Poliovirus Proteins Associated with the Replication Complex in Infected Cells

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(Accepted 4 September 1975)

SUMMARY

Viral polypeptides associated with the membrane-free replication complex of poliovirus RNA were multiple in nature. The structural protein precursors [VPo, VP1, VP3] predominated, and because they were found in a cytoplasmic component with the same S value and density as the replication complex are likely to be attached to it in vivo. They were not present in the form of empty capsids. The electrophoretic polypeptide pattern of the membrane-bound replication complex was similar but showed a predominance of NCVPX or VP1, unless the cells were slightly depleted in amino acids when the non-structural polypeptide NCVP2 became important. Cystine was the only amino acid capable of reversing this depletion effect on its own.

INTRODUCTION

The structures in which poliovirus RNA appears to be replicated are found attached to smooth membranes in the cytoplasm of infected cells (Girard, Baltimore & Darnell, 1967; Caliguiri & Mosser, 1971). After removing the membranes with mild detergent, a ‘replication complex’ of the viral replicative intermediate RNA with protein sediments heterogeneously at about 250S. When virus is grown in the presence of guanidine, the sedimentation coefficient of this structure becomes much greater (Baltimore, 1968; Huang & Baltimore, 1970).

The nature of the proteins in the replication complex is not known. Presumably they include poliovirus-specified replicase or replicase factors, which have not yet been defined, and possibly protein components specified by the host cell. In addition, a regulator protein (the ‘equestron’; Cooper, Steiner-Pryor & Wright, 1973) might also be expected to be associated with replicating poliovirus RNA.

A systematic examination has been made of the poliovirus polypeptides associated with various components of the infected cell in order to extend information on the gene functions of poliovirus. These components include the translation complex (Wright & Cooper, 1974), the DNA (Garwes, Wright & Cooper, 1975) and the nucleus (Abraham & Cooper, 1975a). This paper describes the poliovirus polypeptides associated with the replication complex when this is obtained free of, or still attached to, the smooth membranes.

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METHODS

Cells and virus, and procedures for infection, sucrose and CsCl gradient centrifugation and
gel electrophoresis, were as described previously (Wright & Cooper, 1974). All virus was
poliovirus type I (Mahoney), grown and assayed in U cells, which were cultured in Eagle’s
medium plus calf serum. In all figures, the top of a gradient or the electrophoretic anode is
at the right.

Preparation of replication complexes. Suspensions of cells infected with poliovirus at 0 °C
were incubated at 37 °C in Eagle’s medium lacking all amino acids except glutamine, but
containing 2% calf serum, 0.7 mg/ml NaHCO₃ and 0.2 μg/ml actinomycin D. Guanidine
hydrochloride (2 mM) was added to one half of the cultures after 195 min incubation,
puromycin hydrochloride (100 μg/ml) to all the cultures 20 min later (at 215 min) and
cultures harvested after a further 5 min (at 220 min). The [³H]-uridine (5 μCi/ml) was added
at 200 min, 5 min after the guanidine, but the [³⁵S]-methionine (12 μCi/ml) was added
30 min before the guanidine (at 165 min). Cytoplasmic extracts were centrifuged for 30 min
at 22000 g in the Spinco SW50.1 rotor, the pellets resuspended in RSB (0.1 M-NaCl,
1.5 M-MgCl₂, 10 mM-tris, pH 7.4), sodium deoxycholate and then Brij 58 added to 5 mg/ml
of each, and the preparations finally centrifuged through separate 15 to 30% sucrose
gradients (1.5 h at 50000 g and 5 °C in the Spinco SW25.1 rotor).

