Membrane-dependent uridylylation of the genome-linked protein VPg of poliovirus

(anti-VPg antibodies/HeLa cell membranes/in vitro RNA synthesis/nucleotidyl-protein/repetition model)

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Communicated by Parnell W. Choppin, September 1, 1983

ABSTRACT A small nucleotidyl-protein has been synthesized in vitro in a membrane fraction of poliovirus-infected HeLa cells. Analyses of the nucleotides and polypeptide have shown that the nucleotidyl-protein is VPg-pUPU: the genome-linked protein of poliovirion RNA covalently bound to the first two 5'-terminal nucleotides of poliovirus RNA. Synthesis of VPg-pUPU in vitro was sensitive to nonionic detergent. We suggest that VPg-pUPU is part of the initiation complex in poliovirus RNA replication in a membranous environment.

Previous observations of the structure of intracellular poliovirus RNAs suggested that the genome-linked protein VPg of poliovirus is involved in the initiation of virus-specific RNA synthesis (ref. 1 and references therein). Studies to support this hypothesis have been complicated due to a failure to detect free VPg in the infected cell by isotope labeling (2) or by radioimmunoassays (3, 4). This failure has led us to speculate that VPg is generated from precursor molecule(s) by proteolytic cleavage only at the initiation of RNA synthesis (5). Three putative precursor molecules for VPg (P3-9, 3b/9, and X/9) have been identified by radioimmunoassays (3, 4, 6) and by amino acid sequence analysis (4, 6). All three VPg-containing polypeptides are membrane bound, presumably because they contain a non-polar sequence of 22 amino acids (4, 6). One or all of these polypeptides appear to be the lipophilic carriers of the strongly basic VPg to the membranous site of poliovirus RNA replication (7). The smallest VPg-containing polypeptide (P3-9, M, 12,000) is the most likely candidate for a donor of VPg to the membrane-bound-associated poliovirus RNA synthesizing complex because it is an abundant viral product of proteolytic processing that appears to turn over only slowly (no more than 5% of it would be needed to initiate all newly synthesized RNA strands).

Baron and Baltimore (8) have recently reported the synthesis of polynucleotides in an aqueous system in vitro that appeared to be linked to protein(s) immunoprecipitable with anti-VPg antibodies. However, the yield of this reaction was too low to identify as poliovirus specific the putative nucleotidyl-protein or the newly synthesized RNA.

Using a membrane fraction from poliovirus-infected cells, we report here the synthesis in vitro of VPg-pUPU, a phenomenon strictly dependent upon the presence of intact membranes. The uridine-5'-phosphate residues are the first two 5'-terminal nucleotides of plus- and minus-strand poliovirus RNA (9). We suggest that the VPg-pUPU is a component of the initiation complex of poliovirus RNA synthesis.

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MATERIALS AND METHODS

Virus Infection and Preparation of the Crude Membrane Fractions. HeLa S3 cells were infected with poliovirus type 1 (Mahoney) at a multiplicity of infection of 50. Cells were harvested and homogenized with a Dounce homogenizer 1/2 hr after infection. After separating the nuclei from the cytosol, a crude membrane fraction was prepared by centrifugation of the cytoplasmic extract at 25,000 X g for 30 min as described (6). The crude membrane fraction contains both smooth membranes and rough endoplasmic reticulum.

Conditions for Synthesis of the Nucleotidyl-Protein. The synthesis of the nucleotidyl-protein was carried out in a mixture (40 μl) containing 50 mM Hepes (pH 8.0), 3.5 mM magnesium acetate, 10 mM dithiothreitol, 10 μg of actinomycin D per ml, 0.5 mM ATP, 30 μCi of [α-32P]UTP (ICN, 1,000–2,000 Ci/mmol; 1 Ci = 3.7 X 1010 Bq), and the crude membrane fraction (80 μg of protein). After incubation for 30 min at 30°C, the reaction was stopped by the addition of NaDodSO4 to 2% and 2-mercaptoethanol to 5%, and, after dilution, the components were subjected to immunoprecipitation (see below).

