JOURNAL OF VIROLOGY, Sept. 1982, p. 1113–1117
0022-538X/82/091113-05$02.00/0

Vol. 43, No. 3

NOTES

Purification and Characterization of Bovine Rotavirus Cores

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Received 16 February 1982/Accepted 27 May 1982

Using the chaotropic effect generated by a high concentration of $CaCl_2$, we converted calf rotavirus particles into cores of 40 nm in diameter. These cores were purified by rate zonal centrifugation in sucrose gradients and by isopycnic gradients. They had a sedimentation coefficient of 280S ± 20S and a density of 1.44 g/ml in CsCl. When analyzed by polyacrylamide gel electrophoresis, they contained three polypeptides (VP125, VP89, and VP78). The major internal polypeptide of the virion (VP39) was recovered in a purified and soluble form in the top fractions of the sucrose gradients. From this stepwise degradation, it appears that VP39 is the most external polypeptide of dense particles. In contrast to reovirus cores, calf rotavirus cores did not exhibit transcriptase activity. Purified VP39 also did not exhibit transcriptase activity when tested after being mixed with purified rotavirus genome RNA as a template. Transcriptase activity was partially recovered when ionic conditions were adjusted to permit the reassociation of VP39 with the cores.

Rotaviruses possess several features in common with reoviruses, including a double-stranded RNA genome (23) and a protein shell composed of two layers (4). Several analyses of the structural proteins of human and animal rotaviruses, purified from infected cell cultures or from stool samples, agree on the presence of six to nine polypeptide species falling into three molecular weight groups (7, 10, 11, 16, 17, 21–24).

Treatment of purified complete virions with chelating agents has been used to remove the outer shell of the virion, which consists of glycopeptides of molecular weights 33,000 and 31,000 (7). This treatment transforms the complete particles, also called smooth particles (4) or light particles (6), into incomplete noninfectious particles, called rough particles, dense particles, or even cores (18). This latter designation is somewhat confusing, because the term core has been used by Almeida (1) to describe a smaller rotavirus particle measuring 40 nm in diameter. In the present report, core has the same meaning as in Almeida's studies (1, 2).

Until recently, little information has been available concerning the position of the proteins within the viral capsid, except for the glycopeptides (molecular weights, 33,000 and 31,000) that have been shown to be the most externally exposed polypeptides (8). While the present study was in progress, Novo and Esparza (20) reported results on the localization of polypeptides of calf rotavirus by in vitro iodination of viral particles. Using stepwise degradation with chaotropic agents, we studied the topography of rotavirus particles. Special attention was paid to localizing more precisely the virion-associated RNA transcriptase activity.

In preliminary experiments, we used electron microscopy to visualize the degradation of a mixture of purified light (L) and dense (D) particles treated at pH 8.0 with various concentrations of the mild chaotropic agent $CaCl_2$ (Fig. 1). Four concentrations (0.5, 1, 1.5 and 2 M) were tested. With the lowest concentration, small indentations could be seen at the periphery of D particles, indicating a partial solubilization of the inner capsid. With 1 or 1.5 M $CaCl_2$, an increasing percentage of D particles was converted into cores of a 40-nm diameter, but some L particles remained intact. The resistance of L particles to degradation by $CaCl_2$ was confirmed by using purified L particles. In contrast, 100% of purified D particles were converted into cores at 1.5 M $CaCl_2$ (Fig. 1b). When the concentration of $CaCl_2$ was increased to 2 M, cores were no longer detectable by negative staining and were probably fully disrupted. At this concen-

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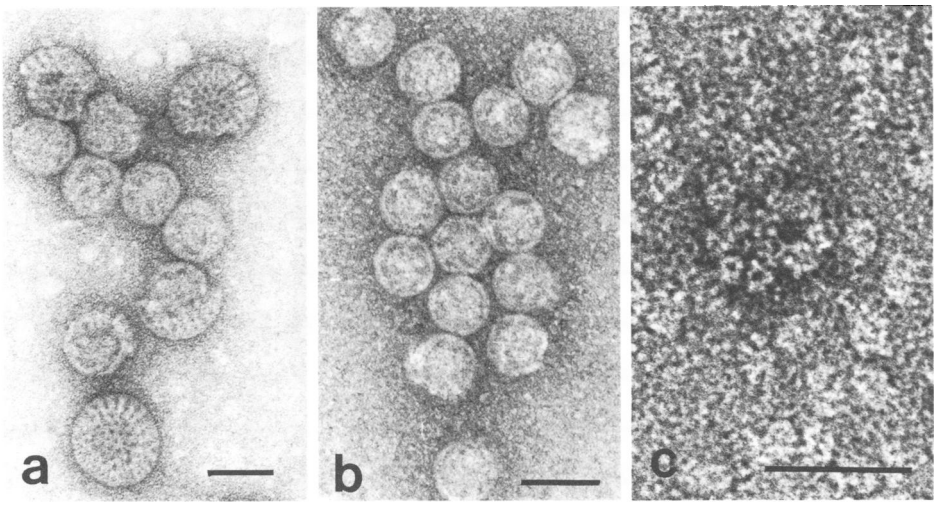

