Fine Structure Analysis of Pichinde Virus Nucleocapsids

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SUMMARY

The structure and organization of the ribonucleoprotein (RNP) complex of an arenavirus, Pichinde virus, was investigated. The basic configuration of the RNP was found to be a linear array of globular subunits or nucleosomes, 4 to 5 nm in diameter, that represent individual molecules of the major N polypeptide. This filament appears to fold progressively through a number of intermediate helical structures, 12 to 15 nm in diameter, that reveal an increasing number of nucleosomes associated with each turn of the helix. They range from a fragile configuration of two or three nucleosomes per turn to a more stable fibre in which the nucleosomes cannot be resolved. The strands were shown to form closed circles and it appeared that the twisting of these circular forms resulted in the formation of 20 nm-thick fibres which were seen in isolated viral core structures. The association of these RNP structures with other viral components is discussed.

INTRODUCTION

The family Arenaviridae is a group of enveloped viruses which have been characterized as containing a single-stranded multicomponent RNA genome of negative polarity (Matthews, 1982). There is general agreement that arenaviruses contain a major nucleocapsid-associated protein of mol. wt. 54000 to 68000 together with one or two glycoproteins located in the outer viral envelope. Five additional minor proteins have also been variably reported (for reviews, see Howard & Simpson, 1980; Pedersen, 1979; Rawls & Leung, 1979).

The morphology and morphogenesis of all members of this group are remarkably similar, with the result that it is not possible to distinguish any individual family member either by application of morphological criteria or by the appearance of virus-related structures within infected cells (Murphy & Whitfield, 1975). Extracellular virus is spherical or pleomorphic with an average diameter in the range of 90 to 120 nm, although particles up to 500 nm in diameter are not uncommon (Pedersen, 1979). Embedded in the viral membrane are surface projections that have been described as 5 to 10 nm-long club-shaped structures with an apparently hollow central axis and which are randomly spaced over the viral surface (Murphy & Whitfield, 1975; Gard et al., 1977; Vezza et al., 1977). Examination of ultra-thin sections of both extracellular virus and infected cells reveals the characteristic presence within mature and budding virions of 20 to 25 nm granular structures indistinguishable from host cell ribosomes (Dalton et al., 1968). The role of these ribosomes in arenavirus infections remains unresolved, however, and although their presence is not essential for viral replication (Leung & Rawls, 1977) it has been suggested (Pedersen, 1979) that they may play a limited role in virion assembly.

In contrast to many other enveloped viruses, morphological evidence of a viral nucleocapsid has not been consistent. Strands with a beaded appearance that are 5 to 10 nm in diameter have been described for nucleocapsid fractions of both Tacaribe and Pichinde viruses (Palmer et al., 1977; Vezza et al., 1977); however, it is not clear as to how this material is organized within the virus particle. In order to investigate the architecture of the internal arenavirus components, structures released from purified Pichinde virus (Trapido & San Martin, 1971) by a variety of techniques was examined. The findings reported in this study suggest that the packaging of the
arenavirus genome is strictly organized as helical nucleocapsids that form closed circles of variable length.

**METHODS**

**Virus.** Pichinde virus (strain Coan 3739 R6053) was obtained from Dr J. Casals (Yale Arbovirus Research Unit, U.S.A.) at its ninth passage in suckling mouse brain, passaged twice more on receipt and then plaque-purified three times in Vero cells. Working virus stocks were prepared by harvesting tissue culture fluids from infected baby hamster kidney cells (BHK-21) 48 h post-infection. After clarification to remove cell debris, virus stocks were stored as aliquots at −80°C.

