we have found that this protein is responsible for the viral ATPase activity (J. L. Pizarro et al., unpublished results).

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