The Formation of Poliovirus Particles in Association with the RNA Replication Complexes

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(Accepted 18 May 1973)

SUMMARY

Poliovirus particles are associated with the RNA replication complexes and can be identified by velocity sedimentation and electron microscopy. After pulse-labelling with \[^{3}H\]-uridine beginning 3.5 h after infection, these virus particles have a radioactivity to infectivity ratio three- to eightfold higher than virus particles from other cell fractions. A specific association between the RNA replication complexes, poliovirus particles and smooth cytoplasmic membranes is shown by isopycnic sedimentation and partial resistance to enzyme digestion. These results strongly suggest that virus RNA replication and particle formation are coupled processes which occur in association with smooth cytoplasmic membranes.

INTRODUCTION

Results of cell fractionation studies indicate that synthesis of poliovirus proteins and replication of virus RNA are associated with distinct membranous components (Caliguiri & Tamm, 1969, 1970a, b). The virus RNA replication complexes are associated with smooth cytoplasmic membranes that proliferate after infection (Caliguiri & Mosser, 1971; Mosser et al. 1972a; Mosser, Caliguiri & Tamm, 1972b). Virus protein synthesis takes place on polysomes associated with the rough microsomal fraction (Caliguiri & Tamm, 1969, 1970a, b). These findings are consistent with the results of the ultrastructural studies that showed the spatial separation of virus polysomes near the periphery of the cell and the smooth membranous bodies in the central region of infected cells (Dales et al. 1965). This evidence excludes the possibility of a single membrane-bounded, virus-synthesizing structure as proposed by Penman, Becker & Darnell (1964); however, these results do not indicate the site of virus formation.

Structural proteins and the structural protein precursor, VPO, are associated with the virus RNA replication complexes (Caliguiri & Mosser, 1971). Electron microscopy indicates that virus particles and empty capsids are in close proximity to the smooth membranes in the centrosphere region of the cell (Dales et al. 1965). Furthermore, results of kinetic experiments show that newly synthesized virus RNA is rapidly incorporated into particles, whereas virus capsid protein is incorporated at a much slower rate (Baltimore, Girard & Darnell, 1966). This evidence suggests but does not prove that particle formation begins at the site of virus RNA replication on the smooth cytoplasmic membranes (Caliguiri & Tamm, 1970b; Caliguiri & Mosser, 1971).

The present report describes an analysis of the structures associated with the virus RNA replication complexes to determine whether particle formation occurs at the site of virus RNA synthesis. After lysis of membranes, poliovirus particles can be isolated from the

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replication complex and identified by electron microscopy and infectivity. The labelled RNA to infectivity ratio is higher in the particles isolated from the replication complexes than in particles isolated from other cell fractions. These results indicate that particle formation and virus RNA synthesis are coupled processes which occur in association with smooth membranes.

**METHODS**

**Cell and virus culture.** The conditions of growth of S 3 HeLa cells and of infection of the cells with poliovirus type 2 (P712-ch-2ab) were described previously (Caliguiri & Tamm, 1970a).

**Chemicals and buffers.** [3H] and [14C]-reconstituted protein hydrolysates and [5-3H]-uridine were obtained from Schwarz/Mann, Orangeburg, New York. [35S]-Methionine was obtained from Amersham Searle, Des Plains, Illinois. Actinomycin D was obtained through the courtesy of Dr A. F. Wagner of Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey. Pancreatic ribonuclease and trypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Ultra-pure sucrose was obtained from Schwarz/Mann, Orangeburg, New York. RSB is 0.01 M-tris pH 7.4, 0.01 M-KCl and 0.0015 M-MgCl₂. SSE is 0.15 M-NaCl, 0.015 M-sodium citrate and 0.01 M-disodium ethylenediaminetetraacetate (EDTA).

**Radioactive labelling.** Poliovirus-infected cultures of S 3 HeLa cells (1.5 to 4.7 x 10⁶ cells) in Eagle's spinner medium containing actinomycin D (5 μg/ml) received [3H]-uridine (10 to 20 μCi/ml) at 3 h 30 min after infection and the cultures were harvested at different times by pouring on to crushed frozen PBS. When cultures were labelled with [35S]-methionine (2 to 5 μCi/ml), medium containing 1/5 of the normal concentration of methionine was used. In pulse-chase experiments, cultures received [35S]-methionine and at the end of the pulse period the cells received 1 mM unlabelled methionine, were centrifuged and resuspended in regular Eagle's spinner medium. In experiments in which [14C]-amino acid mixture (2 μCi/ml) was used, cultures were suspended in medium containing 1/5 th of the normal concentration of amino acids.