**Growth and purification of virus.** Confluent monolayers of BHK-21 cells in 120 cm² glass bottles were inoculated with virus stock at a multiplicity of infection (m.o.i.) of 0.5 to 1.0. Virus was allowed to adsorb for 1 h at 37°C and then 50 ml of DMEM medium (Gibco Europe, Glasgow, U.K.) containing 2% foetal calf serum and antibiotics was added to each bottle. After a further 24 h at 37°C, the medium was removed and the monolayers washed twice with phosphate-buffered saline (PBS) prior to the addition of either (i) fresh DMEM medium, (ii) DMEM medium supplemented with 10 μCi [3H]uridine (Amersham International) per ml or (iii) MEM medium (Gibco Europe; containing one-tenth the normal concentration of methionine) supplemented with 20 μCi [35S]-methionine per ml and 10 μCi [3H]glucosamine-HCl per ml (Amersham International). Culture fluids were harvested 48 h post-inoculation and clarified by centrifugation at 3000 g for 10 min at 4°C in an MSE bench centrifuge. All subsequent procedures were carried out at 4°C unless otherwise stated. The supernatants were made 0.4 M with NaCl and the virus precipitated for 2 h with 6% (w/v) polyethylene glycol 6000 (PEG; Hythe Chemicals Ltd, Southampton, U.K.). The precipitate was pelleted by centrifugation for 20 min at 10000 g and resuspended in GNTE buffer (0.2 M-glycine, 0.2 M-NaCl, 0.02 M-Tris-HCl pH 7.8, 0.002 M-EDTA; Gschwender et al., 1975). After 1 min sonication in a water bath at 20 kHz, followed by centrifugation for 10 min at 3000 g to remove remaining particulate material, the suspension was layered over a discontinuous gradient, consisting of 20% and 50% (w/v) sucrose in GNTE, and centrifuged for 2 h at 50000 g in a Beckman SW27.1 rotor. The visible band at the interface was collected by side-puncture, diluted 1:3 with GNTE and centrifuged to equilibrium in a 20 to 50% (w/v) continuous sucrose gradient for 20 h at 50000 g in a Beckman SW27.1 rotor. Virus bands recovered from these gradients were subjected to gel filtration through Sephadex G-75 (Pharmacia) and peak virus fractions in the eluate, as monitored by either A₂₈₀ measurements or radioactivity, were collected and used immediately or stored at −80°C.

**Virus fractionation.** Pichinde virus was disrupted with the non-ionic detergent Nonidet P40 (NP40; BDH). The detergent was added to 0.5 ml purified virus (approx. 1 mg/ml) in the presence of 0.5 M-NaCl, to a final concentration of 1% and the mixture incubated for 10 min at 37°C. The disrupted virus was layered over a 10 to 60% (w/v) Urografin (Schering Chemicals Ltd., Burgess Hill, Sussex, U.K.) gradient prepared in GNTE buffer and centrifuged for 20 h at 115000 g in a Sorvall SW40 rotor. Fractions were collected and either solubilized for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or processed immediately for electron microscopy as described below.

**Electron microscopy.** For negative-stain electron microscopy, virus preparations in GNTE buffer were stored at 4°C either unfixed or fixed with 0.1% glutaraldehyde and used within 2 days. Samples were applied directly to carbon-Formvar-coated copper grids and allowed to adsorb for 5 min. The grids were either washed by inversion over a drop of distilled water prior to staining with 2% potassium phosphotungstate (PTA) at pH 5-0 or 6.8, or blotted dry and then inverted directly onto a drop of 2% PTA at pH 5-0. All negative stain solutions contained 0.1% bacitracin.

For rotary-shadowing, purified nucleocapsid preparations in GNTE buffer were fixed overnight with 0.1% glutaraldehyde. The samples were applied to carbon-Formvar-coated copper grids and allowed to adsorb for 5 min. The grids were washed with distilled water, dehydrated with ethanol for a few seconds and blotted dry on filter paper. The grids were then rotary-shadowed at an angle of 10° using either gold-palladium or carbon-platinum evaporated from an electron gun at a vacuum below 10⁻⁵ Torr. All samples were examined in a JEOL 100CX electron microscope.

**RESULTS**

**Electron microscopy of purified Pichinde virus**

Although isolated nucleocapsids of both Tacaribe and Pichinde viruses have been described previously (Palmer et al., 1977; Vezza et al., 1977) neither negative stain nor thin-section analysis of either purified preparations or infected cells has revealed much information on the internal organization of the virus. In order to visualize the internal components with minimal dissociation, purified virus in GNTE buffer was subjected to single-step osmotic shock
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Fig. 1. Purified virus particles lysed by osmotic shock and negatively stained with 2% PTA pH 5.0 shows the release of 12 to 15 nm diameter fibres. Twisting and supercoiling of these fibres into thicker 20 nm strands is frequently observed (arrowed in a and d). Bar markers represent 100 nm in (a), (c) and (d) and 200 nm in (b).

and negative staining. This procedure results in the stabilization of the preparation at the moment of particle disruption. Internal structures released in this way appear as an array of convoluted fibres (Fig. 1), approximately 12 nm in diameter, that are similar to the coiled filaments found in the nucleocapsid preparations of Tacaribe virus (Palmer et al., 1977). Twenty nm diameter fibres were also observed and appear to form through the twisting of the 12 nm strands (arrowed in Fig. 1 a, d). Although accurate length measurements are not possible due to the excessive overlap of these strands, estimates of up to 15 μm were obtained for the fibres released from some particles (Fig. 1 b).

