A Poliovirus-induced Cytoplasmic Membrane Complex is Exploited by the RNA Polymerase of Superinfecting Mouse Elberfeld (ME) Virus

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SUMMARY

The preexistence of a cytoplasmic membrane complex in HEp-2 cells, induced by poliovirus when inhibited in its reproduction by guanidine, was a prerequisite for accelerated reproduction of superinfecting Mouse Elberfeld (ME) virus. Guanidine-inhibited poliovirus induced a membrane complex of 470S that was successively modified into a faster sedimenting membrane complex (up to 700S) by superinfecting ME virus and exploited for ME virus reproduction. The modified membrane complex was the site for ME virus-specific RNA polymerization characterized by the existence of in vivo and in vitro activity of ME virus RNA polymerase associated with the modified membrane complex. Proof of membrane-bound RNA polymerase and newly synthesized ME virus RNA including replicative intermediate led to the conclusion that superinfecting ME virus exploits the 'poliovirus/guanidine'-induced complex as the site of action of its replication complex.

Mixed infections with the two picornaviruses, poliovirus and Mouse Elberfeld (ME) virus, revealed that virus-specific membrane alterations of the infected cells may occur independently of virus reproduction. (i) In cells simultaneously infected with both viruses, ME virus reproduction is inhibited by interfering poliovirus. Under these conditions non-reproducing ME virus determines the membrane changes at the cell surface (Zeichhardt et al., 1982). (ii) In a mixed infection in the presence of guanidine, poliovirus reproduction is inhibited (Baltimore et al., 1963); however, reproduction of ME virus is advanced (Diefenthal et al., 1973; Zeichhardt et al., 1981). The reproduction of ME virus is accelerated by 1 h in a simultaneous mixed infection and by as much as 3 h when the cells are preinfected with poliovirus for at least 2 h. Under these conditions poliovirus is not rescued by superinfecting ME virus, i.e. complementation does not take place between ME and poliovirus reproduction. As we were able to show recently (Zeichhardt et al., 1981), poliovirus inhibited by guanidine, nevertheless, induces a cytoplasmic membrane complex of 470S (Fig. 1). In a superinfection with ME virus this complex is successively modified into an ME virus-specific membrane complex of 700S depending on the length of superinfection. In cells preinfected with poliovirus for 4 h the complex sediments at 700S after a 6 h superinfection with ME virus (Fig. 1), whereas in a single infection with ME virus the 700S complex is formed not earlier than 8 h post-infection. From this it has been concluded that ME virus is reproduced with a shortened latent period by exploiting the 'poliovirus/guanidine'-induced and preexisting membrane complex for its own reproduction. The present communication shows that the modified complex serves as a site for ME virus-specific RNA synthesis.

Growth of HEp-2 cells in monolayer cultures, propagation of poliovirus type 1 (strain Mahoney) and ME virus (laboratory strain), double infection experiments with both viruses and separation of cytoplasmic membrane complexes were performed as described previously (Zeichhardt et al., 1981). In brief, the conditions for superinfection were chosen according to the kinetics of reproduction of ME virus in poliovirus-preinfected cells. A division of the total
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Fig. 1. Superinfecting ME virus modification of a membrane complex of 470S induced by poliovirus in the presence of guanidine into an ME virus-specific membrane complex of 700S. Infected HEp-2 cells (m.o.i. of each of the viruses: 20) were Dounce homogenized in 1/15 M-phosphate buffer pH 7.2 with 4 mM-magnesium acetate and separated through 25 to 60% (w/w) sucrose gradients in 10 mM-Tris-HCl pH 7.2, 10 mM-MgCl₂, 80 mM-KCl for 3.5 h at 40000 rev/min (220000 gₑ) in an SW40 rotor (Spinco, Beckman). The gradients were monitored at 260 nm and calibrated with markers (poliovirus, 150S; phage T7, 487S; phage T2, 700S) according to Martin & Ames (1961). The figure is a superposition of two separation profiles. ---, Separation profile of homogenates of cells singly infected with poliovirus in the presence of guanidine for 8 h (P₈ + G); induction of a membrane complex of 470S. ----, Separation profile of homogenates of cells preinfected with poliovirus for 4 h and superinfected with ME virus for 6 h, with guanidine present throughout infection (P₄ME₆ + G); modification of the preexisting 470S complex into an ME virus-specific membrane complex of 700S. A single infection with ME virus for 8 h also leads to the formation of a 700S complex (not shown, see Zeichhardt et al., 1981).

In order to demonstrate that the modification of the 'poliovirus/guanidine'-induced 470S complex was correlated with ME virus-specific reproduction, both the 470S complex and the ME virus-modified 700S complex were isolated by sucrose density gradient centrifugation (Fig. 1) and analysed for ME virus RNA synthesis. The 470S complex, detectable as early as 4 h post-infection and up to 10 h post-infection with poliovirus in the presence of guanidine (Zeichhardt et al., 1981), was isolated after an 8 h infection (P₈ + G). The 700S complex resulted from a 4 h preinfection with poliovirus in the presence of guanidine followed by a 6 h superinfection with ME virus (P₄ME₆ + G). The in vivo activity of the ME virus RNA polymerase was measured by examining the newly synthesized viral RNA associated with the complex in the cell, whereas the in vitro polymerase activity was determined in a polymerase assay with isolated membrane complexes.