**Cell fractionation.** Techniques by which cytoplasmic extracts were prepared and the smooth and rough microsomes separated in discontinuous sucrose gradients have been described previously (Caliguiri & Tamm, 1970a). Isopycnic sedimentation of the smooth membrane fraction in 5 to 20 % potassium tartrate/RSB has been described (Caliguiri & Mosser, 1971). Velocity sedimentation of virus-specific structures in 15 to 30 % sucrose/RSB gradients after deoxycholate (DOC) treatment of membrane pellets has also been described (Caliguiri & Mosser, 1971).

**Electron microscopy.** Samples were applied to grids with carbon-coated formvar films and stained with a saturated solution of uranyl acetate. Specimens were examined in a Philips EM 300 microscope.

**SDS-acrylamide gel electrophoresis.** Techniques for preparation of samples, gel electrophoresis, and determination of radioactivity were described previously (Caliguiri & Mosser, 1971).

**RNA extraction.** The smooth membrane fractions were isolated as described above and after DOC treatment virus specific structures were separated by velocity sedimentation in sucrose gradients. The fractions in the 100 to 300 S regions were pooled and RNA was extracted with phenol, SDS, and diethyl pyrocarbonate as described previously (Caliguiri & Tamm, 1970b). The extracted RNA was centrifuged in 15 to 30 % sucrose/SSE gradients at 246000 g (R₉₀) for 3.5 h in a Spinco SW 56 rotor.
RESULTS

Effects of enzymes on the membrane-bound ribonucleoprotein structures

The RNA-protein complexes associated with smooth membranes are heterogeneous with a buoyant density in the range from 1.04 to 1.10 g/cm³ in potassium tartrate (Caliguiri, & Mosser 1971). Fig. 1A shows the distribution of labelled RNA and protein in a potassium tartrate gradient after isopycnic sedimentation. Treatment of the smooth microsomal pellet with trypsin prior to isopycnic sedimentation reduces the amount of protein in the complexes by 60% with only a 15% reduction in RNA. The buoyant density of the membrane-bound RNA-protein complexes is not altered significantly after trypsin treatment (Fig. 1B). In contrast, ribonuclease digestion of the smooth microsomal pellet before centrifuging reduces the RNA radioactivity by 35% and the ribonucleoprotein peak shifts to a lower buoyant density (Fig. 1C). When membranes are lysed before ribonuclease treatment, over 90% of the labelled RNA is digested. These results suggest that the cytoplasmic membranes partially protect the ribonucleoprotein structures from enzymatic digestion and that the buoyant density of these membrane-bound RNA-protein complexes is in part determined by the amount of RNA present.

Virus-specific RNA and polypeptides associated with smooth microsomes

After a short pulse of labelled uridine, the predominant species of RNA in the smooth microsomal fraction is replicative intermediate RNA (Caliguiri & Tamm, 1970b). It was necessary to determine if completed, 35S virus RNA was present in this fraction after a longer pulse. Fig. 2 compares the virus-specific RNA's in the smooth microsomal fraction after a 2 min and 10 min pulse of [³H]-uridine. The RNA labelled after 2 min sediments heterogeneously as a broad peak from 18 to 60 S and about 20% of the RNA is resistant to ribonuclease digestion (Fig. 2A). This RNA has the characteristics of replicative intermediate RNA as determined by precipitation in high salt. There is more label incorporated after a 10 min pulse and it sediments as a large peak at 35 S that is over 95% ribonuclease-sensitive (Fig. 2B). These results establish the presence of single-stranded, 35S virus RNA in the RNA-protein complexes associated with smooth cytoplasmic membranes.
Both structural and non-structural polypeptides associated with the smooth microsomal fraction of infected cells are labelled after a 1.5 h pulse of radioactive amino acids (Caliguiri & Mosser, 1971). To determine the time course of association of these polypeptides with the smooth membrane fraction, pulse-chase experiments were performed. Fig. 3 shows that after a 5 min pulse of $^{35}$S-methionine a major polypeptide with an estimated mol. wt. of 105,000 is detectable. The mol. wt. of this polypeptide corresponds to that of a precursor of capsid proteins, NCVPI (Jacobson, Asso & Baltimore, 1970). The incorporation of labelled amino acid into the smooth membrane fraction increases during the 60 min chase period and indicates an intracellular pool of labelled virus polypeptides. These results confirm the previous observation that labelled amino acids were incorporated into the smooth membrane fractions throughout the chase period (Caliguiri & Tamm, 1970a). During a 60 min chase period, the large precursor polypeptide disappears and polypeptides that co-migrate with the three major structural proteins are found. The large peak in the region of structural protein 1 probably contains three polypeptides, VPo, VP1, and NCVPX (Jacobson et al. 1970; Caliguiri & Mosser, 1971). There is a peak at fraction 33 that represents a non-structural polypeptide and has an estimated mol. wt. of 67,000. Several other nonstructural polypeptides with estimated mol. wts. of 80,000, 56,000 and 19,000 are also present. Similar results were obtained when an inhibitor of protein synthesis, cycloheximide, was added during the chase period, which confirms the presence of an intracellular pool of virus polypeptides. Thus, the predominant polypeptides associated with the replication complexes are the structural polypeptides and this suggests the presence of virus particles or particle precursors.

