Antibody to a Synthetic Nonapeptide Corresponding to the NH₂ Terminus of Poliovirus Genome-Linked Protein VPg Reacts with Native VPg and Inhibits In Vitro Replication of Poliovirus RNA

CASEY D. MORROW AND ASIM DASGUPTA*

Department of Microbiology and Immunology, Jonsson Comprehensive Cancer Center and Molecular Biology Institute, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

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A synthetic nonapeptide corresponding to the N-terminal sequence of poliovirus genome-linked protein (VPg) was linked to bovine serum albumin and used to raise antibodies in rabbits. The anti-peptide antibodies specifically precipitated the nonapeptide, native VPg, and VPg-linked poliovirion RNA. The anti-peptide antibodies inhibited host factor-stimulated, poliovirus replicase-catalyzed in vitro synthesis of full-length (35S) RNA in response to virion RNA. Oligouridylic acid-stimulated RNA synthesis was not affected by the anti-peptide antibodies. Preincubation of the antibodies with excess nonapeptide reversed the anti-peptide antibody-mediated inhibition of host factor-stimulated RNA synthesis by the poliovirus replicase. A role for VPg in the in vitro replication of poliovirus RNA genome is discussed.

The single-stranded RNA genome of poliovirus is replicated by an RNA-dependent RNA polymerase (replicase) found in cells infected with poliovirus (4). Early attempts to isolate a template-dependent form of poliovirus replicase were unsuccessful because of its tight association with the endogenous template and cellular membranes. A template-dependent form of the enzyme (13) was first isolated by Flanagan and Baltimore (16) as a polyadenylic acid [poly(A)] · oligouridylic acid [oligo(U)]-dependent polyuridylic acid [poly(U)] polymerase. All template-dependent poliovirus replicase preparations reported to date have been cytoplasmic (13, 15, 19, 39). The virus-specific poly(U) polymerase activity copurifies with the template-dependent RNA replicase activity (13). A single viral protein called P63 (NCVP4, P3-4b) is believed to be responsible for poly(U) polymerase activity in vitro as well as for replicase activity in poliovirus-infected cells (17, 39). The template-dependent replicase has been shown to copy an entire virion RNA molecule (plus polarity) in the presence of an oligo(U) primer (6, 11, 40). A host cell protein (host factor) (15) isolated from uninfected HeLa cells can substitute for oligo(U) in poliovirus replicase-catalyzed synthesis of minus-strand RNA molecules (6, 11), suggesting a role for host factor in initiation of RNA synthesis. The observation that an antibody to host factor inhibits host factor-depen-

dent synthesis of poliovirus RNA (minus) but not the elongation of preinitiated RNAs also supports this notion (12, 14). Host factor, a 67,000-dalton protein, has recently been purified and has been shown to physically interact with the virus-specific poly(U) polymerase (5, 11, 12).

Among animal RNA viruses, picornaviruses and calciviruses are known to possess a small protein, VPg, at the 5'-terminus of their genomic RNAs (10, 18, 21, 22, 26-28, 34, 41). VPg, a 22-amino acid-long protein, contains only one tyrosine residue which forms the bridge, via its O⁴ hydroxyl group, to the 5'-terminal phosphate of the nucleotide chain (VPg-Tyr-O⁴-pUAAAAAC . . .) (2, 33). Not only are the 5'-ends of virion RNA (plus strands) VPg linked, but also minus-strand [VPg-(pU)₆₀ . . .] nascent strands of the replicative intermediate and both strands of the double-stranded RNA (replicative form) are VPg linked (18, 28, 31, 43). Based on these findings, it has been proposed that the VPg may act as a primer of RNA synthesis (28). However, free VPg cannot be detected in the virus-infected cells, suggesting that VPg enters the RNA replication complex in the form of a precursor polypeptide (7, 28, 35). Recent studies with antibodies to synthetic peptides corresponding to the C-terminus of VPg have yielded information about the viral precursors to this small protein (7, 35). Furthermore, one study has found that

the antibody to the C-terminal 14 amino acids of VPg would inhibit poliovirus replicase-catalyzed *in vitro* synthesis of minus-strand RNA (8).

The amino acid sequence of VPg is encoded in viral RNA within the portion of the genome that encodes the precursor for the RNA polymerase (24, 25, 29). From a combination of protein and nucleic acid sequence data, the length of VPg has been shown to be 22 amino acids (1, 24, 25, 32, 36). Zabel et al. have isolated sufficient quantities of genome-linked protein (VPg) from cowpea mosaic virus RNA to generate anti-VPg antibodies in rabbits (44). It is difficult to isolate poliovirus VPg in amounts sufficient for immunization of laboratory animals. We have, therefore, followed the strategy of Walter et al. (42) and Sutcliffe et al. (38) and used a synthetic nonapeptide corresponding to the N-terminus of VPg to generate antibodies to VPg in rabbits. The antipeptide antibodies specifically precipitate the nonapeptide and native VPg, as well as VPg-linked poliovirion RNA. Furthermore, the antibodies inhibit host factor-stimulated, poliovirus replicase-catalyzed synthesis of minus-strand RNA, including 35S RNA. Oligo(U)-stimulated copying of either poly(A) or poliovirion RNA is not affected by the antibody.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma Chemical Co., unless otherwise noted. Unlabeled nucleotides were obtained from Calbiochem, poly(A) was from Miles Laboratories, and oligo(U)₁₀₋₂₀ was obtained from Collaborative Research. Poly(U)-Sephacel 4B and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals, Inc. Phosphocellulose was purchased from Whatman. Iodogen was purchased from Pierce Chemical Co. All radioisotopes were purchased from New England Nuclear. The nonapeptide Gly-Ala-Tyr-Thr-Gly-Leu-Pro-Asn-Lys (VPg-N₉), corresponding to the N-terminus of VPg, was made to order from Peninsula Laboratories. Another nonapeptide having the amino acid sequence Thr-Gln-Ser-Gln-Gly-Glu-Ile-Gln-Trp was also purchased from Peninsula Laboratories.