Preparation of smooth membrane fractions. Suspensions of cells infected with poliovirus
at 0 °C were incubated for 1.5 h at 37 °C in modified Eagle’s medium (see below) contain-
ing 2% calf serum, 0.7 mg/ml NaHCO₃, 0.5 μg/ml actinomycin D and 2 mM-guanidine
carbonate. This treatment was included to suppress host protein synthesis while inhibiting
virus replication (Summers, Maizel & Darnell, 1965), so that virus polypeptides could be
examined during the early part of virus replication once its inhibition was reversed by
washing. The medium was either Eagle’s lacking all amino acids except glutamine (but
containing certain other amino acids, if indicated), or Eagle’s lacking only leucine. At 1.5 h,
cells were washed twice in cold PBS, and resuspended at 37 °C in medium of the same
composition as before the wash but omitting actinomycin and guanidine. Label ([³H]-
leucine, 1 to 4 μCi/ml) was added and the cells chilled for harvesting at the times after
reversal that are stated in the figure legends. Smooth membrane fractions were obtained by
equilibrium sedimentation of cytoplasmic extracts in discontinuous isopycnic sucrose
gradients (Caligiuri & Tamm, 1970 a, b). Fractions 2 and 3 (the bands visible between 0 and
30% sucrose, corresponding to densities 1 to 1.15 g/ml and containing the smooth mem-
branes) were well separated from more dense bands; their suspended membranes were
pelleted and portions prepared for gel electrophoresis.

Electrophoretic markers. [¹²⁵I]-labelled standard proteins used for gel electrophoresis
(Abraham & Cooper, 1975 a) were Jasus lalandii haemocyanin (mol. wt. 86000), human serum
albumin (70000), pepsin (33600) and cytochrome c (12500). The [¹²⁵I] behaves in liquid
scintillation counting as intermediate in radiation energy between [²H] and [¹⁴C]; channel
settings of 050 to 175 V with 50% gain and 200 to 1000 V with 10% gain, typically gave
channel efficiencies for [¹²⁵I] of about 4% and 14%, respectively, in the Packard 3320
spectrometer, and with [²H] in three to fivefold excess over [¹²⁵I] only a small cross-channel
correction was necessary. The [³H]-amino acid mixture was ‘Reconstituted Protein Hydro-
lyzate’, Schwarz, Orangeburg, N.Y.
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Fig. 1. Rate zonal sucrose gradient analyses of poliovirus replication complexes, labelled with (a) [3H]-uridine or (b) [35S]-methionine, in the presence or absence of 2 mM-guanidine, as indicated. See Methods for procedures. Pooled fractions 5 to 12 of the guanidine-treated gradient of a were fixed in (c) 8% glutaraldehyde in RSB, or (d) in RSB after addition of 20 mM-EDTA, and banded in CsCl. Pooled fractions 4 to 14 from the guanidine treated (e) and the untreated (f) gradients of b were similarly fixed in RSB and banded in CsCl. The right-hand ordinate of b refers to fractions 20 and above; each division represents 4 ct/min × 10−4.
RESULTS

Characterization of the replication complexes (labelling with [3H]-uridine)

The results of several experiments are illustrated by Fig. 1 (a, c and d); the detailed procedure is given in Methods. In brief, infected cells labelled with [3H]-uridine at mid-cycle, in presence and absence of guanidine, were treated with puromycin to destroy polysomes, and membranous structures bearing the replication complexes (P-20 pellets, Girard et al. 1967) sedimented from the separated cytoplasm. These pellets were treated with mild detergent (DOC/Brij) and analysed on separate sucrose gradients (Fig. 1a). The broad peaks of label between fractions 5 and 15 represent the replication complexes, large multi-stranded RNA components in which labelled uridine first appears. Guanidine inhibits the incorporation of label considerably, and increases the S value of the replication complex.

Fractions 5 to 12 of each gradient were pooled, a portion fixed with glutaraldehyde and analysed in CsCl gradients as previously described (Wright & Cooper, 1974). In each case a single peak at a density of 1.54 g/ml was obtained, although only the guanidine-treated sample is shown in Fig. 1c. Some of this sample was also adjusted to 20 mM-EDTA before fixation (Fig. 1d), when most of the label appeared at 1.44 g/ml, with much less at 1.40 g/ml. The previously reported density of the replication complex, fixed with or without EDTA, or formed in presence of guanidine, was 1.44 g/ml (Baltimore & Huang, 1968; Huang & Baltimore, 1970). The structures found in the present work may contain double-stranded regions and correspondingly less attached protein. The possibility that the material fixed comprised largely polyribosomes (also 1.54 g/ml) could be excluded for two reasons. First, the cells were treated with puromycin at a concentration (100 µg/ml) sufficient to break down polysomes (Latham & Darnell, 1965). Secondly, the cells were incubated in 0.2 µg/ml actinomycin D for over 3 h before labelling, and hence the only labelled RNA in polysomes could be vRNA; if the puromycin treatment had failed for some reason, EDTA would have produced a band at 1.40 g/ml (the density of vRNA).