**Structures associated with the RNA replication complex**

Virus-like particles were observed by electron microscopic examination of the region of sucrose velocity gradients which contained the RNA replication complex released from the deoxycholate-treated smooth membrane fraction (Fig. 4 A, B, C). Fractions from the
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Fig. 3. Electrophoresis of the polypeptides in the smooth cytoplasmic membrane pellet. The smooth membrane pellets were analysed by electrophoresis on 7.5% acrylamide-SDS gels with [3H]-labelled, purified poliovirus as marker. The origin of the gels is to the left.

100 to 250 S region of sucrose gradients were examined after sedimenting, and showed numerous particles 250 to 300 Å in diam. with the morphology of poliovirus (Fig. 4A, C); some of the particles were in clusters associated with strands of thread-like material (Fig. 4C). Some particles appeared to be penetrated by the stain, resembling empty capsids. Prior to centrifuging, similar particles were present in low concentration in the peak fractions of the large replication complex (Fig. 4B). Single particles, rather than clusters, were often found in the unpelleted material, suggesting that aggregation may occur when the particles are centrifuged. In addition to the particles looking like full and empty capsids, structures were also observed which were of similar size but had a less well-defined appearance. For comparison, when the rough microsomal fraction was lysed and the labelled RNA analysed in a similar velocity gradient, the predominant structures were polyribosomes with some virus particles also present (Fig. 4D). This fraction has been shown to contain 60% of the total infective virus (Caliguiri & Tamm, 1969).

Fig. 5 shows the sedimentation pattern of labelled RNA and protein from a DOC-treated smooth microsomal pellet. After velocity sedimentation in sucrose gradients, the
Fig. 4. For legend see opposite page
Fig. 5. Effects of ribonuclease on the structures associated with the membrane-free RNA replication complex isolated by velocity sedimentation. The smooth cytoplasmic membrane fraction was isolated, treated with DOC, and one sample received ribonuclease (10 μg/ml) before both samples were incubated for 15 min at 4 °C. The top of the gradient is to the right. ●—●, [3H]-uridine radioactivity; ○—○, [14C]-amino acid radioactivity.

Fig. 6. Infectivity associated with the membrane-free RNA replication complex isolated by velocity sedimentation. Each fraction of the sucrose gradient was assayed for infectivity on HeLa cell monolayers and expressed as plaque forming units (p.f.u.)/ml. The top of the gradient is to the right.

distribution of labelled RNA is similar to that described previously for virus RNA polymerase activity (Caliguiri & Mosser, 1971). The two peaks of labelled RNA represent two virus RNA replication complexes that differ functionally as well as structurally (L. A. Caliguiri, unpublished results). A large replication complex is present in the 100 to 300 S region and a small complex sediments at less than 70 S. There is a peak of labelled protein near the 150 S

Fig. 4. Virus-specific structures isolated from membrane fractions by velocity sedimentation after lysis of membranes.

(A) Sample pelleted from the 100 S to 250 S region after membrane lysis and velocity sedimentation. Numerous particles 250 to 300 Å in diam., with the appearance of poliovirus particles, are present. Some of the particles are partially penetrated by the stain.

(B) The same sample as described in A but without pelleting. Single virus-like particles and thread-like material are present, as well as particles similar to virus in size but with poorly defined structure (arrows).

(C) The same sample as described in A. A cluster of virus-like particles 250 to 300 Å in diam. is adjacent to thread-like material. Some of the particles have electron-dense centres indicating penetration by uranyl acetate.

(D) Rough microsomal fraction. Sample pelleted from the 350 S region after membrane-lysis and velocity sedimentation. A cluster of virus-like particles 250 to 300 Å in diam. (arrow) is shown as well as the ribosomes in chains and clusters.
Table 1. Infectivity and radioactivity in virus particles isolated from different cell fractions

<table>
<thead>
<tr>
<th>Sample*</th>
<th>2 min pulse†</th>
<th>5 min pulse</th>
<th>30 min pulse</th>
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<tbody>
<tr>
<td></td>
<td>p.f.u. x 10^6</td>
<td>ct/min</td>
<td>p.f.u. x 10^6</td>
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<tr>
<td>Replication complexes</td>
<td>18</td>
<td>48</td>
<td>65</td>
</tr>
<tr>
<td>Polysomal fraction</td>
<td>590</td>
<td>5</td>
<td>900</td>
</tr>
<tr>
<td>Cytoplasmic extract</td>
<td>——</td>
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<td>1100</td>
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* Ribonuclease-resistant 150 S structures were isolated from various cell fractions as described in Fig. 5 and analysed for infectivity and radioactivity.
† Actinomycin-treated infected cultures received [3H]-uridine (30 μCi/ml) at 3.5 h after infection.

region of the gradient with a shoulder extending to the bottom (Fig. 5A). If the sample is digested with ribonuclease before sedimentation, a single peak of labelled RNA remains which coincides with the peak of protein (Fig. 5B). This ribonuclease-resistant peak co-sediments with purified poliovirus at about 150 S.