Amino acid-labeled nucleotidyl-protein was prepared from a crude membrane fraction isolated from HeLa cells that had been labeled with 0.25 mCi of [3H]lysine per ml from 3–4.5 hr after infection. The crude membrane fraction was then incubated for 30 min at 30°C in the presence of 0.5 mM UTP and ATP and the [3H]lysine-labeled nucleotidyl-protein was isolated by immunoprecipitation and polyacrylamide gel electrophoresis (4, 6).

Analyses of the Nucleotidyl-Protein. 32P-Labeled virion RNA (10) or 32P-labeled nucleotidyl-protein was digested with nucleases for 90 min at 37°C as described (11), except that all incubation mixtures also contained 0.5% Nonidet P-40 (NP-40). Antibodies against VPg were raised in rabbits immunized with two different VPg peptides: one peptide corresponded to the COOH-terminal sequence of 7 amino acids (C7) (4), and the other corresponded to the NH2-terminal sequence of 10 amino acids (N10). This latter peptide (H2N-Gly-Ala-Tyr-Thr-Gly-Leu-Pro-Asn-Lys-Lys-COOH) was synthesized using the solid-phase method of Erickson and Merrifield (12). Mono-specific antibodies directed against the COOH and NH2-terminus of VPg are denoted anti-VPg(C7) and anti-VPg(N10), respectively.

Immunoprecipitation was carried out on samples boiled first for 2 min in 2% NaDodSO4/5% 2-mercaptoethanol and then diluted with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, and 5 mM EDTA; samples were incubated with antisera overnight at 0°C (4, 6, 13).

Analyses of the nucleotidyl-protein on NaDodSO4/polyacryl-
amide gels (12.5% or 13.5%) were carried out according to Laemmli (14). The elution of [3H]lysine-labeled material from the gel and the microsequence analysis by automated Edman degradation was as described (15, 16).

RESULTS

Synthesis of a Nucleotidyl-Protein. A crude membrane fraction of poliovirus-infected HeLa cells is highly active in virus-specific RNA synthesis (6) and capable of releasing full-length poliovirus plus strands (ref. 17; unpublished results; see also ref. 18). We believe that RNA synthesis in this system not only reflects “run-off” but also de novo initiation because maps of RNase T1-resistant oligonucleotides of in vitro synthesized RNA show 5’- and 3’-terminal oligonucleotides in nearly equimolar amounts (ref. 18; unpublished results). However, the yield of newly synthesized RNA has been found to be too low to isolate VPg-pUP by nuclease digestion (unpublished results). Because the crude membrane fraction also contains polypeptides thought to participate as precursors to VPg during the initiation of RNA synthesis (6), we incubated the complex with [α-32P]UTP and ATP and immunoprecipitated the 32P-labeled products with antibodies directed against the NH2 and COOH termini of VPg [anti-VPg(N10) and anti-VPg(C7)].

Anti-VPg(C7) antibodies are capable of precipitating VPg-containing polypeptides (Fig. 1B, lane 2), an observation reported previously (4). Similarly, antibodies directed against the NH2-terminal decapptide of VPg also react with these viral peptides (Fig. 1B, lane 3). Also indicated in Fig. 1B are two larger VPg-containing polypeptides (3b/9 and X/9) that are barely visible under the condition of labeling but are intensified if the labeling is carried out in the presence of Zn2+ (6).

Both anti-VPg(C7) and anti-VPg(N10), but not the preimmune serum, immunoprecipitate 32P-labeled material (Fig. 1A, lanes 1 and 2) from incubation mixtures containing membrane fraction, ATP, and [α-32P]UTP. This material migrates faster than the VPg-containing polypeptides shown in Fig. 1B and was found near the position of capsid polypeptide VP4 (M, 7,000).

