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Improved Infectivity of Reassembled Polyoma Virus†

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Polyoma virus was dissociated into capsomeres (18, 12, and 5S) and a DNA-protein complex (48S) with the Ca^{2+} chelator, ethyleneglycol-bis-*N*,*N*'-tetraacetic acid, and the reducing agent, 2-mercaptoethanol. The reaction was maintained at pH 5.0. Reassembly of the dissociated components to complete virions was accomplished by dialyzing these components overnight at 4°C against the reassembly buffer containing $CaCl_2$, dimethylsulfoxide, Triton X-100, and 0.01 M Tris-acetic acid (pH 5.0). Reconstituted particles ranged from 240S complete virions to lighter intermediate species. Approximately 25% of the dissociated particles could be physically reassembled to complete virions. These virions regained 12.5% of their hemagglutination ability and as much as 6.7% of their original infectivity. The infectivity of these reassembled particles represented a 100-fold increase in infectivity compared with that of the particles that were dissociated and reassembled at pH 7.4. Biochemical analysis showed that the polyoma viral receptor of the virions reassembled at pH 7.4 was greatly reduced, whereas virions reassembled at pH 5.0 retained their receptor. Reassembly could be further improved by additions of either exogenous capsomeres or DNA-protein complex to the reassembly reaction mixture.

Analysis of polyoma virions by X-ray fluorometry revealed the close association of Ca^{2+} ions with the virus (7). When polyoma virus was treated with the Ca^{2+} chelator (ethylene glycol-bis-*N*,*N*'-tetraacetic acid [EGTA]) and the reducing agent (dithiothreitol) at pH 7.4, it was dissociated into capsomeres (18, 12, and 5S) and a 48S DNA-protein complex (8). These dissociated components were successfully reassembled at pH 7.4 to infectious viral particles which biophysically resembled the intact polyoma virions. Even though as much as 30% of the physical particles could be reconstituted, only a small amount of infectivity could be regained, as determined by plaque assay (6). The purpose of this report is to present our method to improve the infectious capability of reassembled polyoma virions.

Mouse embryo and mouse kidney cells used in these experiments were prepared as described previously (11, 13, 17). Wild-type polyoma virus was used to infect cells at a multiplicity of infection of 10. Infected cultures were maintained in serum-free Dulbecco-modified Eagle medium (14). Virus was purified from infected cell lysates as described previously (14). CsCl gradients used to purify the virus were prepared as described by Brunck and Leick (9) and were described in greater detail previously (7, 8). The preparation of [^{3}H]thymidine-labeled virions has been described (8, 14). Polyoma virions labeled either with ^{125}I or ^{131}I by the chloramin-T method have been described (4, 5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins, hemagglutination (HA), and plaque assays to polyoma virus have also been described (10, 11, 15, 16).

Dissociation of polyoma virus with EGTA and 2-mercaptoethanol (ME) at pH 5.0 resulted in three species of capsomeres (18, 12, and 5S) and a 48S DNA-protein complex (data not shown). These dissociated products were identical to those obtained when polyoma virus was dissociated at pH 7.4 (8). The neutral sucrose gradient pattern of particles reassembled at pH 5.0 demonstrated the presence of reassembled virions (240S) as well as various intermediate families of partially reassembled virions sedimenting lighter than 240S. The efficiency of polyoma reassembly at pH 5.0 was found to be 25% as determined by radioactivity ([^{3}H]thymidine) in the 240S region of the sucrose gradient (data not shown). Brady et al. (6) demonstrated that the amount of Ca^{2+} present in the reassembly buffer was critical for successful reassembly. A similar effect was observed when reassembly was performed at pH 5.0. Each virus preparation had to be reassembled with various concentrations of Ca^{2+} to determine the concentration of Ca^{2+} for optimum reassembly.