For analysis of viral RNA associated with the membrane complexes the viral RNA was radioactively labelled with [6-3H]uridine. Two h before the infected cells were pulsed, actinomycin D was added to the cultures. After the virus-induced and/or modified membrane complexes had been isolated from the sucrose gradients, the membrane-bound RNA was extracted by phenol (Noble et al., 1969) and examined by standard agarose–polyacrylamide gel electrophoresis according to Yogo & Wimmer (1975). The ME virus-modified 700S complex contained the three RNA species typical for picornaviruses: single-stranded (ss)RNA, double-stranded replicative form (RF) and multi-stranded replicative intermediate (RI) (Fig. 2a).
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Fig. 2. (a) ME virus RNA associated with the modified 700S complex as analysed by agarose-polyacrylamide gel electrophoresis. •, Analysis of the 470S complex from a P8 + G infection; ○, analysis of the 700S complex from a P4-ME6 + G infection. For the analysis of viral RNA synthesized in vivo the infected cells were pulsed with [6-3H]uridine (20 μCi/ml of growth medium, Amersham/Buchler) 3 h before harvest. Two h before pulsing, actinomycin D (Boehringer, Mannheim) was added to the cultures (5 μg/ml medium). The membrane complexes from the sucrose gradients (fractions 21 to 24 for the 470S complex, and fractions 13 to 17 for the 700S complex; see Fig. 1) were precipitated by ethanol (−20 °C). Precipitates were removed by centrifugation at 13 000 g, for 1 h at −15 °C. Pellets were dissolved in 0.5 ml TNE buffer (0.01 M-Tris-HCl pH 7.4, 0.1 M-NaCl, 1 mM-EDTA) and adjusted to 1% SDS. Ribosomal RNA of E. coli (1 A260) was added as carrier (courtesy of Dr R. Garrett). The RNA was phenol-extracted according to Noble et al. (1969), precipitated by ethanol and washed twice in a solution of 7 vol. ethanol/3 vol. 0.9 M-NaCl (−20 °C). The RNA was dissolved in electrophoresis buffer and separated in 0.5% agarose−1.8% polyacrylamide gels according to Yogo & Wimmer (1975). Bromophenol blue (B) was used as front marker. Gels were cut into slices of 2 mm and counted for radioactivity. (b) ME virus RNA polymerase activity associated with the modified 700S complex. This polymerase activity was tested in a standard polymerase assay that was established for detecting and optimizing ME virus-specific RNA polymerase activity bound to crude membranes of infected cells. The assay was based on measuring the conversion of [3H]GTP to an acid-insoluble product (method modified according to Baltimore, 1964; Ehrenfeind et al., 1970; Chinchar, 1978; M. W. Taylor, personal communication). Crude membranes were obtained from ME virus-infected cells grown as monolayers. At maximal polymerase activity (8 h post-infection) the cells were removed by a rubber policeman, washed in TNM buffer (10 mM-Tris-HCl pH 7, 10 mM-NaCl, 1.5 mM-MgCl2) and three times frozen and thawed. After centrifuging the cell debris for 5 min at 10000 g, the pellet was resuspended in 0.2 ml TN buffer (10 mM-Tris−HCl pH 8, 10 mM-NaCl). The total protein content of these crude membranes containing the polymerase was measured by the method of Lowry et al. (1951). A standard reaction mixture of 100 μl contained for maximal incorporation: 25 μl of crude membranes (6 mg total protein/ml), 25 μl 65% (w/w) sucrose in double-distilled water, 80 mM-Tris−HCl pH 8, 8 mM-MgCl2, 25 mM-KCl, 12.5 μg/ml actinomycin D (Boehringer, Mannheim), 7.5 μg/ml pyruvate kinase (Sigma), 0.65 mM-phosphoenolpyruvate (Paesel), 0.6 mM each of ATP, CTP and UTP (Boehringer, Mannheim), and 0.007 μM-[aH]GTP (14 to 23 Ci/mmole, 18 μCi/ml of reaction mixture; Amersham/Buchler). Incubation was performed at 26 °C for 30 min. Controls contained crude membranes of mock-infected cells at the same protein concentration. The reaction was terminated by addition of 5 ml ice-cold 5.7% trichloroacetic acid supplemented with 10 mM-sodium pyrophosphate and 1 M-NaCl. Precipitates were collected on glass fibre filters (GF/C, Whatman) and counted for radioactivity. In this standard polymerase assay the following membrane complexes of the superinfected cells, taken directly from the fractionated sucrose gradients, were tested in 25 μl amounts: 470S complex from a P8 + G infection (8 h single infection with poliovirus in the presence of guanidine); 700S complex from a P4-ME6 + G infection (4 h preinfection with poliovirus in the presence of guanidine, followed by a 6 h superinfection with ME virus); 220S to 400S membranes from mock-infected cells; 600S complex from an ME6 infection (6 h single infection with ME virus); 700S complex from an ME6 infection (8 h single infection with ME virus). The presence of guanidine in mock, ME6 and ME8 infections had no influence on the incorporation rates. All determinations represent an average of three investigations.
Three considerations suggest that these RNAs were ME virus-specific. (i) The RNA of the 700S complex migrated on agarose–polyacrylamide gels similarly to the RNA isolated from control cells singly infected with ME virus for 6 h in the absence of guanidine (not shown). (ii) The 'poliovirus/guanidine'-induced 470S complex was deficient in newly synthesized RNA (Fig. 2a). (iii) As shown recently by several criteria, the reproduction of poliovirus inhibited by guanidine was not complemented by ME virus in this superinfection system (Zeichhardt et al., 1981). The virus progeny in the superinfection was solely ME virus; it had the same growth properties as ME virus, was insensitive to guanidine, became inactivated by treatment with 0-1 m-NaCl pH 6 (Rueckert, 1971) and was not neutralized by antibodies against poliovirus.