Cell culture. HeLa cells were grown in Joklik modified medium supplemented with 5 to 8% calf serum (13, 15).

Poliovirus infection. Suspension cultures of HeLa cells were infected with poliovirus type 1 as described previously (13, 15) and were treated with 5 µg of actinomycin D per ml at 15 min postinfection. The infected cells were collected by centrifugation at 5 to 6.5 h postinfection, washed once in phosphate-buffered saline (PBS), and kept frozen at -70°C.

Purification of poliovirus replicase [poly(U) polymerase] and host factor. A total of 8×10^8 HeLa cells infected with poliovirus were used for preparation of poliovirus replicase. Purification of poliovirus replicase through phosphocellulose (fraction II) and poly(U)-Sephacel 4B (fraction IV) has been described previously (13, 15). Purification of host factor from our laboratory has recently been described (11). Host

factor was purified from a ribosomal salt wash fraction prepared from uninfected HeLa cells which involved 40 to 60% saturated ammonium sulfate precipitation, DEAE-Sephacel, phosphocellulose, hydroxylapatite column chromatography, glycerol density gradient centrifugation, and a second phosphocellulose chromatography. Upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the purified host factor showed a single protein band having an approximate molecular weight of 67,000 (11).

Poly(U) polymerase, replicase, and host factor assays.

Poly(A) · oligo(U)-dependent poly(U) polymerase activity was assayed for 30 min at 30°C as described by Flanagan and Baltimore (16). For poliovirus RNA-dependent replicase activity, the standard incubation mixture contained, in a total volume of 50 µl: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), 5 mM magnesium acetate, 4 mM dithiothreitol, 10 µM of actinomycin D per ml, a 0.2 mM concentration of each of three other ribonucleoside triphosphates, 1 µM [α -³²P]UTP (specific activity, 50 to 100,000 cpm/pmol), 1 µg of poliovirion RNA, and 10 to 15 µg of fraction II or 2 to 3 µg of fraction III replicase as defined previously by Dasgupta et al. (13). The host factor activity was assayed essentially the same way as the replicase activity except that 1 to 2 µg of fraction IV replicase and 0.03 µg of fraction VII host factor (11) were used. RNA synthesis in the presence of only fraction IV replicase served as the control. Incubation was for 1 h at 30°C. The labeled products were either collected on membrane filters (0.45 µm; Millipore Corp.) after precipitation with 7% trichloroacetic acid (one-third saturated with sodium pyrophosphate) in the presence of 100 µg of added carrier RNA and counted in a scintillation counter, using 5 ml of Bray scintillation fluid, or analyzed on 1% agarose gels containing 15 mM methylmercuric hydroxide (3).

Poliovirion RNA. Unlabeled poliovirion RNA was prepared by the method described by Spector and Baltimore (37).

Immunizations. The nonapeptide (VPg-N₉) was coupled to bovine serum albumin (BSA) with glutaraldehyde according to the technique of Kagan and Glick (23). Glutaraldehyde (1 ml; 20 mM) was added dropwise to a 2-ml solution containing 30 mg of BSA and a 40-fold molar excess of peptide in 0.1 M sodium phosphate buffer (pH 7.5). The reaction was allowed to continue for 1 h at room temperature with constant stirring. The peptide-BSA conjugate was then dialyzed against PBS for 42 h with five changes of buffer.

One- to 2-month-old New Zealand white rabbits were immunized with 750 µg of VPg-N₉ peptide emulsified in complete Freund adjuvant by intradermal injections at multiple sites. A sample of serum was obtained from animals before immunization. Rabbits were boosted with 200 µg of VPg-N₉ in incomplete Freund adjuvant 4, 6, and 8 weeks after the primary immunizations. Blood was drawn 8 to 10 days after immunization, allowed to coagulate, clarified by centrifugation at $20,000 \times g$ for 15 min, and stored at -20°C. Anti-BSA antibodies were removed by chromatography on BSA-agarose. BSA was first coupled to agarose, using cyanogen bromide. A 50-mg amount of BSA was incubated with 25 ml of packed cyanogen bromide-activated Sepharose overnight at 4°C. BSA coupled to the resin was centrifuged, and the resin was thoroughly washed with PBS. The coupling efficiency

FIG. 1. Amino acid sequences of the synthetic VPg peptide and the poliovirus genome-linked protein VPg. The amino acid sequence of VPg is taken from previously published data (24, 36). VPg (22/22) represents the entire 22-amino acid sequence, whereas VPg (9/22) includes only the 9 amino-terminal amino acids. VPg is found to be covalently linked to RNA through its one tyrosine hydroxyl group. The linkage between tyrosine and the first nucleotide (U) of RNA is known to be a phosphodiester bond (2, 33).

was approximately 90%. Antisera were mixed with BSA-agarose beads and incubated overnight at 4°C. BSA-agarose was then removed by centrifugation, and this process was repeated twice with the supernatant. The supernatant obtained after the final centrifugation was then applied to a protein A-agarose column.