The concentrations of EGTA and ME used in the virion dissociation reaction at pH 5.0 were

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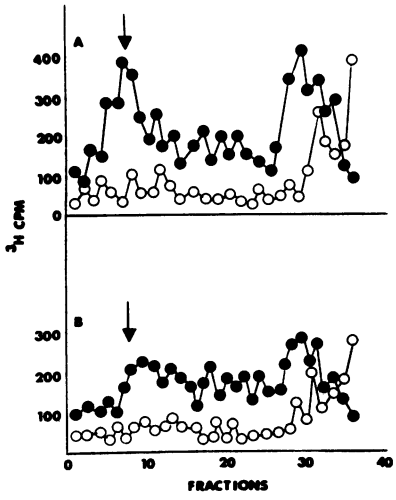

FIG. 1. Effect of EGTA and ME concentrations on polyoma reassembly. [^{3}H]thymidine-labeled polyoma virions in 0.01 M Tris-hydrochloride (pH 7.4) were treated with EGTA at pH 7.4 for 8 h at 4°C. The acid pH (5.0) was not used because at this pH EGTA has minimum ability to chelate Ca^{2+}. At this stage, it was found that the EGTA treatment described above did not cause any dissociation of the virion preparation. The pH of the reaction was then adjusted to 5.0 with 0.01 M Tris-acetic acid (pH 2.0). Dissociation was completed by additions of 0.15 M NaCl (final concentration) and ME at 4°C overnight. Concentrations of EGTA-ME used were 20 mM EGTA–2.5% ME (A) and 25 mM EGTA–3% ME (B). At the end of the dissociation reaction, half of the reaction volume was removed from the reaction mixture and stored at 4°C. To the other half of the dissociation products, $CaCl_2$ (10^{-6} M) and dimethylsulfoxide (20%) were added. After a 30-min incubation at room temperature, the dissociation products were dialyzed against a reassembly buffer containing 0.15 M NaCl–0.01% Triton X-100–10% dimethylsulfoxide–10^{-6} M $CaCl_2$–0.01 M Tris-acetic acid (pH 5.0). Dialysis was carried out at 4°C overnight with stirring. The reassembled virus and the dissociation products (removed earlier from reaction mixture) were analyzed in 10 to 30% neutral sucrose gradients (0.15 M NaCl–0.01% Triton X-100–0.01 M Tris-hydrochloride [pH 7.4]). Velocity sedimentation was performed in an SW50.1 rotor at 35,000 rpm for 45 min at 4°C. Fractions were collected and counted in toluene-Triton (3:1) scintillation liquid with a Beckman LS-233 scintillation counter. Symbols: arrows indicate the position of the 240S native polyoma virions; ●, reassembled virus; ○, dissociated virus.

shown to affect the efficiency of reassembly. Figures 1A and B show two reassembly patterns obtained when different concentrations of EGTA-ME were used for virion dissociation. Whereas there was no significant difference in the virus dissociation, 20 mM EGTA–2.5% ME produced a better reassembly pattern (Fig. 1A). To optimize virion reassembly, appropriate concentrations of EGTA-ME must also be determined for each purified virus preparation.

Polyoma virions that were dissociated and reassembled at pH 5.0 or 7.4 were compared to determine their biophysical and biological activities based on reassembled particles sedimenting in the 240S region of the sucrose gradient, ability to hemagglutinate guinea pig erythrocytes, and ability to infect mouse cells as determined by plaque assay (Table 1). Virions that were dissociated at either pH were not capable of meeting any of the above requirements. However, particles reassembled at both pHs were found to regain 12.5% of the original HA activity. However, particles reassembled at pH 5.0 were found to be 100 times more infectious than those reassembled at pH 7.4, even though their HA recoveries were identical.