In order to prove that the ME virus-specific RNA associated with the 700S complex was actually synthesized by ME virus RNA polymerase at this membrane complex, the membrane complexes from infected cells were isolated on sucrose gradients. Evidence for membrane complex-associated ME virus RNA polymerase in vitro, in combination with the detection of newly synthesized viral RNA at the ME virus-modified membrane complex in vivo, would mean that this membrane complex is the site of action of the replication complex of ME virus (see Girard et al., 1967; Lundquist & Maizel, 1978). Therefore, the in vitro activity of the membrane complex-associated polymerase of ME virus was determined by measuring the conversion of [3H]GTP to an acid-insoluble product. For this purpose, a standard assay for ME virus-specific RNA polymerase was developed according to methods described for membrane-bound RNA polymerases of poliovirus and mengovirus (Ehrenfeld et al., 1970; Chinchar, 1978; M. W. Taylor, personal communication). In this standard assay the polymerase in crude membrane preparations of ME virus-infected cells yielded a maximum incorporation rate 8 times higher than membranes of mock-infected cells (see legend to Fig. 2b).

When the ME virus-modified membrane complex of 700S (P4ME6 + G) was tested for ME virus RNA polymerase activity in comparison to the 'poliovirus/guanidine'-induced 470S complex (P8 + G), only the 700S complex showed ME virus-specific polymerase activity (Fig. 2b). The activity of the 700S complex was 5-6 times higher than the activity of the 470S complex. The 470S complex yielded the same low incorporation rate as membranes of mock-infected cells (220S to 400S). The polymerase of the 700S complex from the P4ME6 + G infection was 3-1 times more active than the polymerase activity of a 600S complex isolated from cells after 6 h single infection with ME virus (ME6). The 700S complex had an incorporation rate 1.5 times higher than the 700S complex produced after a single infection with ME virus for 8 h (ME8). Proof of in vitro and in vivo activity of the ME virus-specific RNA polymerase at the ME virus-modified membrane complex, i.e. proof of simultaneous existence of polymerase and newly synthesized viral RNA including RI at the membrane complex, led to the conclusion that this membrane structure contained the ME virus-specific replication complex.

The consistently higher polymerase activity at the 700S membrane complex obtained after 6 h superinfection with ME virus compared to complexes from single infections with ME virus lasting either 6 h (ME6) as in the superinfection experiments, or even 2 h longer (ME8), was in good agreement with other observations that several ME virus-specific effects are raised or advanced in cells preinfected with poliovirus in the presence of guanidine (Zeichhardt et al., 1981, 1982). (i) The reproduction of ME virus is accelerated by 3 h. (ii) The formation of the ME virus-specific 700S membrane complex is advanced by 2 h. (iii) The ME virus-induced cytolysis of the infected cells is advanced by 2 h. We conclude that these effects are correlated at least indirectly to the exploitation of the preexisting 'poliovirus/guanidine'-induced membrane complex for the reproduction of ME virus.

A comparison with the large quantity of data in the literature about double infections with picornaviruses in the presence of guanidine (for detailed references, see Zeichhardt et al., 1981) revealed that the interdependencies observed between polio- and ME viruses seem to reflect a special situation. In only one other system (encephalomyocarditis virus and poliovirus) was the reproduction of the mixedly infecting virus advanced by non-reproducing poliovirus (Shirman et al., 1973); however, an involvement of membranes was not documented. In superinfections with polio- and mengoviruses the reproduction of mengovirus was not influenced (McCormick & Penman, 1968). Most other investigations with closely related or unrelated picornaviruses
revealed either genetic recombination (King et al., 1982), phenotypic mixing (Holland & Cords, 1964) or complementation (Trautman & Sutmoller, 1971).

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REFERENCES


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