Protein A-agarose chromatography. Rabbit immunoglobulin G (IgG) was purified from sera, using protein A-agarose chromatography as described previously (D. R. Blanco and J. N. Miller, presented at the 1st Sexually Transmitted Diseases World Congress, San Juan, P.R., 1981). Briefly, rabbit sera were loaded onto a protein A-agarose column in 0.1 M Tris-hydrochloride (pH 8.0). The column was extensively washed with 0.14 M PBS (pH 8.0) to remove unbound serum proteins. The IgGs were eluted with 0.1 M sodium citrate-citric acid buffer (pH 3.0). The eluent was immediately neutralized with 1 M Tris base. Fractions with optimal optical density at 280 nm were pooled and exhaustively dialyzed against PBS. The samples were adjusted to give a protein concentration of 10 mg/ml and stored in aliquots at -20°C.

Iodinations. Nonapeptide (VPg-N₉) and BSA were iodinated by using the Iodogen method previously described (20). A 50-μg portion of nonapeptide and BSA were iodinated with 100 μCi of Na¹²⁵I. Protein (or peptide)-bound ¹²⁵I was separated from free ¹²⁵I by Sephadex G-25 chromatography. Efficiency of iodination was either by direct counting of the iodinated samples or by determining the acid-insoluble radioactivity.

In vitro labeling of VPg RNA with ¹²⁵I was performed according to the procedure described by Bolton and Hunter (9). ¹²⁵I-labeled VPg RNA was separated from free ¹²⁵I by passage through a Sephadex G-25 column. Iodinated poliovirus RNA was further purified by centrifugation through a 10 to 30% sucrose density gradient in 0.01 M Tris-hydrochloride (pH 7.6)-0.1 M NaCl-0.001 M EDTA-0.5% SDS for 4.5 h at 200,000 × g and 25°C, using an SW41 rotor. The peak of RNA at approximately 35S was pooled and ethanol precipitated twice at -20°C.

Isolation of VPg from poliovirus RNA. ¹²⁵I-labeled VPg RNA was digested with 0.4 U of RNase T₁, 1 U of RNase T₂, and 0.4 U of RNase A, and 2 μg of snake venom phosphodiesterase in a total volume of 50 μl containing 10 mM Tris-hydrochloride (pH 7.5)-10 mM MgCl₂-10 mM β-mercaptoethanol for 1 h at 37°C. The product VPg was separated from mononucleotides by gel filtration on Sephadex G-25 as described before (2).

Immunoprecipitations. All immunoprecipitations

were carried out in PBS (treated with diethyl pyrocarbonate) containing 1% Triton X-100, 0.5% Nonidet P-40, and 0.5% SDS (IP buffer). The incubation conditions for antibody-antigen binding are described in appropriate figure legends. Protein A-agarose was added at 5 mg per reaction to bind IgGs. After further incubation of the reaction mixture at 4°C for 60 min, unbound IgGs and antigen were removed from the protein A-agarose by centrifugation in an Eppendorf centrifuge. The pellets were washed three to four times with IP buffer. The protein A-agarose pellet was resuspended in 30 μl of electrophoresis sample buffer (60 mM Tris-hydrochloride, pH 6.8, 2% [wt/vol] SDS, 20% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 min. The supernatants were recovered after centrifugation and analyzed by SDS-polyacrylamide gel electrophoresis.

Electrophoresis. Immunoprecipitates containing labeled VPg were analyzed on 15% gels containing 0.37 M Tris-hydrochloride (pH 8.8)-0.1% SDS-0.1% N,N'-methylenebisacrylamide. The stacking gel contained 4% acrylamide-0.1% methylenebisacrylamide-0.125 M Tris-hydrochloride (pH 6.8)-0.1% SDS. Electrophoresis was carried out in 0.05 M Tris-0.384 M glycine-0.1% SDS at 120 V for 5 to 6 h. The gel was fixed for 15 min in 10% ethanol-10% acetic acid and dried, and the labeled polypeptides were visualized by autoradiography.

Immunoprecipitates containing ¹²⁵I-labeled VPg RNA were analyzed on 1% agarose gels containing 15 mM methylmercuric hydroxide as described by Bailey and Davidson (3).