FIG. 1. Negative staining of various steps of degradation of virus particles. (a) A mixture of purified D and L particles was treated with 1 M $CaCl_2$ for 10 min, then adsorbed for 1 min on carbon-coated grids, transferred onto a drop of uranyl acetate (2%, pH 7) for 1 min, and immediately examined. Virus treated with 1.5 M $CaCl_2$ and purified in sucrose gradient (Fig. 2) was also visualized by negative staining of (b) fractions 10 and 11 (core suspension) or (c) top fractions 1 to 5 of the same gradient. Bar, 50 nm.

tration of the chaotropic agent, however, some L particles remained intact. As estimated by electron microscopy, the kinetics of degradation were fairly rapid. For each concentration of $CaCl_2$, the degradation effect reached a maximum after 10 min.

With potassium thiocyanate as the chaotropic agent, the results were similar. The same concentrations gave almost the same effects; however, the kinetics were much slower with potassium thiocyanate compared with $CaCl_2$.

After rate zonal centrifugation, core preparations migrated as a single peak in the middle of a 30 to 45% (wt/vol) linear sucrose gradient. This migration corresponded to a sedimentation constant of 280S ± 20S, calculated by the method of McEwen (19). Part of the disrupted D particles remained at the top of the gradient (Fig. 2). When examined by negative staining (Fig. 1c), these top fractions contained some semiamorphous material that apparently was outer structural units of D particles. Cores further purified by isopycnic centrifugation banded as a single peak corresponding to a buoyant density of 1.44 g/ml in CsCl.

Three polypeptides were associated with the cores (Fig. 3). These polypeptides had molecular weights of 125,000, 89,000, and 78,000. They corresponded to the largest proteins of the six polypeptides (designated VP or VGP for glycopeptides) of L particles (VP125, VP89, VP78, VP39, VGP33, and VGP31). Profiles of stained gels (Fig. 3, lane d) showed that a very high

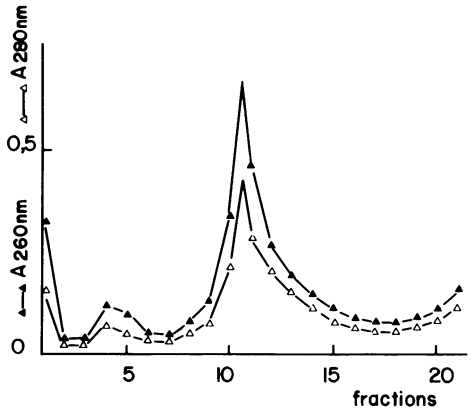

FIG. 2. Purification of cores in sucrose gradients. The bovine rotavirus used in this study was extracted from the feces of experimentally infected, colostrum-deprived newborn calves and was purified in CsCl gradients as previously described (6). Purified viral particles suspended in 5 mM Tris-hydrochloride–50 mM NaCl (pH 8.0) were treated at room temperature with 1.5 M $CaCl_2$. Then the mixture was layered very carefully on the top of a 30 to 45% (wt/vol) sucrose gradient (because the density of 1.5 M $CaCl_2$ is very close to the density of 30% sucrose) and centrifuged for 120 min at 154,000 × *g* in a Beckman SW40 rotor. Fractions were collected from the top and monitored for optical density at 280 and 260 nm with an ISCO UA5 spectrometer. See Fig. 1 for pictures of the material in fractions 1 to 5 and fractions 10 and 11.