The protein composition of the reassembled virions was analyzed by SDS-PAGE (Table 2). Protein patterns of the 240S virions reassembled at either pH were similar to those observed for the native polyoma virions. The intermediate sedimenting reassembled particles (170 and 120S)

TABLE 1. Biological and physical properties of dissociated and reassembled virus

pH and conditions[a]	% Physical particles at 240S[b]	% HA[c]	% PFU[d]
pH 7.4			
Dissociation	0	0	0
Reassembly	30	12.5	0.06
pH 5.0			
Dissociation	0	0	0
Reassembly	25	12.5	6.7

[a] ^{125}I-labeled polyoma virions were dissociated and reassembled at either pH 7.4 or 5.0.

[b] Percentages of physical particles reassembled were calculated from the ratio of the ^{125}I-labeled virus present in the 240S region of 10 to 30% sucrose gradients to the total radioactivity in the gradients.

[c] HA of guinea pig erythrocytes was performed in microtiter plates. Percentages were determined by comparing the HA titer of the intact native virus with that of the dissociation and reassembly mixtures.

[d] Biological infectivity was determined by comparing the PFU from native untreated virions with that of dissociated and reassembled particles in the 240S region of the sucrose gradients. Biological activity obtained in separate experiments of the reassembled particles at pH 7.4 gave PFU ranges from 5.0×10^3 to 3.0×10^4/ml, whereas the reassembled particles at pH 5.0 gave PFU ranges from 5.5×10^5 to 3.35×10^6/ml. These values were then compared with that of untreated polyoma, which had a titer of 5.0×10^7/ml. The percentages of reassembled particles at pH 7.4 and 5.0 represent the highest values obtained.

TABLE 2. SDS-PAGE of ^{125}I-labeled reassembled virus[a]

pH (sedimentation)	% VP1	% VP2	% VP3	% Histones
Native virus standard	81.5	7.4	5.1	6.0
7.4 (240)	80.5	7.6	3.1	8.8
7.4 (170)	62.7	11.1	4.1	22.1
7.4 (120)	65.0	11.2	4.8	19.0
5.0 (240)	79.0	10.0	3.6	7.4
5.0 (170)	72.6	12.6	5.5	9.3
5.0 (120)	70.0	13.2	5.0	11.8

[a] ^{125}I-labeled polyoma virions were dissociated and reassembled at either pH 7.4 or 5.0. Particles in 240S (fractions 7 through 9 [Fig. 1A]), 170S (fractions 12 through 14), and 120S (fractions 18 through 20) regions of the sucrose gradients were isolated. Fractions from each region were pooled and concentrated by speed-vacuuming the samples. These samples were analyzed on 17.5% SDS-PAGE, and autoradiography was performed on the slab gel. Percentages were calculated by weighing areas under the peaks obtained from densitometer tracings of the autoradiograms by using a Gilford spectrophotometer with a gel transport. The autoradiograms were scanned at 660 nm.

were also analyzed and found to have lower percentages of VP1 content but higher percentages of the other structural proteins (VP2 and VP3). This observation suggested that the intermediate particles were lacking VP1 protein. The exceptionally high percentages of radioactivity in the histone region of the gels found with the intermediates of the pH 7.4 reassembled particles are probably breakdown products of the major capsid protein, VP1, which migrates with the histones. This is quite likely, since our laboratory (5, 16) as well as others (12) has previously demonstrated that dissociation of polyoma virions at slightly alkaline pHs causes VP1 protein to fragment to peptides of 29,000, 16,000, and 14,000 daltons.