Poliovirus polypeptides associated with the replication complexes
(labelling with [35S]-methionine)

Experiments closely similar to those using uridine were repeated with [35S]-methionine as label (Fig. 1b, e and f). In this case the label was added before the guanidine to allow for its slower entry into the replication complex (see Methods). In Fig. 1b, guanidine is seen to inhibit the incorporation of amino acid slightly; in contrast to the result with uridine label, there was no noticeable peak of labelled protein in the region of the replication complexes. However, some label was present, and fractions 4 to 14 of each gradient were pooled, a portion of each pool fixed and banded in CsCl (Fig. 1e, f) and the remainder co-electrophoresed with [3H]-labelled empty capsids as a marker for pure VPo, VP1 and VP3 (Fig. 2). In the gel of the no-guanidine culture (Fig. 2b), 63% of the recovered label was in the VPo, VP1 and VP3 region, while in the CsCl gradient of the same preparation (Fig. 1f), 63% of the label was in the component of density 1.54 g/ml, the density of the replication complexes in these experiments. Thus at least some VPo, VP1 and VP3 existed at the density of the replication complex in untreated cultures. Since the identical comparison for the guanidine-treated cultures (Fig. 2a and 1e) gave values of 45% and 56%, a similar conclusion for the guanidine-modified replication complex is not certain.

The complex [VPo, VP1, VP3] attached to the 45S ribosomal subunits in infected cells was released as a 6S particle on disruption of the subunit with ribonuclease, EDTA, LiCl and DOC, but the same treatment given to incipient polyribosomes also carrying [VPo,
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VP1, VP3] resulted only in heterogeneous structures that appeared to be non-specific aggregates of [VPo, VP1, VP3] monomers (Wright & Cooper, 1974). Fig. 3a shows that similar treatment of protein-labelled replication complexes grown in the absence of guanidine met with a similar lack of success, namely that the bulk of the label after disruption sedimented in a broad band at 100 to 120S, with very little material at 6S or at 65S; Fig. 3c shows a marker gradient of purified empty capsids (65S). The guanidine-modified replication complex gave a variety of products (Fig. 3b). However, gel electrophoresis of the 110S peak of Fig. 3a (Fig. 2c) showed that it now represented almost exclusively VPo, VP1 and VP3 (with some VP2 presumably obtained by cleavage of VPo; Jacobson, Asso & Baltimore, 1970), and had lost virtually all the other polypeptides present in the starting material (Fig. 2b). As with the 45S and 60 to 80S ribosomal structures, the experiment of Fig. 3 provides important additional evidence that, although the replication complex contained VPo, VP1 and VP3, these polypeptides were not present in the form of empty capsids.

Poliovirus polypeptides associated with smooth membrane fractions from infected cell cytoplasms

The results of Caliguiri & Tamm (1970a, b) led to the expectation that poliovirus polypeptides associated with the smooth membrane fractions (isolated from infected cell cytoplasms at a density of 1.10 to 1.15 g/ml by isopycnic sucrose gradients) would include those involved with replicase activity, as these membranes appear to carry the replication complex. However, two independent gel electrophoretic analyses of such polypeptides specifically labelled with amino acids gave different gel patterns: those of Cooper et al. (1971) contained predominantly large mol. wt. material (mainly NCVP2, about 80000) while those of Caliguiri & Mosser (1971) showed smaller molecules (mainly NCVPX, about 30000). In both cases, intermediate sized polypeptides were also present.

