Comparison of the Properties of Enveloped and Naked Frog Virus 3 (FV 3) Particles

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SUMMARY

Enveloped and naked frog virus 3 particles have been separated on sucrose gradients and their morphology checked by electron microscopy. Naked nucleocapsids sedimented at a density of 1.25 g/ml and enveloped virions at a density of 1.20 g/ml. The enveloped virions were found to possess an additional 58 K mol. wt. polypeptide. The ratio between the number of infectious particles, determined by plaque titration, and that of physical particles established by radioactive labelling as well as by electron microscopic counts, showed that the infectivity of the enveloped particles could be up to 150-fold greater than that of naked ones.

INTRODUCTION

It is generally agreed that in the case of most of the enveloped RNA viruses, the envelope is required for infectivity of the virus particle (Hoyle, 1952; Waterson & Cruickshank, 1963; Bose & Sagik, 1970). A correlation between the presence of an envelope and virus infectivity has been established for some of the enveloped DNA viruses, particularly members of the herpes group (Abodeely et al. 1970; Stein et al. 1970; Roizman & Furlong, 1974) and vaccinia virus (Payne & Norrby, 1978), although it has never been shown that the corresponding non-enveloped particles are non-infectious.

Concerning frog virus 3 (FV 3), a cytoplasmic DNA virus (for review, see McAuslan & Armentrout, 1974), enveloped virions which bud at the plasma membrane appear to be released in far smaller quantities than the naked particles released by cell lysis (Bingen-Brendel et al. 1971). Moreover, during purification, the envelopes may be damaged; thus only a very small percentage of enveloped virions are present in virus suspensions (Tripier & Kirn, 1973). Under these conditions, one may question whether this occasional envelope plays a role in infectivity.

This paper describes a method for the isolation of suspensions containing either naked or enveloped FV 3 particles which have been further analysed by SDS–polyacrylamide gel electrophoresis and tested for virus infectivity. Enveloped particles have been found to be more infectious than naked nucleocapsids.
METHODS

Cells and viruses. We used Chinese hamster ovary cells (CHO), baby hamster kidney cells (BHK21) and primary chick embryo fibroblasts (CEF). Stock FV 3 was grown on the chorioallantoic membranes of 10-day-old embryonated eggs and was purified as previously described (Aubertin et al. 1973). The virus titre was determined by the plaque assay at 29 °C on monolayers of BHK21 cells. The Lister strain of vaccinia virus was cultivated by infection of BHK21 cells.

Isolation of naked and enveloped particles

Production of radioactive virus. Cells grown for 24 h on roller bottles were infected at an input multiplicity of 0·1. After a 1 h adsorption period at room temperature, BHK21 and CEF cells were incubated at 29 °C in MEM medium (Eurobio, Paris, France) supplemented with 5% calf serum, while CHO were covered with alpha medium (Eurobio) containing 2% calf serum. Three h later, a radioactive medium containing 1 μCi of 3H-thymidine per ml (25 Ci/mmol; Commissariat à l’Energie Atomique, Saclay, France) and 0·5 μCi of 14C-amino acids per ml (Chlorella vulgaris protein hydrolysate, 45 mCi/mAtoms C; Commissariat à l’Energie Atomique) was placed on the cell monolayers. The medium used was MEM with a 10-fold-diluted concentration of amino acids and supplemented with 5% dialysed calf serum. The infection was allowed to proceed for about 60 to 72 h at 29 °C until complete necrosis; the roller bottles were then frozen at -20 °C.

Purification of labelled virions. The virus particles were collected both from the culture fluid and from the cells. For this purpose, the cells were scraped from the glass with a rubber policeman, broken by three ultrasonic treatments of 1 min each and then centrifuged at 2500 g for 20 min to remove cell debris. The pellet was discarded and the supernatant, which contained virus particles, was further purified. First it was layered on to cushions of 43% sucrose (w/w) in 10⁻³ M-tris-HCl, pH 8.9, and centrifuged at 4 °C for 4 h at 82500 g in a Spinco ultracentrifuge with an SW27 rotor. The pellets were resuspended in 10⁻³ M-tris-HCl, pH 8.9, and the virus suspension obtained was applied to 20 to 65% sucrose gradients (w/w). These gradients were centrifuged for 20 min at 30000 g in an SW27 rotor. Virus bands were collected and subjected to equilibrium centrifugation at 4 °C for 15 h at 82500 g on linear 35 to 65% (w/w) sucrose gradients in an SW27 rotor (longer centrifugations gave the same results). One ml fractions were collected from the bottom of the gradient, radioactivity was counted in each sample and refractive indices were determined in a Zeiss refractometer to obtain the densities.