The genome-linked protein VPg of poliovirus has a Mr of only 2,200 (2, 19), yet it migrates aberrantly slowly in polyacrylamide gels with an apparent Mr of 5,000–7,000 (10, 11). Because of its antigenicity and its electrophoretic behavior, the small 32P-labeled complex seen in Fig. 1A could be a nucleotidyl-protein of the structure VPg-pU, or VPg-p(U,A), or a crude membrane fraction isolated from mock-infected HeLa cells did not form this material (Fig. 2A, lane 3), the 32P-labeled complex is likely to be poliovirus specific (see below).

Synthesis of the small 32P-labeled complex increased over an incubation period of 30 min (Fig. 2B, lanes 1–4) and was dependent upon the presence of UTP and ATP. After only 3 min of incubation, a complex of higher mobility (smaller) can be observed (Fig. 2B, lane 2), which we assume is VPg-pU and which is subsequently converted to VPg-pUpU (see below). This smaller complex is also occasionally seen when the membrane fraction is incubated with [α-32P]UTP only (data not shown). The 32P-labeled complex is not formed with [α-32P]CTP and ATP (Fig. 2B, lane 6) or with UTP and [α-32P]ATP (not shown), whereas it can be detected after incubation with [α-32P]CTP, [α-32P]UTP, and unlabeled ATP (Fig. 2B, lane 7). In 12.5% polyacrylamide gels (Figs. 1 and 2) uridylylated VPg is known
to migrate as a narrow band very close to the front. Large quantities of [α-32P]NTPs in the reaction mixture appear occasionally as a band just below the VPG-(pU)n. In Fig. 2B, this unspecified band has been cut off from the autoradiogram, although traces of it can be seen in lanes 6 and 7. In higher percentage gels (>13.5%), VPG-(pU)n separates well from the front but migrates as a broad band (see Fig. 3).

Characterization of the Nucleotidyl-Protein. The following experiments show that the 32P-labeled complex identified in Fig. 1A or Fig. 2 is VPG-pUpU. (i) The material was sensitive to proteolytic cleavage with Pronase (data not shown). (ii) Incubation of the reaction mixture with a mixture of RNase T1 and T2 yielded a product whose electrophoretic migration was slightly increased (Fig. 3A, lane 2) and was similar to that of authentic VPG-pUpU (Fig. 3A, lane 5; see refs. 10 and 11). (iii) Incubation of the reaction mixture with nuclease P1 yielded material with an electrophoretic mobility (Fig. 3B, lane 2) of authentic VPG-pUpU (Fig. 3A, lane 5; see refs. 10 and 11). (iv) Digestion of gel-purified VPG-pU, with Pronase yielded a single product (Fig. 4A Middle; Fig. 4B, lane 1) with the assumed structure (aa)n-(pU)n.

Cleavage of this product with RNase T2 and snake venom exonuclease yielded pUpU (Fig. 4B, lane 2) as the only labeled product, which suggests that (aa)n-(pU)n is (aa)n-pUpU. In agreement, cleavage of (aa)n-(pU)n with nuclease P1 produced (aa)n-pU and pU (Fig. 4B, lane 3), two components that comigrated on a DEAE-Sephadex column at pH 5.5 (Fig. 4A, Bottom; ref. 11). These results prove that the nucleotidyl moiety of the nucleotidyl-protein is pUpU. (c) Microsequence analysis of [3H]lysine-labeled complex isolated by preparative gel electrophoresis produced [3H]lysine at positions 9, 10, and 20, as counted from the NH2 terminus (Fig. 5). Such release of [3H]lysine corresponds to the known amino acid sequence of VPG (2, 19). In addition, stepwise Edman degradation of 32P-labeled nucleotidyl-VPG yielded 32P radioactivity at cycle 3 (data not shown), as expected from the known site of uridylylation in VPG (19).
Conditions of VPg-pUpU Synthesis. Virus-specific RNA synthesis—that is, polynucleotide chain elongation—is dependent upon Mg²⁺ ions, with an optimal concentration of 3 mM if the incorporation of NTPs is measured (21) and of 5–7 mM if full poliovirus-specific transcripts are to be synthesized (22, 23). In contrast, the formation of VPg-pUpU occurs at a very low Mg²⁺ concentration (0.2 mM). Of the three concentrations measured (0.2, 3.7, and 7.2 mM), 3.7 mM Mg²⁺ yielded the most VPg-pUpU (data not shown). Incubating the crude membrane fraction with trypsin (50 μg/ml for 20 min at 30°C) or heating the complex to 60°C for 1 min abolished its ability to synthesize VPg-pUpU.