Bolen et al. (3) have recently demonstrated that VP1, the major capsid protein, was separable into six distinct species by isoelectric focusing (IEF). These different forms of virion VP1 appeared to be generated by modifications (phosphorylation and acetylation) of the initial translation products. Various biological functions were relegated to the VP1 polypeptide species: association with the minichromosome, hemagglutination activity, receptor for infection, and their possible associations in hexon and penton structures. Species E (pI 5.9) was identified as the polypeptide having the receptor function responsible for cell infection. Virions that were dissociated and reassembled at pH 7.4 were found to have little infectivity and relatively high HA activity (Table 1), and their intermediate reassembled particles were found to be different in VP1 content (Table 2). Thus, it was of interest to analyze the IEF pattern of VP1 to determine whether the E species was affected by the pH of reassembly. The IEF patterns of VP1 are shown in Fig. 2A and B, and the calculated percentages of the VP1 species are shown in Table 3. The percentages of the VP1 species of the pH 5.0 reassembled particles were similar to those of the species found for native virions. However, a 70% decrease in the E species was found in particles that were reassembled at pH 7.4. It should be mentioned that, in other, similar experiments, the E species was found to be completely missing. In this particular experiment, other species, i.e., A and F, were found to exhibit slightly higher percentages than that found with the native virions. However, these differences with A and F species were not con-

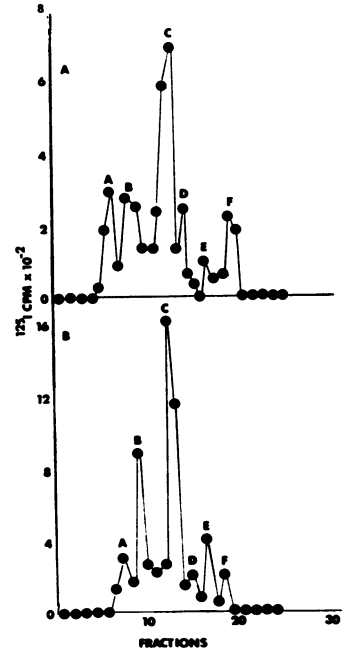

FIG. 2. Isoelectric focusing analysis of reassembled particles. Polyoma virions were labeled with either ^{125}I or ^{131}I by the chloramin-T method. ^{125}I-labeled virions were dissociated and reassembled at either pH 5.0, as described in the legend to Fig. 1, or pH 7.4, as described by Brady et al (6). ^{125}I-labeled reassembled particles in the 240S regions of the sucrose gradients were applied to 15% SDS-PAGE tube gels. ^{131}I-labeled native virion proteins were resolved in parallel tube gels. The ^{125}I- and ^{131}I-labeled VP1 slices were analyzed by IEF, as described by Bolen et al. (3). Each IEF tube gel consisted of a ^{125}I-labeled VP1 slice from reassembled virus cofocused with a ^{131}I-labeled VP1 slice from native virions, with the latter serving as an internal standard. The labeled slices from IEF were counted in an LKB 1275 minigamma counter.

TABLE 3. Percentages of VP1 species of reassembled particles[a]

VP1 species	% ^{131}I-labeled native virions	% Reassembled ^{125}I-labeled virions (pH 5.0)	% Reassembled ^{125}I-labeled virions (pH 7.4)
A	8	10	14
B	20	18	18
C	50	50	45
D	8	6	9
E	7	9	2
F	7	7	12

[a] Details of treatment of ^{125}I- and ^{131}I-labeled polyoma virions are described in the legend to Fig. 2. Percentages of VP1 species were calculated by determining the area under each peak shown in Fig. 2.

sistently observed, whereas the reduction or complete loss of E was always observed. These findings appear to indicate that the low infectivity found with particles that were dissociated and reassembled at pH 7.4 may be attributed to the loss of E, the virus receptor.

During reassembly of polyoma virus, intermediate families (170 and 120S) of reassembled particles are always observed sedimenting in regions lighter than the 240S particles. If these intermediates are partially reassembled particles, they should form complete virions upon addition of exogenous capsomeres. On the other hand, exogenous DNA-protein complex should interact with the free capsomeres from the original dissociated virions to form intermediates as well as complete virions. Experiments were designed to determine whether exogenous addition of capsomeres or DNA-protein complex could aid partially reassembled particles to form complete virions (Table 4). Additions of either exogenous capsomeres or DNA-protein complex resulted in a twofold increase in the amount of physical particles reconstituted, as determined by radioactivities sedimenting in the 240S region of neutral sucrose gradients. There was a 92% increase in infectivity when exogenous DNA-protein complex isolated from a pH 8.5 sucrose gradient was added to the reassembly mixture, but additional capsomeres from the same gradient did not have any effect on increasing infectivity. To determine the effect of the gradient pH on the capsomere viral receptor, capsomeres and DNA-protein complex were separated on a pH 5.0 sucrose gradient. Under these conditions, both exogenous capsomeres and DNA-protein complex were able to enhance infectivity of the reassembled particles by 120%.