RESULTS

Generation of antibodies specific for the synthetic nonapeptide. Our primary interest in obtaining an antibody to VPg was to study the role of VPg in poliovirus RNA replication. For the following reason, an N-terminal peptide was chosen against which antibodies would be raised. It was known that the linkage of VPg with the polyribonucleotide chain of RNA was through a tyrosine molecule (third amino acid from the N-terminus of VPg; Fig. 1), and if VPg (or precursors) was involved as a primer in the initiation of RNA synthesis, the initial reaction could presumably be the linkage of a UMP residue with this tyrosine molecule. We therefore thought that an antibody to an N-terminal

FIG. 2. Analysis of iodinated nonapeptide (VPg-N₉) before and after immunoprecipitation on SDS- and SDS-urea polyacrylamide gels. ¹²⁵I-labeled VPg-N₉ was analyzed by 15% polyacrylamide (A), 10 to 20% polyacrylamide gradient plus 8 M urea (B), and 15% polyacrylamide plus 8 M urea (C) gel electrophoresis. For immunoprecipitation of ¹²⁵I-labeled VPg-N₉, approximately 20,000 cpm of the labeled peptide was incubated with 1 μ l of immune or preimmune IgG in a total volume of 200 μ l (IP buffer). Incubation was for 1 h at 0°C. A 5-mg portion of protein A-agarose was then added to the incubation mixture. After an additional 60 min of incubation at 4°C, the protein A-agarose pellet was recovered by centrifugation and washed three times with IP buffer. The protein A-agarose pellet was resuspended in 30 μ l of electrophoresis sample buffer and boiled for 5 min. Supernatant recovered after centrifugation was analyzed by SDS-polyacrylamide gel electrophoresis. (A) Lane 1, ¹²⁵I-labeled VPg-N₉ peptide (PEP) (not immunoprecipitated); lane 2, ¹²⁵I-labeled VPg-N₉ plus preimmune IgG; lane 3, ¹²⁵I-labeled VPg-N₉ plus immune IgG. The positions of migration of ¹²⁵I-labeled VPg and ³²P-labeled VPg-p are indicated. ³²P-labeled VPg-p was prepared as described before (2). (B) Lane 1, ¹²⁵I-labeled VPg-N₉ (not immunoprecipitated); lane 2, ¹²⁵I-labeled protein markers. (C) Lane 1, ¹²⁵I-labeled protein markers; lane 2, ¹²⁵I-labeled VPg-N₉ (not immunoprecipitated). The following molecular weight markers were analyzed: bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α -lactalbumin (14,000).

peptide containing the tyrosine moiety would have the potential to interfere with poliovirus RNA-dependent synthesis of RNA. To increase the antigenicity of the nonapeptide, it was coupled to BSA before immunization (23). Antibodies specific for the peptide were detected in the serum at 1 week after the last booster immunization. Antipeptide antibodies increased over the first 10 weeks, but then began to level off from

weeks 10 to 12. As expected, we observed a rapid increase in the anti-BSA antibodies as detected by immunoprecipitation with iodinated BSA. To reduce the possibility of nonspecific effects of the antiserum by contaminating anti-BSA antibodies, we purified the immune serum by repeated passage through a BSA-agarose column. This treatment removed >95% of the anti-BSA antibodies, whereas very little anti-peptide antibody was lost (data not shown). To remove contaminating proteins (mainly RNases and proteases) from the sera, we purified both preimmune and immune sera by affinity chromatography, using protein A-agarose (Blanco and Miller, 1st Sexually Transmitted Diseases World Congress, 1981). Fractions enriched in IgG were concentrated and dialyzed against PBS.

The concentrated IgG fractions were tested for capacity to immunoprecipitate the VPg-N₉ nonapeptide. ¹²⁵I-labeled peptide was incubated with immune IgG, and the immunoprecipitates were analyzed on a 15% SDS-polyacrylamide gel (Fig. 2). The preimmune IgG did not precipitate any labeled material (Fig. 2A, lane 2). The immune IgG precipitated a radiolabeled band which migrated with an approximate molecular weight of 35,000 (Fig. 2A, lane 3). The actual molecular weight of the nonapeptide was estimated to be 920. We therefore expected that it would migrate further on a 15% polyacrylamide gel. However, when the ¹²⁵I-labeled nonapeptide was analyzed on the same gel, it comigrated with the immunoprecipitated material (Fig. 2A, lane 1). Analysis of the ¹²⁵I-labeled nonapeptide on urea-SDS gels (15% and 10 to 20% polyacrylamide gradient) did not change its mobility (Fig. 2B, lane 1, and C, lane 2). In both gels, the peptide migrated between ovalbumin (43,000 daltons) and carbonic anhydrase (30,000 daltons). A large quantity of ¹²⁵I-labeled peptide was analyzed on these gels to check the purity of the synthetic peptide. In all three types of gels, the nonapeptide moved much slower than VPg (molecular weight, 2,354). We do not know the reason for the anomalous movement of this peptide on polyacrylamide gels. An unrelated nonapeptide (see Materials and Methods for the sequence) did move faster than VPg on identical gels (data not shown). Although SDS-polyacrylamide gel electrophoresis did not give an accurate reflection of molecular weight of the peptide, it was clear from the data that the antibody did immunoprecipitate the radiolabeled peptide.

Antipeptide IgGs react with native VPg. To determine whether antibody raised against the nonapeptide could react with native VPg, we assayed its ability to immunoprecipitate VPg purified from poliovirus RNA. Highly purified poliovirus RNA was labeled with ¹²⁵I-Bolton-Hunter reagent, and the ¹²⁵I-labeled VPg was

cleaved from the RNA by treatment with RNases T₁, T₂, and A and snake venom phosphodiesterase. ¹²⁵I-labeled VPg was purified by chromatography on Sephadex G-25. The native VPg was then incubated with immune IgG, and the precipitates were analyzed through 15% SDS-polyacrylamide gels (Fig. 3). The affinity-purified immune IgG precipitated ¹²⁵I-labeled VPg (lane 3), whereas the preimmune IgG did not show any reactivity towards ¹²⁵I-labeled VPg (lane 2). The immunoprecipitated VPg comigrated with native ¹²⁵I-labeled VPg (lane 1) and ³²P-labeled VPg-p (data not shown). The specificity of the immune IgG was confirmed by the ability of unlabeled nonapeptide to compete out the immunoprecipitation of ¹²⁵I-labeled VPg (lanes 4 to 7). In the presence of 10 µg of unlabeled nonapeptide, immunoprecipitation of ¹²⁵I-labeled native VPg was completely inhibited (lane 6).