When this material was sufficiently dispersed, other levels of organization were observed. In all preparations examined, ribonucleoprotein (RNP)-like structures resembling those found in other enveloped RNA viruses (Compans & Choppin, 1973) were occasionally seen (Fig. 2 a to d); however, their scarcity suggests that they are relatively fragile. These complexes were composed of 4 to 5 nm globular subunits arranged as helical strands, 12 to 15 nm in diameter
with a pitch of between 7 and 9 nm (Fig. 2a). Their appearance in electron micrographs varied according to the specific arrangement of nucleosomes within each fibre. Fig. 2(a) and (b) are compatible with a helically organized filament containing two or three nucleosomes per turn while Fig. 2(c) and (d) reveal structures in which there is an increased number of nucleosomes associated with each turn of the helix. This increase in the number of nucleosomes per turn is accompanied by a loss in the ability to resolve individual subunits. Fragmentation of these strands was also observed (arrowed in Fig. 2d). The ring-like structures that were released appeared to consist of a single turn of the helix that surrounded a hollow core 5 to 7 nm in diameter. The most stable helical configuration appears to be the 12 nm fibres which are
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Fig. 3. Electron micrograph of Pichinde virus cores prepared by sonication of a purified virus preparation followed by centrifugation in a linear 20 to 50% sucrose gradient. The cores appear to be composed of a loosely interwoven network of thick fibres. Negatively stained with 2% PTA pH 6.8. Bar marker represents 200 nm.

regularly seen in spontaneously disrupted virus preparations (Fig. 1). This further structural condensation results in little increase in the diameter of the fibre (compare Fig. 1 and 2). Unfolding of these higher order structures reveals the basic configuration of the nucleocapsid as a linear array of nucleosomes 4 to 5 nm in diameter that are arranged approximately 6 nm apart (Fig. 2e, f). The mol. wt. of each subunit may be estimated at between 40000 and 60000 based on direct measurements of electron micrographs (Green, 1969). As the major nucleocapsid-associated polypeptide has a mol. wt. estimated at 64000 by SDS–PAGE (Young et al., 1981) it is probable that the 4 to 5 nm beads may represent individual molecules of the major N polypeptide.

Disruption of purified virus using a sonicator probe released cores that were separated intact by centrifugation in a linear sucrose gradient. These cores have a buoyant density of 1.22 g/ml, were 80 to 120 nm in diameter and appeared as a complex arrangement of interwoven convoluted strands 20 to 25 nm thick (Fig. 3).

Virus fractionation

Purified virus was solubilized by incubation with 1% Nonidet P40 (NP40) in 0.5 m-NaCl for 30 min at 37 °C. The disrupted components were then separated by isopycnic centrifugation in a linear Urografin gradient (Fig. 4a). Both the solubilized components remaining at the top of the gradient and material recovered at a density of 1.25 g/ml were analysed by SDS–PAGE. The two glycoproteins, G1 and G2, were present in the solubilized fraction and reflects their association with the viral envelope. Analysis of the dense fraction revealed that the non-glycosylated N polypeptide was the major protein component. The minor p72 and p15 proteins were also found in this fraction (Young et al., 1981). Although a trace of [3H]glucosamine label was found to co-sediment with the nucleocapsid fraction (Fig. 4a) neither glycoprotein was resolved. The association of virion RNA with this material, identifying it as the nucleocapsid, was shown after a similar dissociation analysis was performed on [3H]uridine-labelled purified virus (Fig. 4b). Examination of this nucleocapsid fraction by electron microscopy revealed strands of tightly packed beads (Fig. 5a) that are most probably globular condensations of the 12 nm strands shown in Fig. 1. These globules, approximately 15 nm in diameter, may arise in a manner analogous to the appearance of 'superbeads' in cellular chromatin, which Thoma et al. (1979) suggest form as a result of an association between neighbouring turns of the DNA helix. The addition of a monoclonal antibody directed against the N protein aggregated this material,
Fig. 4. Isolation of Pichinde virus nucleocapsids. Purified virus preparations labelled with either (a) \(^{35}S\)methionine (●) and \(^{3}H\)glucosamine (○) or (b) \(^{3}H\)uridine (▲) were solubilized with NP40 and then separated in linear Urografin gradients as described in Methods. The distribution of radioactivity and the refractive indices of selected fractions (□) were determined. The direction of sedimentation is from left to right. Nucleocapsids were recovered at a density of 1.25 g/ml. Material recovered at a density of 1.14 g/ml in (b) was found to be associated with an undisrupted virus fraction.