Analysis of a similar gradient for infectivity shows a single peak of infectivity in the 150 S region (Fig. 6). In other experiments, the sedimentation and infectivity of this peak did not change after trypsin or ribonuclease treatment. The infective structures probably represent the ribonuclease-resistant ribonucleoprotein structures described above and co-sediment with purified, labelled poliovirus, indicating that the structures observed after ribonuclease treatment are poliovirus particles.

It seemed likely that virus assembly may be initiated in association with the replication complexes. To establish the site of formation of virus particles, infected cells were pulse-labelled with [3H]-uridine for varying lengths of time beginning at 3.5 h after infection. The ribonuclease-resistant 150 S particles were isolated from various cell fractions and analysed for radioactivity and infectivity. Virus particles isolated from their site of formation should have the highest radioactivity to infectivity ratio. Table 1 shows that during the interval from 3.5 to 4 h after infection there is an increase in the amount of infectivity in each fraction analysed. During each of the labelling periods, the ratio of radioactivity to infectivity is highest in the particles isolated from the replication complexes. The radioactivity to plaque forming unit ratio of virus particles from the replication complexes is about eightfold higher than that of particles associated with the polysomal fraction and about threefold higher than that of particles in the cytoplasmic extract. These results support the view that the replication complexes are also the site of particle formation.

DISCUSSION

The infectivity, morphology, and sedimentation properties of the ribonuclease-resistant structures associated with the poliovirus RNA replication complexes show that these structures are poliovirus particles. The strong binding of the RNA replication complexes and particles to membranous structures is indicated by their continued association after isopycnic sedimentation in potassium tartrate. In addition, the membrane-bound RNA replication complexes are partially resistant to digestion by ribonuclease and trypsin, which also may result from intimate association with membranes. Furthermore, the particles associated with the replication complexes contain the highest proportion of newly synthesized virus RNA. Taken together, the evidence strongly suggests that virus RNA replication
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and particle formation are coupled processes that occur in association with smooth cytoplasmic membranes. The membranes may provide a fixed point of attachment for the efficient interaction of nascent virus RNA and virus polypeptides during the process of particle formation. Previous results of kinetic studies on the incorporation of RNA and protein into particles (Baltimore et al. 1966) and ultrastructural studies showing virus particles and empty capsids in close proximity to membranes (Dales et al. 1965) are also consistent with this view.

A major problem in the isolation of a purified virus RNA polymerase is the presence of virus particles. In previous studies it was shown that the predominant polypeptides associated with the RNA replication complex after membrane lysis are structural proteins (Caliguiri & Mosser, 1971). The present results suggest that most structural virus polypeptides associated with the replication complex may be components or precursors of virus particles, although the possibility remains that they may also participate in the polymerase reaction. A recent report indicates that a polycytidylate-dependent polymerase can be isolated from EMC virus-infected cells free of structural polypeptides after treatment of the smooth membrane fraction with nuclease and SDS (Rosenberg et al. 1972).

The mechanism by which virus formation occurs is not entirely clear. Jacobson & Baltimore (1968) suggest that empty capsids are the immediate precursors of virus particles. However, results with poliovirus grown in MiO cells showed that empty capsids were not detected in these cells (Ghendon, Yakobson & Mikhejeva, 1972). Electron microscopic examination reveals structures resembling empty capsids associated with the RNA replication complex; however, it has not been possible to isolate empty capsids from the RNA replication complex (Caliguiri & Mosser, 1971, and Fig. 5). Our findings suggest that particle formation occurs by association of structural subunits with nascent RNA. After a short pulse of labelled amino acids the structural polypeptide precursor, NCVP1, is the major polypeptide associated with the RNA replication complex and it may associate with nascent RNA before being cleaved during virus formation. Such a mechanism would provide for aggregation of protein subunits on nascent RNA in the membrane-bound replication complex. However, the present evidence does not exclude the possibility that empty capsids are the functional precursors of virus particles but are unstable under certain conditions. More evidence is needed to decide which model of virus formation is correct.

We thank Dr Igor Tamm for his encouragement and many helpful discussions. The excellent technical assistance of Toyoko Kikuchi, Roberta Taylor, and Ann Erickson is gratefully acknowledged.

This investigation was supported by Research Grant AI-03445 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and by Contract AT (11-1)-3504 from the U.S. Atomic Energy Commission.

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(Received 2 April 1973)