SDS–polyacrylamide gel electrophoresis of polypeptides. Polypeptides were separated on slab gels using the discontinuous method described by Laemmli (1970). Mol. wt. were determined by A. M. Aubertin with the following protein standards: phosphorylase b (mol. wt. 94000), bovine serum albumin (mol. wt. 68000), catalase (mol. wt. 60000), glutamate dehydrogenase (mol. wt. 53000), ovalbumin (mol. wt. 45000), DNase I (mol. wt. 31000), chymotrypsinogen (mol. wt. 25700), trypsin inhibitor from soybean (mol. wt. 21500), RNase (mol. wt. 13700) and cytochrome c (mol. wt. 11700). A 5% SDS–polyacrylamide stacking gel and 12.5% SDS–polyacrylamide running gel were used. Electrophoresis was carried out at 40 V for about 6 h until the bromophenol blue dye marker reached the bottom of the running gel. After electrophoresis, gels were stained for 30 min at 37 °C with 0·25% Coomassie brilliant blue in 50% methanol–10% acetic acid, destained at room temperature in several changes of 7.5% acetic acid–5% methanol, prepared for fluorography as described by Bonner & Laskey (1974) and fluorographed at -80 °C with Kodak X-Omat R film, using an appropriate exposure time.
Properties of enveloped and naked FV 3

Electron microscopy. Fractions corresponding to the radioactive peaks from each gradient were observed with the electron microscope in order to check their content. Owing to the high concentration of sucrose which impedes staining and to the low number of virions in these samples, a direct observation was unsuitable. We had to use the pseudo-replication method of Sharp (1958) modified for negative staining by Smith & Melnick (1962), which allowed better staining and concentration of the particles. In order to prevent the virions from collapsing, this filtration was followed by fixation for several hours with 5% formol (Tripier & Kirn, 1973). Negative staining was performed with 2% sodium phosphotungstate, and preparations were observed with an electron microscope (Philips EM 300).

Virus particle counts. In order to determine the concentration of virus particles, a reference suspension of vaccinia virus, devoid of aggregates, was added to the FV 3 samples to be counted. Vaccinia virus, easy to distinguish from FV 3 owing to its morphological features, stained in the same way as FV 3. Before mixing the two suspensions, occasional FV 3 and vaccinia aggregates were dissociated by two ultrasonic treatments of 15 s, and the monodispersity of virus suspensions checked by negative staining. In order to minimize the standard error, the relative concentration of each suspension was chosen so that the number of particles of both types was about the same. Random fields were photographed and the number of FV 3 and vaccinia particles were counted on prints. For each sample,
we counted from 1000 to 2000 virions after having checked that the standard deviation was lower than 5% under these conditions. We could then calculate the mean ratio between FV 3 and vaccinia particles, considering their proportions in the observed preparation.

RESULTS

Isolation of enveloped FV 3

Suspensions of FV 3 obtained from infected cells as well as from the culture medium contained both naked and enveloped particles. To separate the two types of particles, virus suspensions were layered on to equilibrium sucrose gradients, as indicated in Methods. Fig. 1 shows the profiles of ³H-thymidine-labelled DNA and of ¹⁴C-amino acid-labelled polypeptides in the different fractions of the gradient. We could thus observe two peaks, at densities of 1·25 ± 0·01 g/ml and 1·20 ± 0·04 g/ml.

By observation after the pseudo-replication and negative staining techniques, we found that the heavier peak contained unenveloped virus nucleocapsids (Fig. 2) and the lighter one a mixture of naked and enveloped particles (Fig. 3). The fraction of naked nucleocapsids was generally homogeneous whereas the lighter fraction, in which cell fragments

Fig. 2. Electron micrographs of naked nucleocapsids present in the heavy peak of sucrose gradients. (a) In this highly purified preparation, the virus population is quite homogeneous. (b) At higher magnification, the icosahedral morphology is clearly visible.
Fig. 3. Electron micrographs of the virus population in the light peak of sucrose gradients. (a) All the particles shown are enveloped, but while some envelopes are intact, many others are discontinuous (arrows). In other fields, naked particles were present. (b) Isolated enveloped particles. The upper virion displays a fairly intact envelope whereas the lower one has envelope fragments only. (c) Three virions, having probably budded in the same cell protrusion, are present in a unique envelope. (d) 'Twin' virions released in the same envelope.

were often present, was far less homogeneous. Some particles displayed an intact envelope, some envelope fragments only, while others were devoid of any envelope. In some instances, we could also observe several virions within the same envelope (Fig. 3c, d). The proportion of enveloped particles in this fraction was difficult to evaluate due to the poor preservation of the envelopes. The number of enveloped particles varied from 20 to 80%.