Fig. 5. Purified nucleocapsid preparation in GNTE buffer, negatively stained with 2% PTA pH 6.8 to which has been added (a) normal mouse ascitic fluid and (b) monoclonal antibody directed against the N protein of Pichinde virus. Bar marker represents 100 nm.
Fig. 6. Electron micrographs of purified nucleocapsids rotary-shadowed with (a to e) gold–palladium or (f) platinum–carbon, at an angle of 10°. (a) Large nucleocapsid aggregates; (b) 'spider-forms'; (c), (d) and (e) isolated circles in various stages of supercoiling; (f) nucleocapsid fibres as linear arrays of 15 nm beads (compare with Fig. 6a). Bar markers represent 200 nm in (a) and 100 nm in (b) to (f).

thereby confirming the specificity of the protein component, and resulted in further condensation with the formation of beaded structures up to 25 to 30 nm in diameter (Fig. 5b). An association between adjacent turns in the helically organized Pichinde virus nucleocapsids which is enhanced by immune complex formation may also explain the production of these more highly condensed globular forms. In low ionic strength buffers (below 50 mM-NaCl) this fraction unfolds to give the characteristic 'beads-on-a-string' appearance of nucleosomes. The beads are 4 to 5 nm in diameter and are spaced on average 5 to 7 nm apart (Fig. 2f).

Visualization of the RNP is enhanced after rotary-shadowing with gold–palladium (Fig. 6a)
and reveals an array of strands approximately 15 nm in diameter. The presence of ‘spider-forms’ in these preparations reflects the spread of RNP from discrete cores (Fig. 6b) and, when sufficiently dispersed, closed circles either twisted or in an open configuration (Fig. 6c, d, e) and ranging in length from 450 to 1300 nm were also observed. Localized disintegration of some of these strands into 15 nm beads reveals their higher order structure. These beads are clearly seen when the nucleocapsids are rotary-shadowed with platinum–carbon (Fig. 6f).

DISCUSSION

Pichinde virus nucleocapsids were released from purified virions by a variety of techniques and then examined by electron microscopy. The most common structure observed was a 12 to 15 nm diameter fibre which was seen in both disrupted virions (Fig. 1) and in isolated nucleocapsid preparations (Fig. 6). The organization of this strand as a condensed helix composed of an array of subunits that appear to be single molecules of the major N polypeptide was revealed when this structure was relaxed in low ionic strength buffer (Fig. 2). Indeed, all of the different structural forms of the viral nucleocapsid that are described in this study, from the linear array of nucleosomal subunits, their arrangement as variably condensed helical fibres, supercoiling and finally to their packaging as discrete cores appear to be directly related to ionic strength. As the ionic strength is reduced (below 100 mM-\( \text{NaCl} \)) a progressive unfolding of the higher order structures is observed. Like cellular chromatin (Thoma et al., 1979) this may be explained as a consequence of electrostatic repulsion between adjacent charges on the helically organized RNA which is progressively neutralized with increasing ionic strength. This is supported by limited studies showing the apparent requirement for divalent cations to stabilize the higher order core structures which are rarely recovered from nucleocapsid preparations isolated in the presence of EDTA (P. R. Young, unpublished observations). The stability of these structures at ionic strengths comparable with the cellular cytoplasmic environment would suggest a relatively energy-free self-assembly process for nucleocapsid maturation.

Rotary shadowing of isolated nucleocapsids clearly demonstrates their arrangement as closed circles (Fig. 6c, d, e). The variability in the size of these circular forms (from 450 to 1300 nm), however, shows that there is no direct relationship between the two unique viral RNA segments, L and S (Carter et al., 1973; Vezza et al., 1978a) and their packaging as discrete RNP structures. The frequent appearance in these electron micrographs of panhandled forms and linear fibres and the observation that denatured viral RNA is predominantly linear (Vezza et al., 1978b) suggest that these circular nucleocapsids are not covalently closed but may arise as a result of base-pairing. A similar hypothesis for the formation of the circular nucleocapsids of the bunyaviruses (Obijeski & Murphy, 1977) has been supported by the demonstration of sequence complementarity between the 3' and 5' termini of the viral RNA segments of Uukuniemi virus (Parker & Hewlett, 1981). Assuming, then, that circularization results from end sequence base-pairing, the recent report showing homology between the 3' terminal nucleotide sequences of the two RNA species of Pichinde virus (Auperin et al., 1982) would further suggest that base-pairing between either the L and/or S RNAs may occur. The variable lengths of the nucleoprotein structures observed in this study support the possibility that multiple copies of either one or both RNA species are present within individual nucleocapsids.

