Nucleocapsid Protein Subunits of Simian Virus 5, Newcastle Disease Virus, and Sendai Virus¹

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Received for publication 17 July 1970

Helical nucleocapsids of each of the paramyxoviruses simian virus 5 (SV5), Newcastle disease virus (NDV), and Sendai virus have been isolated in two different forms. One form contains larger protein subunits and is obtained from mature virions or infected cells dispersed by ethylenediaminetetraacetic acid. The other form possesses smaller subunits and is obtained from infected cells dispersed by trypsin. The estimated molecular weights of the larger subunits in the three viruses are similar: SV5, 61,000; Sendai virus, 60,000; NDV, 56,000. The smaller nucleocapsid subunits are also very similar: SV5, 43,000; Sendai virus, 46,000; NDV, 47,000. The helical nucleocapsid composed of the smaller subunit appears to be less flexible and more stable than that formed by the larger subunit. There is suggestive evidence that conversion of the larger subunit to the smaller by proteolytic cleavage may occur intracellularly. The possibility that such a mechanism could be involved in the accumulation of nucleocapsid in cells persistently infected with paramyxoviruses is discussed.

Simian virus 5 (SV5), Newcastle disease virus (NDV), and Sendai virus are members of the paramyxovirus group. They possess a lipoprotein envelope covered with surface projections and a helical nucleocapsid. The nucleocapsids of these viruses are morphologically very similar, each having a unit length of $\sim 1 \mu\text{m}$ and a diameter of $\sim 18 \text{ nm}$ (8, 9, 16, 19, 20). They contain single-stranded ribonucleic acid (RNA) with a molecular weight of 6×10^6 to 7×10^6 daltons (2, 10, 13) which is intimately associated with multiple identical protein subunits arranged in a single helix.

In a previous study reported from this laboratory, the protein subunit of SV5 nucleocapsid isolated from infected cells dispersed by trypsin was found to be a single polypeptide with a molecular weight of approximately 43,000 (5). The nucleocapsid protein of NDV has been reported to have a molecular weight of 54,000 (17) or 62,000 (3, 15). Since the nucleocapsids of SV5 and NDV are structurally similar (8, 9, 20), this degree of difference in the molecular weight of the protein subunits seemed unlikely. This communication describes the nucleocapsids of SV5, NDV, and Sendai virus and their protein subunits isolated and compared under identical

conditions. The results indicate that the nucleocapsid proteins of each of these three paramyxoviruses can exist in at least two different forms: a large form with a molecular weight near 60,000, and a smaller form with a molecular weight near 45,000.

MATERIALS AND METHODS

Cells. Monolayer cultures of a variant of the MDBK line of bovine kidney cells were grown on plastic surfaces in reinforced Eagle's medium (REM) (1) with 10% fetal calf serum as described previously (7). The BHK21-F line of baby hamster kidney cells was grown in REM with 10% calf serum and 10% Tryptose phosphate broth (18).

Viruses. The W3 strain of SV5 was grown in MDBK cells. The isolation, growth, and assay of this virus have been described (6, 18). Seed stocks of the Hickman strain of NDV and of Sendai virus were grown in the allantoic sac of 10- to 11-day-old embryonated chicken eggs inoculated with $\sim 10^6$ EID₅₀; allantoic fluid was harvested at 24 hr.

Purification of virus. For electrophoretic studies of virion proteins, all three viruses were grown in MDBK cells in serum-free REM. To label viral proteins, $5 \mu\text{Ci}$ of ^3H -leucine per ml or $1 \mu\text{Ci}$ of ^{14}C -amino acid mixture per ml was added. Released virus was harvested 24 to 42 hr after inoculation with 2 to 20 infective virions per cell, and cell debris was sedimented from the supernatant medium at $3,000 \times g$ for 20 min. Virus was precipitated with an equal volume of saturated ammonium sulfate, banded

¹ Presented in part at 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., 20 April-1 May 1970.

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twice in linear 15 to 40% (w/w) potassium tartrate gradients at 23,000 rev/min for 2.5 hours in a Spinco SW 25.1 rotor, dialyzed overnight against REM or deionized distilled water, and either used immediately or stored at -60°C .

Purification of nucleocapsid. MDBK cells (or BHK21-F cells in the experiment shown in Fig. 9) were infected as above, and after 24 to 42 hr the monolayers were washed twice with phosphate-buffered saline (PBS; 14) without Ca^{2+} and Mg^{2+} and dispersed from the monolayer by 3.0 ml of one of the following solutions: (i) 0.05% ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin (Difco, 1:250); (ii) 0.1% trypsin (Worthington, $2 \times$ crystallized); or (iii) 0.07% EDTA. After addition of one of the above solutions, the bottles were placed at 37°C for 5 to 20 min to dislodge the cells. The cells were then pelleted, resuspended in distilled water, and disrupted by Dounce homogenization; the nucleocapsid was purified by CsCl density gradient centrifugation as described in detail previously (8).