**Comparison of the polypeptide composition of enveloped and naked particles**

The polypeptide composition of the two particle populations was determined by polyacrylamide gel electrophoresis. Fig. 4 presents the electrophoretic patterns obtained from
four suspensions of FV 3 grown on CEF cells, two containing enveloped particles and two containing naked ones. Comparison of these patterns showed that the enveloped particles contained an additional major polypeptide of approx. 58,000 mol. wt. A corresponding band was present in only minute amounts in the profile from the ‘naked’ fraction. There seemed to be no significant difference in the relative amounts of any of the other polypeptides. These modifications in the polypeptide profile of enveloped particles are independent of the cells used to grow the virus since the gel patterns of enveloped FV 3 grown on BHK or CHO cells were identical (results not shown).

Enveloped particles display enhanced infectivity

As shown in Fig. 1 the light peak containing enveloped particles had the highest infectivity titre. Nevertheless a shoulder in the infectivity profile could be observed at the level of the denser naked particles. In order to compare more precisely the infectivities of naked and enveloped FV 3, we determined for each gradient the number of infectious units per physical particle in the ‘enveloped’ fraction and in the ‘naked’ fraction. The number of infectious particles in each sample was determined by titration of sonicated virus suspensions and expressed in p.f.u./ml (after sonication, both enveloped and naked virus suspensions were devoid of aggregates). The number of physical particles was determined both by measuring in each peak the ³H-thymidine and ¹⁴C-amino acid incorporations which are proportional to the number of virions and by counting the number of virus particles in the electron microscope with vaccinia as an internal standard. The ratio between the number of infectious and physical particles is proportional to the specific infectivity ratio of each fraction. Three different experiments are reported in Table 1. From the experimental
Properties of enveloped and naked FV 3

Table 1. Comparison between infectivity ratios of ‘enveloped’ and ‘naked’ fractions obtained from three different gradients

<table>
<thead>
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<th>Expt. I</th>
<th>Expt. II</th>
<th>Expt. III</th>
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<tr>
<td></td>
<td>‘Enveloped’</td>
<td>‘Naked’</td>
<td>‘Enveloped’</td>
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<td>P.f.u./ml</td>
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<td>2.2 × 10⁷</td>
<td>6.8 × 10⁷</td>
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<td>P.f.u./ct/min AA*</td>
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<td>9 × 10⁸</td>
</tr>
<tr>
<td>R₃†</td>
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<td>33</td>
<td>86.7</td>
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* Ratio between p.f.u./ml and ct/min/ml of ¹⁴C-amino acid incorporation.
† R₁, Quotient between p.f.u./ct/min amino acid in the ‘enveloped’ and in the ‘naked’ fraction; R₂, quotient between p.f.u./ct/min thymidine in the enveloped and in the naked fraction; R₃, quotient between p.f.u. × X in the ‘enveloped’ and in the ‘naked’ fraction.
‡ Ratio between p.f.u./ml and ct/min/ml of ³H-thymidine incorporation.
§ n, Number of FV 3 physical particles/ml; X, unknown concentration of vaccinia virus suspension, used for virus counts (see Methods).

data, we calculated for each gradient the quotients R₁, R₂, R₃ established in three different ways. These values represented the ratio between the infectivities in the ‘enveloped’ fraction and in the ‘naked’ fraction. This ratio should be in the range of one if the infectivity of both fractions is the same; however it was very high in our experiments, thus indicating that the enveloped particles were far more infectious than the unenveloped ones.

DISCUSSION

When virus was purified from the culture medium of infected cells, both enveloped and naked particles were observed. Moreover, virus suspensions prepared from the infected cells after discarding the medium also contained significant amounts of enveloped virions. In the first case, the suspensions contained naked nucleocapsids probably as a result of lysis of many infected cells at late times of infection and also as a result of envelope damage. In the second case, enveloped particles were associated with infected cells, either as a consequence of their budding at the moment of recovering cells or due to trapping of virions within membrane folds. Therefore purification of enveloped and naked particles was carried out on culture fluids, cells or both, with essentially the same results.

The technique we used allowed us to separate one fraction containing only naked nucleocapsids and another composed of a mixture of naked and enveloped particles. Enveloped particles present in this fraction appeared to be surrounded by either intact or fragmented envelopes of various sizes. In some instances several particles were enclosed within a single envelope. The relative number of enveloped virions, difficult to evaluate due to the poor preservation of the envelopes, ranged from 20 to 80% of the total number of virus particles. Other authors have also separated two populations of FV 3 particles during purification. Smith & McAuslan (1969) demonstrated by electron microscopy that their heavier peak contained subviral particles, while the lighter one contained typical FV 3 particles; however these authors did not detect the presence of enveloped particles. Tan & McAslan (1971) also reported the separation of two populations of virus particles, but they did not determine their morphology.
The two fractions we obtained in sucrose gradients had a density of 1.25 ± 0.01 g/ml for unenveloped particles and of 1.20 ± 0.04 g/ml for enveloped ones. The determination of the density, very accurate for the heavier peak, was less precise for the lighter one; this can be explained by the fact that whereas the population of the first peak was very homogeneous, the second peak contained a heterogeneous population of enveloped particles. The density of 1.25 determined in sucrose gradients for nucleocapsids was slightly lower than the values 1.265, 1.275 and 1.285 obtained in caesium chloride gradients by Smith & McAuslan (1969), Vilagines & McAuslan (1970) and Morris et al. (1966), respectively. This may be due to different physical characteristics of the virus in the solutions used. The fact that the densities obtained were relatively low for naked icosahedral particles can be explained by the 9% lipid content of FV 3 particles (Willis & Granoff, 1974).