The supercoiling apparent in many of the circular structures visualized by rotary-shadowing indicates that it may function as a step in the packaging of nucleocapsids during virus maturation. This is supported by the finding of regularly sized 20 nm strands in virus cores (Fig. 3) and is compatible with the presence of supercoiled nucleocapsid fibres seen released from spontaneously disrupted virions (Fig. 1a, d). The core component thus appears to be organized as a result of the extensive convolution of these supercoiled fibres. The mechanisms involved, however, in determining the degree of association and subsequent packaging of individual nucleocapsids as discrete core structures are unknown. The variability of the amount of nucleocapsid material which is packaged within single particles, especially from late harvests (unpublished observations), suggests that if any specific controls are present they are somewhat limited.

Viral proteins other than the major N polypeptide have been shown in association with the nucleocapsids of arenaviruses. Ramos et al. (1972) reported that the G1 glycoprotein component
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of Pichinde virus remained bound to nucleocapsids prepared by solubilization in low salt conditions (100 mM-NaCl). A similar association between G1 and the N polypeptide was observed during the course of the present study (P. R. Young, unpublished observations). The importance of low salt on the interaction between spike proteins and nucleocapsid has also been demonstrated for Semliki Forest virus (SFV; Helenius & Kartenbeck, 1980) and Sindbis virus (Burke & Keegstra, 1976). Investigations on the topology of the membrane-associated SFV glycoprotein (Ziemiecki et al., 1980; Smith & Brown, 1977) for example, have shown that there is a specific, ionic strength-dependent transmembrane association between the short carboxy-terminal end of the spike protein, exposed on the inner face of the plasma membrane, with the underlying nucleocapsid. The attachment, through electrostatic interactions, between the Pichinde virus nucleoprotein and transmembrane domains of the G1 glycoprotein as in SFV may explain the observed association between these two proteins. The formation of such bonds may prove to be an important factor both in the initiation of virion assembly and in the limited control of nucleocapsid packaging.

A minor 72,000 mol. wt. polypeptide was also found to be associated with the core component of Pichinde virus (Young et al., 1981) and remained bound to nucleocapsids separated in a range of ionic strengths. This finding is consistent with reports of a similar association between the purified nucleocapsids of Tamiami and Tacaribe viruses and a non-glycosylated protein designated P (Gard et al., 1977; Vezza et al., 1978a). Although this polypeptide was not found in immune precipitates of either infected cell extracts or purified virus preparations (Harnish et al., 1981), its specific association with the nucleocapsids of Pichinde, Tacaribe and Tamiami viruses and its presence within purified virus grown in different host cells (Pedersen, 1979) suggests that it does possess some functional role in the virus. Whether it contributes to the overall structure of the nucleocapsid, however, is unknown. Apart from the appearance of low molecular weight proteins, which have been shown to be cleavage products of the N polypeptide (Harnish et al., 1981), no other proteins were consistently observed in nucleocapsids isolated in this study.

The importance of the relationship between the viral nucleocapsid and host ribosomes remains unresolved. Their association is clearly seen in the large cytoplasmic aggregates found in infected cells (Abelson et al., 1969) and may be responsible for the incorporation of ribosomes within mature virions. However, in the present study, there was no indication that host ribosomes were integral features of nucleocapsid structures released directly from virus particles. Furthermore, the finding that the core component of lymphocytic choriomeningitis virus could be separated from the denser host ribosomes by gradient centrifugation in high salt (Pedersen & Konigshofer, 1976) indicated that ultrastructural studies on gradient-fractionated material would also be free of contaminating ribosomes. Similarly, host cell ribosomes were not observed in the nucleocapsid preparations of Gard et al. (1977) and Palmer et al. (1977).

It is interesting to note, however, that the association between the nucleocapsid of Pichinde virus and incorporated ribosomal material is maintained following detergent disruption in the same ionic conditions (Farber & Rawls, 1975) that favour both the formation of the higher order structures of the nucleocapsid described in this study, and its interaction with G1. The specificity of these inter-relationships and their role in virion assembly is presently under investigation.

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