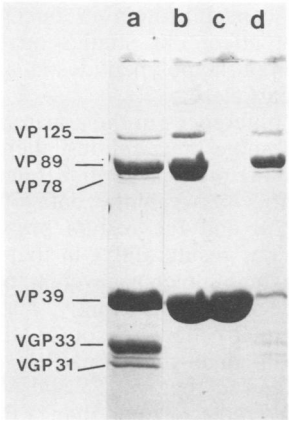

FIG. 3. Polypeptide composition of cores. Polyacrylamide gel electrophoresis was performed by using 12.5% polyacrylamide slab gels and the Laemmli (14) discontinuous buffer system. Cores purified in CsCl gradients (density 1.44 g/ml) were pelleted and analyzed (lane d) in comparison with L particles (lane a) and D particles (lane b). The polypeptides present in the top fractions of sucrose gradients described in the text were precipitated with ethanol, and the suspended pellet was analyzed (lane c). This gel was overloaded to demonstrate the efficiency of the purification procedure of VP39. When this amount of virus (120 μg/lane) was analyzed, minor bands were occasionally detected. These bands were not found reproducibly and, therefore, were not considered as viral proteins.

proportion (about 90%) of the most abundant structural polypeptide (VP39) was absent from purified cores. This result and morphological evidence clearly indicated that the major polypeptide VP39 corresponded to the outer layer of the D particles.

It should be noted that this procedure of degradation of single-shelled particles constitutes a simple method for the purification of the major structural polypeptide of the virion without the use of a detergent. One can readily obtain a pure preparation of VP39 that can be used as a source of group-specific antigen (Fig. 3, lane c).

In contrast to D particles, purified cores exhibited no polymerase activity (Table 1). A mixture of purified VP39 and double-stranded genome also did not stimulate the incorporation of tritiated UMP into acid-precipitable products. To determine whether isolated cores retained the capacity for polymerase activity, we also attempted reconstruction of this function and of these particles with VP39 by dialysis experiments, using a slow variation of chaotropic agent. Initially, the concentration of $CaCl_2$ was increased from 0 to 1.5 M and then decreased to 0 during a 3-h period. Samples of the virus suspension were withdrawn from the upper chamber and pelleted to remove the solubilized proteins and the chaotropic agent. The suspended virus pellets were then assayed for polymerase activity and for VP39 content by polyacrylamide gel electrophoresis. Typical results are shown in Fig. 4. It appeared clearly that a concentration of 1.5 M $CaCl_2$ was critical for the stability of the outer layer of D particles, as well as for its polymerase activity. After treatment with 1.1 M $CaCl_2$, 75% of VP39 remained associated with D particles, and 100% of the transcriptase activity was recovered, but at 1.5 M $CaCl_2$, VP39 was almost totally solubilized, and the polymerase activity was no longer detectable. When the concentration of the chaotropic agent was subsequently decreased, the polymerase activity was partially recovered. However, the level of the polymerase activity remained low and never exceeded 25% when the reassociation of VP39 with the core reached 95%.

The results presented here allow a further localization of rotavirus polypeptides in the virus architecture. It has been shown previously (7, 8) that the glycopeptides of molecular weights 33,000 and 31,000 constitute the outer shell of L or smooth particles. It appears now that the major polypeptide of the virion (VP39) constitutes the outer shell of D particles and that the three polypeptides (VP125, VP89, and VP78) are the most internally located ones.

It should be noted that our results were ob-

TABLE 1. Polymerase activity of cores[a]

Prepn tested	cpm	% of activity
D particles	42,127	100
Cores purified in CsCl gradient	829	2
Major polypeptide, mol wt 39,000 (25 μg) + double-stranded RNA (5 μg)	127	0
Cores purified in sucrose gradient	483	1
D particles + 15% sucrose	41,930	99

[a] In this experiment, the amount of each viral preparation was based on the double-stranded RNA content. RNA concentration was measured by a spectrofluorometric method using ethidium bromide as described by Le Pecq and Paoletti (15). Polymerase activity was determined as previously described (6). The values indicated above were the averages of three determinations, and the background (457 cpm) from the same assay incubated at 0°C was subtracted. D particles or cores, purified in CsCl, were pelleted before the assay, but cores purified in sucrose gradients were added directly to the reaction mixture, leaving a final concentration of 15% sucrose in the reaction mixture. This concentration of sucrose had no effect on the polymerase activity of D particles. The amount of VP39 was determined by the method of Bradford (3) with bovine serum albumin as standard.