Since the immune IgG reacted with native, virion RNA-derived VPg, we next determined whether the antibodies would recognize VPg covalently attached to full-length (35S) virion RNA. We anticipated that immunoprecipitation of 35S virion RNA would be difficult for the following reasons. First, VPg attached to approximately 7,500-nucleotide-long virion RNA might not be accessible to the antibody. Second, even if the antibody recognized VPg, it might be technically difficult to immunoprecipitate the relatively large RNA molecule attached to very small VPg. In this experiment, highly purified virion RNA was iodinated with ¹²⁵I-Bolton-Hunter reagent and the ¹²⁵I-labeled VPg RNA was purified by density gradient centrifugation and passage through a Sephadex G-75 column. Labeled RNA was heated at 90°C for 2 min and then quickly chilled. The labeled RNA was incubated with the antibody in ice in the presence of human placental RNase inhibitor. Inclusion of an RNase inhibitor in IP buffer was helpful because the affinity-purified IgGs were not completely free of RNases (unpublished data). The immunoprecipitates were then analyzed on denaturing agarose gels (3). Under these conditions, we could detect precipitation of labeled 35S RNA by the immune IgG (Fig. 4, lane 3) but not by the preimmune IgG (lane 2). Some degradation of labeled RNA was evident even in the presence of RNase inhibitor. Addition of increasing concentrations of unlabeled nonapeptide (VPg-N₉) inhibited the immunoprecipitation of viral RNA (lanes 4 and 5). However, an unrelated nonapeptide having the amino acid sequence Thr-Gln-Ser-Gln-Gly-Glu-Ile-Gln-Trp could not compete out the immunoprecipitation of 35S RNA (lane 6). The result suggested that immunoprecipitation of VPg-linked 35S virion RNA by the immune IgG was specific

FIG. 3. Immunoprecipitation of ¹²⁵I-labeled VPg by antibody raised against the synthetic nonapeptide. ¹²⁵I-labeled VPg was prepared from ¹²⁵I-labeled poliovirion RNA as described in the text. Approximately 25,000 cpm of ¹²⁵I-labeled VPg was used for immunoprecipitation with 1 µl of preimmune or immune IgG. (Lane 1) ¹²⁵I-labeled VPg (not immunoprecipitated); (lane 2) ¹²⁵I-labeled VPg, plus preimmune IgG; (lane 3) ¹²⁵I-labeled VPg, plus immune IgG; (lane 4) ¹²⁵I-labeled VPg, 100 ng of VPg-N₉ peptide, plus immune IgG; (lane 5) ¹²⁵I-labeled VPg, 1 µg of VPg-N₉ peptide, plus immune IgG; (lane 6) ¹²⁵I-labeled VPg, 10 µg of VPg-N₉ peptide, plus immune IgG; (lane 7) ¹²⁵I-labeled VPg, 100 µg of VPg-N₉ peptide, plus immune IgG. Immunoprecipitations were carried out as described in the legend to Fig. 2. Precipitates were analyzed on a 15% SDS-polyacrylamide gel, and labeled proteins were detected by autoradiography. Positions of poliovirus capsid proteins VP1, VP2, and VP3 are marked at the left. 0 = origin.

and was not due to nonspecific binding of RNAs to the immunoglobulins.

Inhibition of host factor-stimulated replicase activity by immune IgG. Poliovirus-specific poly(U) polymerase utilizes an oligo(U) primer to copy a synthetic poly(A) template (16). The same poly(U) polymerase preparation, in the presence of poliovirion RNA template, is capa-

FIG. 4. Immunoprecipitation of ^{125}I -labeled VPg-RNA. Purified poliovirus RNA was iodinated with ^{125}I -Bolton-Hunter reagent (9). Approximately 300,000 cpm (trichloroacetic acid precipitable) of ^{125}I -labeled VPg RNA was used for immunoprecipitation. Immune and preimmune IgGs were first incubated with 10 μg of yeast tRNA overnight at 0°C . ^{125}I -labeled VPg RNA was heated at 90°C for 2 min and chilled quickly in ice. Labeled RNA was then incubated with IgGs (treated with tRNA) in ice for 18 h in 50 μl of IP buffer containing 0.1 U of human placental RNase inhibitor. A longer preincubation time (18 h) was necessary for efficient immunoprecipitation of RNA. A 5-mg portion of protein A-agarose per reaction was used to bind the antigen-antibody complex. The protein A-agarose pellet was washed three times with IP buffer, resuspended in 50 μl of electrophoresis sample buffer, and boiled for 5 min. The supernatant recovered after centrifugation was phenol extracted. ^{125}I -labeled VPg RNA was precipitated from the aqueous phase in the presence of 5 μg of carrier yeast tRNA. Labeled RNA was then denatured by 15 mM methylmercuric hydroxide. (Lane 1) ^{125}I -labeled VPg RNA (not immunoprecipitated); (lane 2) ^{125}I -labeled VPg RNA, plus preimmune IgG; (lane 3) ^{125}I -labeled VPg RNA, plus immune IgG; (lane 4) ^{125}I -labeled VPg RNA, 10 ng of VPg-N₉ peptide, plus immune IgG; (lane 5) ^{125}I -labeled VPg, 50 ng of VPg-N₉ peptide, plus immune IgG; (lane 6) ^{125}I -labeled VPg RNA, 10 μg of an unrelated nonapeptide, plus immune IgG. The position of 35S RNA marker was determined by analyzing an unlabeled sample of poliovirus RNA and visualizing it by staining with ethidium bromide.