Our investigation of possible reasons for this discrepancy showed that it appeared to result from the amino acid composition of the medium in which the cells were incubated before addition of label. Cooper et al. (1971) pre-incubated in absence of all amino acids save glutamine, while Caliguiri & Mosser (1971) pre-incubated in 1/10 concentration of all amino acids. Fig. 4a shows a typical gel pattern of the smooth membrane fraction from infected cells pre-incubated in Eagle's amino acid mixture lacking only leucine. Despite an extended pre-incubation (4 h including the time in the presence of guanidine) before adding [3H]-leucine, the result is the same as that presented by Caliguiri & Mosser (1971), namely a predominance of viral protein migrating close to pepsin, i.e. NCVPX or VP1 (which could not be distinguished in these gels). In contrast, Fig. 4b shows a typical gel pattern of identical fractions from a similar experiment in which infected cells had been pre-incubated for a lesser time (2.5 h total) but in absence of all amino acids except glutamine. The result is the same as that published by Cooper et al. (1971), namely a predominance of NCVP2 and other viral polypeptides bigger than NCVPX/VP1.

Experiments not presented here have shown that other variables, e.g. the degree of suppression of host protein synthesis, the presence or absence of guanidine or actinomycin, the use of leucine as label (tested using [3H]-lysine in lysine-free medium) or the duration of the labelling period, were not responsible for the difference in polypeptide compositions shown in Fig. 4a and b. However, the difference in gel pattern appeared to follow the progressive depletion of amino acid(s), since 0.5 h pulses of [3H]-leucine given early in infection (in absence of all amino acids except glutamine) could result in an undepleted pattern (Fig. 4c), whereas a 0.5 h pulse given 2 h later in the same experiment was beginning to show the depleted pattern (Fig. 4d). Intermediate times of labelling gave intermediate patterns.
For legends see facing page.
Fig. 2. Polypeptide composition of poliovirus replication complexes labelled with \(^{35}S\)-methionine. (a) and (b), pooled fractions 4 to 14 from, respectively, the guanidine-treated and untreated gradients of Fig. 1 (b); (c), the 110S peak (pooled fractions 2 to 6) of the gradient of disrupted replication complex shown in Fig. 3 (a). Samples were concentrated by acid precipitation and co-electrophoresed in polyacrylamide gels with purified empty capsids labelled with \(^{3}H\)-amino acid mixture.

Fig. 3. Rate zonal sucrose gradient analyses of \(^{35}S\)-methionine-labelled poliovirus replication complexes after a disruption procedure. Samples were treated at 18 °C for 1 h with RNase (5 µg/ml), made 0.5% in Brij-58 and 45% in CsCl, and centrifuged at 170,000 g for 20 h in the Spinco SW65 rotor, after which all label was in the top two fractions. These fractions were dialysed for 1 h against 10 mM-tris, pH 7.4, 0.1 M-NaCl, 0.5% Brij-58, then centrifuged in parallel through 10 to 30% sucrose gradients in the same buffer for 47 h at 90,000 g in the Spinco SW27 rotor. (a) and (b), pools of fractions 4–14 from an experiment identical to that of Fig. 1 b, respectively labelled in the absence or presence of 2 mM-guanidine; (c) purified labelled empty capsids.

Fig. 4. Effect of amino acids on the poliovirus polypeptides associated with the smooth membrane fractions of infected cells. See Methods for procedures. Amino acids present and \(^{3}H\)-leucine labelling times (min at 37 °C after reversal of guanidine inhibition): (a) arg, cys, gln, his, ile, lys, met, phe, thr, trp, tyr, val, 150–180; (b) gln, 60–150; (c) gln, 30–60; (d) gln, 150–180; (e) cys, gln, 60–180; (f) gln, phe, thr, trp, tyr, val, 60–180. (c) and (d) are from one experiment, and (e), (f) and (g) from another. Left-hand ordinates are ct/min \([^{3}H]\)-leucine (solid line). Right-hand ordinates are ct/min \([^{125}\text{I}]\) (broken line) where p25I-labelled marker proteins were included in co-electrophoresis; these were: (a) haemocyanin and pepsin; (b) haemocyanin, human serum albumin and pepsin; (c), (d) and (f), haemocyanin, pepsin and cytochrome c.