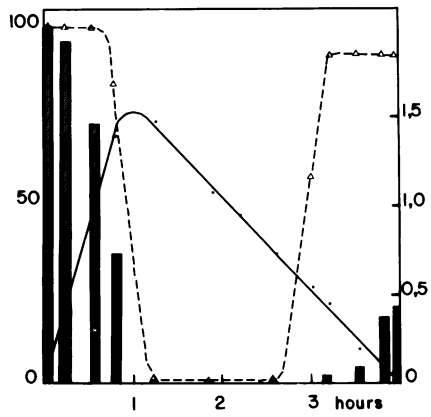

FIG. 4. Relationship between the amount of VP39 associated with cores and the polymerase activity. This experiment was performed in a small flow dialysis cell similar to that described by Colowick and Womack (9). The gradient of $CaCl_2$ in the lower chamber was generated by a gradient maker (Gilson) connected to a peristaltic pump. The flow rate was adjusted to obtain an equilibrium between the two chambers in 5 min as monitored by ^{45}Ca labeling. For each time point, two samples were withdrawn from the upper chamber. These two samples were pelleted without dilution in a Beckman Airfuge. One pellet was suspended in water and washed again before being assayed for polymerase activity (vertical boxes); the other was dissolved in 2% sodium dodecyl sulfate–5% 2-mercaptoethanol–62 mM Tris-hydrochloride (pH 6.8), boiled for 2 min, and analyzed by electrophoresis. The percentage of VP39 associated with the pellet (△) was determined by densitometry scanning after Coomassie blue staining. The concentration of $CaCl_2$ (●) in the lower chamber was determined with an automatic osmometer.

tained with wild virus produced in vivo. However, its polypeptide composition is similar to that of cell culture-adapted strains of calf rotavirus produced in vitro (5, 20). When viruses from these different sources are compared, it appears that the major differences are the presence of four polypeptides in the high-molecular-weight group (instead of three in our study and in that of Novo and Esparza [20]) and the proportion of one of these polypeptides. It has been shown with bovine (5) and simian (10, 12) rotaviruses that a much higher proportion of an outer polypeptide is associated with virus particles grown in the absence of proteolytic enzymes. Consequently, it seems (i) that our preparations of virus particles are almost free of the outer high-molecular-weight peptide and its cleavage product after being exposed to proteolytic enzymes in the gut or during virus maturation in the intestinal cells and (ii) that our VP78 could correspond to the minor inner capsid polypeptide called VP4 (5, 12) or VP3 (18). The lack of the outer capsid protein in our preparations should not affect our studies on the relative localization of the polypeptides in single-shelled and core particles.

Despite differences in the apparent molecular weights of some polypeptides, there is general agreement on the polypeptide composition of calf rotavirus between the data of Novo and Esparza (20) and the results presented here. However, our results differ in that we did not detect the high-molecular-weight outer capsid polypeptide. More significantly, our results differ on the relative localization of VP125, VP89, and VP39 in single-shelled particles. Novo and Esparza showed that VP125 and VP89 are labeled to a greater extent than VP39 when D particles are iodinated in vitro with lactoperoxidase, and they concluded that VP39 is partially covered by VP125 and VP89. In contrast, we found that VP125 and VP89 are inner components of D particles, because they are associated with cores that are freed of VP39. Two hypotheses may explain this discrepancy: (i) lactoperoxidase-catalyzed iodination which labels tyrosine residues might not label VP39 efficiently in D particles if tyrosine residues of VP39 are less exposed than tyrosine residues of VP125 and VP89, or (ii) VP125 and VP89 might pass through the VP39 layer and, therefore, be partially exposed for labeling by lactoperoxidase.

Our degradation method used to obtain and disrupt cores had yielded information on the interactions among the various polypeptides of D particles. The solubilization of VP39 by chaotropic agents could indicate that the outer layer of D particles is maintained by hydrophobic interactions. Indeed, the major polypeptide VP39 seems to be very hydrophobic, as it precipitates easily in low-salt buffers when the concentration of VP39 is higher than 0.5 mg/ml (data not shown).

The rotavirus cores that we have obtained have a buoyant density very close to that of reovirus cores (13). However, in contrast to reovirus cores, calf rotavirus cores do not exhibit any polymerase activity. Polypeptide VP39 does not possess any transcriptase activity; however, its presence on the core surface is apparently needed for the expression of polymerase activity. It is possible that the functioning of the polymerase requires a precise conformation of the core which may only be obtained in the presence of VP39.

We express our appreciation to M. K. Estes and B. Petrie for suggestions in revising the manuscript.

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