ble of synthesizing RNA molecules, including full-length RNA (35S), when provided with host factor (6, 11). Oligo(U) can replace host factor in the synthesis of RNA molecules (6, 11) by binding to the poly(A) tract at the 3'-end of viral RNA and serving as a primer for RNA synthesis (6, 40). To explore the possibility that one or more VPg-related protein(s) might be involved in poliovirus cRNA (minus) synthesis, we tested the effects of antipeptide antibodies on RNA synthesis catalyzed by the viral replicase-host

factor combination. In this experiment, aliquots of poliovirus replicase were preincubated with various concentrations of preimmune and immune IgGs before use in reactions containing host factor, virion RNA, and other components necessary for the transcription of viral RNA. Addition of increasing concentrations of immune IgG progressively inhibited RNA synthesis (Fig. 5A). At higher concentrations of immune IgG, almost 90% of total RNA synthesis was inhibited. The preimmune IgGs did not significantly inhibit RNA synthesis. As predicted, the immune IgGs did not inhibit oligo(U)-dependent copying of either poly(A) or poliovirus RNA (Fig. 5B), indicating the lack of involvement of VPg or VPg-related proteins in these reactions.

The nonapeptide does not substitute for host factor or oligo(U) in RNA synthesis. Since the nonapeptide sequence includes the "tyrosine" moiety, which in the native VPg molecule is used in the linkage to poliovirus RNA (2, 33), we determined whether the free peptide would stimulate *in vitro* RNA synthesis catalyzed by the replicase. Poliovirus replicase was incubated with the nonapeptide in the absence or presence of host factor [or oligo(U)] under standard RNA synthesis assay conditions, and the products were analyzed on a denaturing agarose gel. In the presence of oligo(U) (Fig. 6, lane 2) or host factor (Fig. 6, lane 3), the viral replicase synthesized labeled RNA, which included full-length (35S) RNA molecules. In both host factor- and oligo(U)-stimulated reactions, we routinely observed 35S RNA molecules along with smaller RNA products which appeared as smears on the gel. Presumably the smaller RNAs would be those which had not completed the synthesis at the termination of the reaction. In the presence of replicase alone, no RNA synthesis was detected (lane 1). Addition of pure, synthetic nonapeptide to the reaction containing the viral replicase did not stimulate RNA synthesis (lane 4). Identical results were obtained when various amounts of nonapeptide (1 ng to 100 μg) were used in this reaction (data not shown). However, addition of either oligo(U) (lane 5) or host factor (lane 6) to the reactions completely restored RNA synthesis in the presence of the nonapeptide.

Nonapeptide (VPg-N₉) reverses the immune IgG-mediated inhibition of RNA synthesis. In the experiment described in Fig. 5, we showed that the antibody to VPg peptide inhibited host factor-dependent replicase activity but not oligo(U)-dependent replicase activity. If the immune IgG-mediated inhibition of host factor-stimulated replicase activity was due to inactivation of one or more viral proteins containing the nonapeptide sequence, it should be reversed by

FIG. 5. Effect of anti-peptide antibodies on poliovirus-specific replicase and poly(U) polymerase activities. Aliquots of 1 μg of poliovirus replicase (fraction IV; 15) were incubated with various concentrations of preimmune (○, △) and immune (●, ▲) IgGs for 12 h in ice. The aliquots of replicase were then assayed for either host factor-stimulated replicase (A; ○, ●) and oligo(U)-stimulated replicase (B; ○, ●) activities or poly(A) · oligo(U)-dependent poly(U) polymerase activity (B; △, ▲). The conditions used for RNA synthesis were as described in the text [α - ^{32}P]UTP (specific activity, 10,000 cpm/pmol) was used as the labeled nucleoside triphosphate. Incubation was for 1 h at 30°C. Reactions were stopped by adding 5 mM EDTA, and the acid-insoluble radioactivity was determined as described previously (13, 15).

preincubating the immune IgG with excess nonapeptide. Experiments were performed in which the replicase preparations were first incubated with the immune IgG in the presence and absence of excess nonapeptide and then used in a host factor- or oligo(U)-dependent RNA synthesis assay. Labeled RNA products synthesized in response to virion RNA were denatured with methylmercuric hydroxide and analyzed on denaturing agarose gels. Preincubation of viral replicase with preimmune IgG did not have any effect on the replicase-catalyzed synthesis of RNA (including 35S RNA) in the presence of oligo(U) or host factor (Fig. 7, lanes 2 and 3, respectively). As shown earlier, viral replicase alone could not synthesize any detectable RNA (lane 1). Preincubation of the replicase with immune IgG completely inhibited the host factor-stimulated RNA synthesis, including 35S RNA molecules (lane 6). Addition of excess nonapeptide (VPg-N₉) during preincubation of the replicase with immune IgG could completely restore the ability of replicase to synthesize labeled RNA in the presence of host factor (lane 9). However, inhibition of RNA synthesis could not be reversed by addition of an unrelated peptide (lane 12). These results suggested that, in the presence of excess nonapeptide, the immune IgG probably could not interact with a protein(s) containing the nonapeptide sequence,

which is presumably utilized during the initiation of RNA synthesis.