For isolation of nucleocapsid from mature virions, the medium containing released virus was harvested 42 hr after infection, and cell debris was removed by centrifugation at $6,500 \times g$ for 15 min. Virus was pelleted at $52,000 \times g$ for 45 min and suspended in 2.0 ml of tris(hydroxymethyl)aminomethane (Tris)-EDTA-NaCl buffer, pH 7.2 (10); 0.5 ml of 5% Triton-X 100 in buffer was added; the mixture was held at 37% for 20 min, and 0.1 ml of 10% sodium deoxycholate in buffer was added. A 1-ml amount of this suspension was layered onto a discontinuous gradient containing 0.5 ml of 40% CsCl (w/w), 1.0 ml of 30% CsCl, 1.0 ml of 25% CsCl, and 1.5 ml of 5% (w/v) sucrose in buffer (to separate the detergents from the CsCl). Gradients were centrifuged for 90 min at 34,000 rev/min in a Spinco SW 50 rotor, and the visible nucleocapsid band in the 30% CsCl layer was collected. This was dialyzed against distilled water for 2 to 3 hr at 4°C before examination by electron microscopy and electrophoresis.

Chemicals and isotopes. Components for polyacrylamide gels were obtained from Canal Industrial Corp., Rockville, Md.; ^3H -leucine and ^{14}C -amino acid mixture from either New England Nuclear Corp., Boston, Mass., or Schwarz BioResearch, Inc., Orangeburg, N. Y.; bovine serum albumin (BSA) from Armour Pharmaceutical Co., Chicago, Ill.; trypsin, $2 \times$ crystallized, from Worthington Biochemical Corp., Freehold, N.J.; Triton X-100 from Beckman Instruments, Inc., Fullerton, Calif.; sodium deoxycholate from Mann Research Laboratories, New York, N.Y.; and Nuclear Chicago Solubilizer (NCS) from Amersham/Searle Corp., Des Plaines, Ill.

Polyacrylamide gel electrophoresis. Procedures employed in the preparation of gels and in electrophoresis have been described (5). Acrylamide gels (7.5%) approximately 10 cm long with a 0.5-cm stacking gel of 2.5% acrylamide were used. In coelectrophoretic studies, samples were mixed and treated with sodium dodecyl sulfate (SDS) and mercaptoethanol. The processing of gels for determination of radioactivity was as previously described (5),

except that an additional 0.2 ml of NCS was added just before the scintillation fluid.

Electron microscopy. After dialysis, material from the CsCl gradients was placed on grids with carbon-coated Formvar films, stained with 2% sodium phosphotungstate, pH 6.2, and examined in a Philips EM 300 electron microscope.

RESULTS

Identification of two forms of SV5 nucleocapsid protein subunit. Coelectrophoresis of SV5 virion proteins with purified SV5 nucleocapsid from trypsin-dispersed infected cells showed that the single nucleocapsid protein, which has a molecular weight of $\sim 43,000$, migrated with a rate similar, but not identical, to that of the smallest virion protein (Fig. 1A). This small difference in the relative migration of these proteins was found consistently in repeated experiments. Thus, the protein subunit of SV5 nucleocapsid isolated from trypsinized infected cells, although very similar in size to the smallest virion protein, is not identical to this protein, as was previously thought (5). Figure 1B shows that when SV5 nucleocapsid isolated from virions was coelectrophoresed with virion proteins, the nucleocapsid protein migrated precisely with the second major virion protein with a molecular weight of $\sim 61,000$, thereby identifying this protein as the nucleocapsid subunit of the virion. Identical results have been obtained with nucleocapsid isolated from infected cells which were not exposed to trypsin (see Fig. 6A).

The relatively larger size of the protein of SV5 nucleocapsid isolated from virions as compared to that of nucleocapsid isolated from trypsinized infected cells was also clearly shown by coelectrophoresis of these two proteins (Fig. 2). The small form of nucleocapsid protein (molecular weight $\sim 43,000$) was isolated from cells dispersed by either crude Difco 1:250 trypsin or crystalline trypsin. The molecular weights of the proteins indicated above were estimated on the basis of coelectrophoresis with BSA, ovalbumin, and trypsin as markers by the method of Shapiro et al. (24).

Nucleocapsid morphology. Negatively stained SV5 nucleocapsids obtained from disrupted mature virions are shown in Fig. 3. The helical structures are loosely coiled, very flexible, and somewhat fragmented. Similar nucleocapsids were also obtained from SV5-infected cells dispersed from the monolayer by EDTA without trypsin (Fig. 4), and nucleocapsids from both sources possess the larger, $\sim 61,000$ molecular weight, protein subunit. However, when SV5-infected cells were dispersed by EDTA and trypsin prior to disruption, and the intracellular nucleocapsid purified,

FIG. 1. Coelectrophoresis in a polyacrylamide gel of SV5 virion proteins with SV5 nucleocapsid isolated from trypsinized infected cells, or with nucleocapsid isolated from mature virions. (A) Virion proteins labeled with ^{14}C -amino acids and nucleocapsid protein isolated from trypsinized cells labeled with ^3H -leucine. (B) Virion proteins labeled with ^{14}C -amino acids and nucleocapsid protein from virions labeled with ^3H -leucine. Symbols: ●, nucleocapsid protein; ○, virion proteins. In this and subsequent figures, the origin is on the left and the anode on the right.

a morphologically distinct form of nucleocapsid was obtained (Fig. 5). These nucleocapsids are straighter, more tightly coiled, and less flexible than those seen in Fig. 3 and 4; they have a subunit with a molecular weight of $\sim 43,000$. It should be emphasized that all of the preparations of nucleocapsid shown in Fig. 3 to 5 were purified by density gradient centrifugation in CsCl . This is a rigorous procedure and probably accentuates the differences in the stability of the two forms of nucleocapsid. In negatively stained paramyxovirus preparations, nucleocapsids are usually less fragmented than this purified material.