(results not shown). Attempts to identify the amino acid concerned showed that, when amino acids were added back to Eagle’s medium lacking all amino acids save glutamine, only cystine was capable of preventing this depletion effect on its own (Fig. 4 e). The other amino acids of Eagle’s medium were added back two or three at a time, and gave a pattern for the smooth membrane fraction like that of Fig. 4 f. However, a mixture of all other amino acids except cystine (and leucine) was also capable of preventing this depletion effect (Fig. 4 g), and so the specificity does not appear to be absolute.
DISCUSSION

The presence of the virus proteins [VP0, VP1, VP3] apparently attached to ribosomal structures in the translation complex of poliovirus RNA (Wright & Cooper, 1974) has implicated this complex of proteins as the candidate for the equestron, a hypothetical regulator of poliovirus (Cooper et al. 1973). This paper confirms the prediction of the equestron model that [VP0, VP1, VP3] are also to be found with the same S value and density as the replication complex. However, their regulatory role, if any, remains undemonstrated in either the replication or the translation complex. In the replication complex, the presence of some VP2 (Fig. 2) indicates that some cleavage of VP0 has occurred, so that some or all of the structural unit precursors attached to the RNA may well be in the process of specific aggregation with vRNA and cleavage to form the capsid of mature virus particles (Caliguiri & Compan, 1973). At all events, their chemical properties indicate that they are not present as empty capsids, implying that empty capsids are not the precursors of virus particles; in support of this, Caliguiri & Mosser (1971) have shown that empty capsids do not bind to the replication complex.

Similarly, several other polypeptides are implicated by their presence in the smooth membrane fraction as candidates for the replicases or replicase factors of poliovirus. These polypeptides resemble those implicated as replicases by the correspondence between the sequence of genes in the genetic map (Cooper et al. 1971) and the sequence of translation products suggested by pactamycin experiments (Summers & Maizel, 1971; Taber, Rekosh & Baltimore, 1971), namely NCVPX and NCVP2 or cleavage products thereof. Unfortunately, none of these experiments can demonstrate which polypeptides actually function as replicases.

The effect of amino acid depletion on the virus proteins present in the smooth membrane fraction is unexplained, and introduces a complicating factor in interpreting the polypeptides found. It is an effect specific to this fraction, as the composition of whole cytoplasm was not noticeably affected (results not shown). The most effective amino acid in reversing this depletion effect was cystine, and cystine has been shown in other experiments to have an effect, presumably by means of conformational changes, on products of the structural protein gene of poliovirus (Pohjanpelto, 1958; McCahon & Cooper, 1970; Steiner-Pryor & Cooper, 1973). In addition, the pattern of RNA synthesis of cystine-dependent (ts) structural protein mutants is disturbed under restrictive conditions (Wentworth, McCahon & Cooper, 1968; Cooper, Stancek & Summers, 1970). Cystine could conceivably modify the polypeptide content and/or replicase activity of the replication complex by altering the loci attacked by the cleavage mechanism, or by a direct effect on any regulator that may affect replicase activities (Cooper et al. 1973), or by selectively altering the translation of NCVPX, which may be specified by a separate cistron (Abraham & Cooper, 1975).

Thus, there are several incompletely defined factors influencing the activities of poliovirus replicases. Such considerations make it clear that this system is unsatisfactory for analysis as it stands at present. Further progress on all the aspects discussed above is likely to depend on the availability of purified enzyme preparations in which replicase I and replicase II activities (Cooper et al. 1971), their templates and any regulator component have been separated chemically. Recently, Lundquist, Ehrenfeld & Maizel (1974) have observed a single poliovirus polypeptide (NCVp4) associated with a selected fraction having polymerase activity, but its relation to template or product (i.e. which replicase activity), other viral proteins or any other function remains unclarified.

P.J.W. was supported by a studentship of the C.S.I.R.O.
REFERENCES


(Received 10 March 1975)