DISCUSSION

We have shown that antibodies raised against a synthetic peptide, which corresponds to the N-terminal nine amino acids of poliovirus genome-linked protein VPg, react with the nonapeptide virion RNA-derived VPg and recognize VPg covalently attached to full-length (35S) virion RNA. Furthermore, anti-VPg antibodies inhibit host factor-stimulated RNA synthesis by the poliovirus replicase but not oligo(U)-primed synthesis.

Our initial characterization of the anti-peptide antibodies began with immunoprecipitation of radiolabeled nonapeptide (VPg-N₉). As expected, the anti-peptide antibodies immunoprecipitated radiolabeled nonapeptide (Fig. 2). We found, though, that the iodinated peptide migrated on SDS-polyacrylamide gels between 30,000- and 43,000-dalton protein markers. Three different gel conditions are presented with the same results (Fig. 2). We also found that the unlabeled nonapeptide (not iodinated) migrated similarly to the ^{125}I -labeled peptide (unpublished data). Our result is not surprising because VPg, a protein with a molecular weight of 2,354 (1), migrates on those gel systems like a protein of 10,000 to 12,000 molecular weight. Regardless of

FIG. 6. Effect of VPg-N₉ nonapeptide on *in vitro* RNA synthesis by the poliovirus replicase. A 1- μ g amount of poliovirus replicase (fraction IV; 15) was used to synthesize labeled RNA products in response to virion RNA, in the absence and in the presence of either 15 pmol of oligo(U)₁₀₋₂₀ or 0.03 μ g of host factor (fraction VII; 11). [α -³²P]CTP was used as the labeled nucleoside triphosphate. Conditions used for RNA synthesis were as described before (11). Labeled products, after phenol extraction, were precipitated from aqueous phases in the presence of 5 μ g of yeast tRNA. Product RNA was denatured with 15 mM methylmercuric hydroxide and analyzed on a 1% agarose gel containing 15 mM methylmercuric hydroxide. (Lane 1) Replicase alone; (lane 2) replicase plus oligo(U); (lane 3) replicase plus host factor; (lane 4) replicase plus 50 μ g of VPg-N₉ peptide; (lane 5) replicase, 50 μ g of VPg-N₉ peptide, plus oligo(U); (lane 6) replicase, 50 μ g of VPg-N₉ peptide, plus host factor.

the inaccurate molecular-weight estimate of the nonapeptide, it is clear that the antipeptide antibody does immunoprecipitate the nonapeptide.

The antibody to the nonapeptide was found to reproducibly precipitate virion RNA-derived VPg (Fig. 3). Similar results were obtained by other workers who generated antibodies to the C-terminal 7 (35) and 14 (7) amino acids of VPg and in one case to the entire VPg sequence (7). The specificity of our antibody was confirmed by the ability of unlabeled nonapeptide to compete out the immunoprecipitation of ¹²⁵I-labeled, native VPg by the antibody.

We further analyzed our antipeptide antibodies for the capacity to immunoprecipitate full-length poliovirion RNA, which is known to have VPg covalently attached to the 5'-terminal nucleotide of the RNA (18, 26). Under appropriate conditions, our antipeptide antibodies could specifically immunoprecipitate 35S virion RNA (Fig. 4). We have used ¹²⁵I-labeled VPg RNA in these experiments, but similar results were obtained when ³²P-labeled poliovirus RNA was used instead (data not shown). In our preliminary experiments, we found nonspecific binding

of the labeled RNA to both immune and preimmune IgGs. However, incubation of the IgG fractions with yeast tRNA before incubation with the labeled RNA prevented nonspecific binding of viral RNA to IgGs. Degradation of the labeled viral RNA was minimized by inclusion of human placental RNase inhibitor in the buffer used for immunoprecipitation. However, immunoprecipitation of virion RNA was not as efficient as that of free VPg molecules by the antibody. More labeled RNA had to be used to detect precipitation of virion RNA compared with the amount of labeled VPg used in the immunoprecipitation. Also, less unlabeled nonapeptide was required to compete out the immunoprecipitation of labeled RNA than that required to inhibit precipitation of labeled VPg. Presumably, the relatively large RNA molecule (~7,500 nucleotides) attached to a small protein (VPg) could restrain an efficient interaction between the antibody and the VPg. The binding of immune IgG to virion RNA was specific, though, since addition of unlabeled nonapeptide (VPg-N₉) completely inhibited the precipitation of virion RNA whereas an unrelated nonapeptide could not compete out the precipitation of labeled RNA. It is not known at present whether the antibodies to C-terminal VPg peptides would immunoprecipitate 35S virion RNA (7, 35).