Whereas poliovirus-specific RNA synthesis (polynucleotide chain elongation) readily occurs in membrane-free solutions in vitro in the absence or presence of mild detergent (7, 17, 21, 23, 24), the synthesis of VPg-pUpU was exquisitely sensitive to the presence of detergents. Addition of NP-40 to 0.1% or higher or of deoxycholate to 0.01% completely inhibited the transfer of label from [α-32P]UTP to VPg. This observation strongly suggests that intact membranes are essential for the synthesis of nucleotidyl-VPg, possibly because the precursor(s) to VPg can only accept uridylate and be cleaved to VPg if they are partially embedded in the membrane. The synthesis of single-stranded RNA in a crude replication complex has previously been observed to be sensitive to the presence of detergents (18), but it is unclear whether this phenomenon is related to the one described here.

**DISCUSSION**

We have shown that a crude membrane fraction isolated from poliovirus-infected cells can synthesize a nucleotidyl-protein, the properties of which identify it as VPg-pUpU. To the best of our knowledge, the formation of the nucleotide-linked poliovirus-specific protein has not been observed in vitro previously.

The yield of in vitro synthesized VPg-pUpU is low. This is surprising because the crude membrane fraction contains all of the P3-9, the putative precursor for VPg, synthesized during viral replication (4, 6). We do not know the activity that catalyzes the formation of the uridylyl-tyrosine bond, nor do we know whether this activity is virus encoded or of cellular origin. The low yield of the nucleotidyl-protein suggests that during the preparation of the crude membrane fraction, some factor(s) required for the formation of the phosphodiester bond between the O⁶-hydroxyl of tyrosine and pU (25) is diluted or inactivated. Reconstitution experiments of various cellular components have not increased the yield of VPg-pUpU. Our present data do not exclude the possibility that the VPg-pUpU was formed by some unknown exchange reaction between preexisting nucleotidyl-protein and [α-32P]UTP. We consider this highly unlikely because we have consistently failed to detect uridylylated poliovirus-specific proteins in various fractions of infected HeLa cells (2, 4, 6).

The observation that the synthesis in vitro of VPg-pUpU is dependent upon intact membranes and that it can occur at a low Mg²⁺ concentration suggests that this process is distinct from RNA synthesis catalyzed by the viral RNA polymerase P3-4b. We have been unable to chase the VPg-pUpU into detectable, larger RNA strands, although in the presence of UTP and [α-32P]ATP very small amounts of a larger oligonucleotidyl-protein, thought to be VPg-pU-U-A-A-A-A, can be observed (unpublished results). Our inability to chase the VPg-pUpU into RNA strands is unexplained. It may result from the action of an unlinking activity (26, 27) that in vitro rapidly cleaves VPg from poliovirus RNA but not from a mono- or oligonucleotidyl-protein (28, 29). Alternatively, it may reflect an inefficiency of the system to utilize the small amount of VPg-pUpU for elongation. Such inefficiency has also been reported for the in vitro synthesis of protein-linked adenovirus DNA; replication in this in vitro system also utilized only a very small fraction of newly formed nucleotidyl-protein [pTP-p(dC)] primer (30). Moreover, abortion of RNA synthesis immediately after initiation (at the level of di-, tri-, or tetrancleotides) appears to be a phenomenon observed in different transcription systems (for a recent discussion, see ref. 31).