The present report describes methods to improve the infectivity of reassembled polyoma virus in vitro. It was found essential to maintain the virus dissociation and reassembly reactions at pH 5.0. In addition, concentrations of EGTA-ME for dissociation and Ca^{2+} in the reassembly buffer were found to be critical for the reassembly process. Particles reassembled at pH 5.0 were found to have biophysical properties identical to that of intact virions. They also exhibited much higher (100-fold) infectivity than particles reassembled at pH 7.4. When VP1 of these particles was analyzed by IEF, the viral receptor was shown to be greatly reduced on pH 7.4 reassembled particles, as compared with particles reassembled at pH 5.0 and the ^{131}I-labeled native polyoma virions. The disappearance of the receptor at pH 7.4 could be due to a protease-like activity described by Friedmann (12) and Bolen and Consigli (5). Similar protease activities have been observed in other viruses (1, 2, 18). However, conventional protease inhibitors were not able to inhibit the activity (data not shown). An alternative explanation would be that the receptor protein becomes fragile upon dissociation of the virus at slightly alkaline pHs.

TABLE 4. Improved polyoma reassembly by additions of exogenous capsomeres and DNA-protein complex

Conditions[a]	% Physical particles reassembled	% PFU in 240S region at following pH[b]:	
		8.5[b]	5.0[c]
Normal reassembly[d]	100	100	100
Exogenous capsomeres	272	95.5	220
Exogenous DNA-protein complex	296	192	223

[a] ^{125}I-labeled polyoma virions were dissociated at pH 5.0 as described in the legend to Fig. 1. Exogenous capsomeres or DNA-protein complex were added to the dissociation products. Reassembly was carried out at pH 5.0, as described in the legend to Fig. 1.

[b] Exogenous capsomeres and DNA-protein complex were isolated from a 5 to 20% sucrose gradient (0.15 M NaCl–0.25% Triton X-100–0.01 M Tris-hydrochloride [pH 8.5]). Velocity sedimentation was performed in an SW50.1 rotor at 35,000 rpm at 4°C for 3 h. Percentages of physical particles were determined by comparing amounts of radioactivity in the 240S peaks of reassembled virus (with additions of exogenous capsomeres or DNA-protein complex) with that of the control (normal reassembly). Biological infectivity was determined by comparing the PFU from the 240S region of the sucrose gradient of the normal reassembly with that of the 240S regions after exogenous additions of capsomeres and DNA-protein complex.

[c] Same as described in footnote *b*, except the pH of the 5 to 20% sucrose gradient used to isolate the capsomeres and DNA-protein complex was lowered to 5.0.

[d] Normal reassembly was done as described in the legend to Fig. 1.

Intermediate families (170 and 120S) present during reassembly were shown to be capable of forming complete virions when exogenous capsomeres or DNA-protein complex were added. Capsomeres isolated from sucrose gradients at pH 8.5 were not able to improve infectivity of reassembled virus, whereas exogenous capsomeres from a pH 5.0 sucrose gradient were quite effective. These findings indicate that the viral receptor protein was being destroyed at neutral or alkaline pH but less so in acidic conditions (Fig. 2) and demonstrates the importance of the viral receptor in the infection process. Ultimately, this fundamental information may allow us to better understand the complex mechanism(s) of in vivo assembly and in particular allow us to reassemble polyoma in vitro more efficiently.

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