Anti-VPg antibodies inhibited host factor-stimulated synthesis of RNA by the poliovirus replicase. However, the oligo(U)-primed replicase activity was not inhibited by the antibody. Identical results were obtained by Baron and Baltimore with an antibody to a 14-amino acid-long peptide corresponding to the C-terminus of VPg (8). We have shown, by analyzing the labeled RNAs synthesized in a host factor-stimulated replicase assay on denaturing gels, that the anti-VPg antibody would inhibit *de novo* synthesis of RNA including full-length (35S) RNA. Preincubation of the antibody with excess nonapeptide reversed the immune IgG-mediated inhibition of RNA synthesis, whereas an unrelated nonapeptide failed to reverse the inhibition of RNA synthesis by the immune IgG.

The binding of immune IgG to the 5'-terminal VPg in viral RNA (which is used as the template in RNA synthesis) is probably not the reason for inhibition of RNA synthesis since in the presence of immune IgG oligo(U) can still stimulate the synthesis of RNA by the poliovirus replicase. If RNA synthesis inhibition was due to the interaction of immune IgGs with the 5'-terminal VPg in the template RNA, then one would expect inhibition of both host factor- and oligo(U)-stimulated replicase activities.

The results from the inhibition of *in vitro* RNA synthesis by the anti-VPg antibodies (8; this paper) suggest that *de novo* initiation by the

FIG. 7. Antipeptide antibody-mediated inhibition of full-length RNA synthesis and its reversal by VPg-N₉ peptide. Aliquots of poliovirus replicase (1 µg, fraction IV; 15) were preincubated with 0.5 µg of immune IgG (or preimmune IgG) in the presence and absence of 50 µg of VPg-N₉ peptide (or an unrelated peptide) for 12 h in ice. The aliquots of replicase were then used in poliovirus RNA-dependent RNA synthesis assay in the presence of 15 pmol of oligo(U) or 0.03 µg of host factor (fraction VII; 11). [α -³²P]UTP was used as the labeled nucleoside triphosphate. Each reaction contained 0.1 U of human placental RNase inhibitor. Incubation was for 1 h at 30°C. Labeled RNA products, after phenol extraction, were ethanol precipitated from the aqueous phase and analyzed on a denaturing agarose gel. (Lane 1) Replicase plus preimmune IgG; (lane 2) replicase, oligo(U), plus preimmune IgG; (lane 3) replicase, host factor, plus preimmune IgG; (lane 4) replicase, plus immune IgG; (lane 5) replicase, oligo(U), plus immune IgG; (lane 6) replicase, host factor, plus immune IgG; (lane 7) replicase, 50 µg of VPg-N₉ peptide, plus immune IgG; (lane 8) replicase, oligo(U), 50 µg of VPg-N₉ peptide, plus immune IgG; (lane 9) replicase, host factor, 50 µg of VPg-N₉ peptide, plus immune IgG; (lane 10) replicase, 50 µg of an unrelated nonapeptide, plus immune IgG; (lane 11) replicase, oligo(U), 50 µg of unrelated nonapeptide, plus immune IgG; (lane 12) replicase, host factor, 50 µg of unrelated nonapeptide, plus immune IgG. Only one-third of the total sample was analyzed on lane 11. The other two-thirds of the sample was lost. An identical experiment in the presence of the unrelated nonapeptide did not show any difference between lanes 8 and 11 (data not shown). The unrelated nonapeptide (over a concentration range of 1 ng to 100 µg) did not have any effect on oligo(U)- or host factor-stimulated RNA synthesis by the poliovirus replicase (data not shown).

poliovirus replicase in the presence of host factor may utilize VPg or VPg-related proteins. We do not believe that VPg itself is used in the *in vitro* RNA synthesis for the following reasons. First, free VPg cannot be detected in the poliovirus replicase preparations (unpublished data). Second, neither the VPg-N₉ nonapeptide nor full-length synthetic VPg (8) was effective as a primer in the *in vitro* RNA synthesis assay. VPg, then, is probably utilized in the form of a precursor molecule (7, 8, 30, 35). Baron and Baltimore, using an antibody to a synthetic peptide corresponding to the C-terminal 14 amino acids of VPg, were able to immunoprecipitate (from infected cells) five precursor polypeptides to VPg having approximate molecular weights of 149,000 (NCVP0), 84,000 (NCVP1b), 48,000 (pre-VPg-1), 45,000 (pre-VPg-2), and 12,000 (pre-VPg-3) (7), whereas Semler et al., using an antibody to a seven-amino acid-long C-terminal VPg peptide, could immunoprecipitate two polypeptides having approximate molecular weights of 12,000 (P3-9) and 45,000 (35). We were able to immunoprecipitate two viral proteins of approximate molecular weights 49,000 and 14,000 from infected cells with our antibody (data not shown). Thus far, we have not been able to immunoprecipitate VPg-related proteins from the replicase preparations used in this study. However, our preliminary experiments using a solid-phase radioimmunoassay indicate the presence of VPg-related proteins in the replicase preparations. Furthermore, we can immunoprecipitate *in vitro* synthesized RNA molecules from a host factor-stimulated reaction by using anti-VPg antibodies (C. D. Morrow and A. Dasgupta, manuscript in preparation). We are currently fractionating viral replicase preparations to further separate the VPg-containing protein(s) from the poly(U) polymerase. These studies should allow us to fine tune the *in vitro* RNA synthesis and aid in an understanding of the mechanisms involved in the replication of poliovirus RNA.

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