The polypeptide composition of the virions of each population was analysed by autoradiography after electrophoresis on SDS-polyacrylamide gels. The main difference observed was the presence of an additional 58 K polypeptide in the fraction of enveloped particles compared to naked particles. The presence of a faint band in the 'naked' fraction may be due to contamination with a small amount of enveloped particles or to the actual presence of another polypeptide with a similar mol. wt. in FV 3 particles. This 58 K polypeptide present in the gels of enveloped particles derived from CEF as well as CHO or BHK cells, was most likely a virus-induced polypeptide, for under our experimental conditions, during the period when radioactive labelling was performed, the synthesis of cellular proteins was inhibited by 70%. This 58 K polypeptide is thus a likely candidate for being a major envelope constituent. It should be pointed out that Tan & McAuslan (1971) found that a 54 K polypeptide was present in a light virus peak on top of the gradients used during virus purification. It seems likely that this polypeptide is the same as the 58 K polypeptide that we have described and that their light virus peak may have contained enveloped particles. The FV 3 particles analysed by Goorha & Granoff (1974) did not contain a large amount of a polypeptide equivalent to our 58 K polypeptide, which suggests that the method of purification these authors used selected mostly naked particles. The presence of an additional polypeptide in the enveloped virions may be related to the finding that cell membranes are markedly modified at the morphological level when budding of FV 3 occurs (Tripiet et al. 1974; Kelly, 1975). Using specific stainings, we have previously shown that the most striking event is the appearance of an internal dense layer bound by bridge structures to the outer trilaminar membrane, which is the continuation of the cytoplasmic membrane. Moreover, immunocytochemical techniques have allowed us to demonstrate the presence of virus antigens in the same areas.

The ratio between the number of infectious and physical virus particles led us to assume that the relative infectivity in the peak containing enveloped virions is much higher than in the fraction of naked nucleocapsids. This variation of the infectivity ratios was observed when related both to the number of physical particles determined from electron microscopical counts and to the incorporation of \(^{3}H\)-thymidine and \(^{14}C\)-amino acids. The ratio between infectivities in 'naked' and 'enveloped' fractions varied from one gradient to another, which may reflect differences in the proportion of enveloped particles and in the degree of preservation of the envelopes. This study, which demonstrates that enveloped particles can be up to 150 times more infectious than naked ones, does not allow us to state whether naked nucleocapsids are infectious or not. The low number of infectious units in the fraction of unenveloped particles can indeed be explained either by a lower infectivity of these particles or, in the second hypothesis, by the presence of a few enveloped particles. A similar relationship between the presence of an envelope and the infectivity of FV 3 was previously suggested by Morris et al. (1966). These authors observed that thymidine-
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labelled FV 3 banded in a CsCl gradient at a density of 1.305 g/ml whereas infectious virus was found at 1.287 g/ml (on the light side of the unique radioactive band). They suggested a heterogeneity of the population with an average density of 1.305 g/ml, which may be composed of enveloped virions on the light side and of naked particles at higher densities, the latter being non-infectious. In this paper we have now shown that it is possible to separate two kinds of FV 3 particles and that the presence of the envelope enhances infectivity.

Several hypotheses have already been proposed to clarify the role of the virus envelope for the infectivity of some DNA viruses. Concerning herpes virus, two specific functions were found: one to facilitate the adsorption step (Holmes & Watson, 1963; Dales & Silverberg, 1969), and another to protect the enclosed capsid from the deleterious effects of the extracellular medium (Nii et al. 1968, Stein et al. 1970). In the case of vaccinia virus (Payne & Norrby, 1978), for which extracellular enveloped virions were found to be twice as infectious as naked ones, it was shown that whereas the adsorption kinetics were unafected by the presence of the envelope, the penetration step was favoured for enveloped virions. The envelope of FV 3 may also facilitate either the adsorption step, or the penetration step, or more simply the unenveloped capsid may be unstable in the extracellular environment. Choosing between these different hypotheses requires further investigation.

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