Several distinct bands migrating reproducibly at positions slower than VPg-pUpU can be observed in electropherograms of materials immunoprecipitated from the membrane fraction with anti-VPg(N10) or anti-VPg(C7) (see Fig. 2B). These bands do not immunoprecipitate again with these antisera after they have been electroeluted from the gels. Because they can be labeled also with [α-32P]CTP (Fig. 2B, lane 5) and because they are sensitive to treatment with RNase (data not shown), we assume that they are RNAs that coprecipitate out of the reaction mixture together with VPg-pUpU and other unidentified materials seen at the upper portion of the gels. The yield of the RNAs is too low to identify them as virus-specific sequences, a phenomenon also observed by Baron and Baltimore (8).

Poliovirus RNA replication is thought to occur in a membranous environment (6, 7), and the product of replication is...
FIG. 6. A model of events leading to the initiation of poliovirus plus-strand RNA synthesis. P3-9 is the membrane-bound poliovirus protein, the COOH terminus of which (wavy line) is VPg (4, 6). Tc is the viral proteinase (P3-Tc; refs. 9 and 30); 4b is the viral primer-dependent RNA polymerase (P3-4b; refs. 9, 13, 15, 24, and 33); X is a viral polypeptide (P2-X; ref. 9) of unknown function, and the host factor is a cellular protein thought to be involved in initiation of minus-strand RNA synthesis (21, 23). This model may account also for the initiation of minus strands that occur on the 5’-terminal poly(U) of virion RNA leading to the synthesis of the 5′-terminal poly(A) of minus strands. For details, see text.

VPG-linked plus-strand and minus-strand RNA. Our model of the events leading to initiation of plus-strand RNA synthesis is shown in Fig. 6. This model accommodates all facts known to us that have accumulated during the last few years (reviewed in ref. 1). We speculate that the first step in initiation is the uridylylation of a Tyr residue near the COOH terminus of the membrane-bound polypeptide P3-9. This modification may activate the Gln-Gly cleavage site within P3-9, possibly by conformational change, such that VPg is cleaved from P3-9 by the virus-encoded proteinase P3-Tc (4, 6, 32). The newly formed VPg-pU or VPg-pUpU can now function as a primer for the primer-dependent, virus-encoded RNA polymerase P3-4b (23, 24, 33). Whether cleavage by P3-Tc occurs prior to or after initiation may not matter, but this cleavage is probably essential for the nascent RNA strand to be released from the membrane anchor. In agreement with this hypothesis, we have never found polypeptides larger than VPg attached to nascent RNA. A host factor has been shown to be involved in the initiation of minus-strand synthesis in vitro (23, 34), but whether it is involved in plus-strand synthesis is unknown. The function of the virus-encoded polypeptide P2-X is obscure but this protein has been found to always copurify with P3-9 (6).

The VPg-pUpU described in this communication may be the primer molecule necessary to initiate strand elongation by the viral polymerase P3-4b. In this regard, poliovirus RNA replication may resemble a process described for adenovirus DNA replication (refs. 1 and 35 and references therein).

While this manuscript was being prepared, preliminary data were published that suggested that a nucleotidyl-protein similar to the one described here can be isolated from a crude membrane fraction of encephalomyocarditis virus-infected cells (36).

Note Added in Proof. Crawford and Baltimore (37) have found VPg-pUpU in poliovirus-infected HeLa cells.

We are indebted to Bruce Erickson and Bruce Merrifield for instructing us in peptide synthesis and Bert L. Semler for anti-VPg(CT) antibodies. We thank Bert Semler and Joan Wellink for stimulating discussions and Marlies Schmidt for laboratory assistance. This work was supported by U.S. Public Health Service Grants AI 15122 and CA 28146 and by the U.S. Department of Energy.