Conversion of the large form of nucleocapsid protein to the small form by trypsin treatment of cells. Figure 6 shows that the large form of nucleocapsid can be obtained from infected cells not exposed to trypsin and that the isolation of the small form is not confined to the MDBK cells which were employed in the experiments described above. Replicate monolayers from a single passage of BHK21-F cells were infected with SV5; after 21 hr, one lot of cells was dispersed with EDTA alone, and the remainder was dispersed by treatment with trypsin and EDTA for ~ 5 min at room temperature. Figure 6A shows that the nucleocapsid protein subunit found in the cells not exposed to trypsin was the larger form of nucleocapsid. In contrast, the nucleocapsid protein isolated from the cells which were dispersed by trypsin (Fig. 6B) migrates as a single sharp peak which is precisely coincident with the small form of nucleocapsid included as a marker. This demonstrates the complete conversion of the nucleocapsid subunit from the large to the small form by a brief treatment of the cells with trypsin to remove them from the monolayer.

Effect of trypsin treatment of purified nucleocapsid in vitro. Treatment of the large form of

FIG. 2. Coelectrophoresis of SV5 nucleocapsid protein isolated from virions labeled with ^3H -leucine (○) and SV5 nucleocapsid protein from trypsinized infected cells labeled with ^{14}C -amino acids (●). ^3H -labeled SV5 virions were mixed with ^{14}C -labeled nucleocapsid isolated from trypsinized infected cells, the mixture was treated with detergents, and the nucleocapsids were purified in CsCl as described in Methods. The purified nucleocapsids were then subjected to polyacrylamide gel electrophoresis.

SV5 nucleocapsid protein with trypsin in vitro converts the protein subunits to smaller-molecular-weight species (Fig. 7). In this experiment, ^3H -labeled nucleocapsid isolated from disrupted virions was incubated at 37°C for 30 min with crystalline trypsin at a concentration of 0.5 mg/ml . The treated nucleocapsid was then rebanded in CsCl prior to electrophoresis. Figure 7A shows that, before treatment with trypsin, the ^3H -labeled virion nucleocapsid protein migrated as the large form relative to ^{14}C -labeled small form of nucleocapsid protein included as a marker. After trypsin treatment (Fig. 7B), the nucleocapsid protein migrated heterogeneously in approximately the same region as the marker.

FIG. 3–5. Electron micrographs of negatively stained SV5 nucleocapsids isolated from virions (Fig. 3), infected cells dispersed by EDTA alone (Fig. 4), and infected cells dispersed by EDTA and trypsin (Fig. 5). In each case, the nucleocapsids were purified in a CsCl gradient, a procedure which may cause fragmentation or uncoiling of the more fragile nucleocapsids. $\times 145,000$.

NDV. To determine if the occurrence of two different forms of nucleocapsid is a general property of paramyxoviruses, two other members of the group were investigated. The results obtained on coelectrophoresis of NDV virion proteins and NDV nucleocapsid obtained from trypsinized infected cells is shown in Fig. 8A. This pattern of NDV polypeptides is, in general, similar to those reported previously (3, 15, 17). There are three major protein peaks and, in addition, two or three minor proteins. The protein subunit of NDV nucleocapsid isolated from trypsinized infected cells has a molecular weight of $\sim 47,000$ and migrated just behind one of the minor virion proteins. However, the protein of nucleocapsid obtained from virions migrated precisely with the second major viral protein (Fig. 8B), a result similar to that of others (3, 15). On the basis of its migration with marker proteins, this NDV nucleocapsid protein has an estimated molecular weight of 56,000.

Sendai virus. Figure 9A shows the polyacrylamide gel electrophoresis pattern of Sendai virion proteins. Four major protein peaks are seen; three of these peaks are very close together and represent proteins with estimated molecular weights of 60,000 to 67,000. The other major protein is much smaller, with a molecular weight of $\sim 39,000$. Between the third and fourth major peaks, there are at least two minor protein components which are seen here and are easily identified in a stained gel. Figure 9A is a comparison by coelectrophoresis of the proteins of Sendai nucleocapsid isolated from trypsinized infected cells with the proteins of the virion. This nucleo-

capsid protein, which has a molecular weight of $\sim 46,000$, clearly does not migrate with any of the virion proteins. However, the protein subunit of nucleocapsids obtained from Sendai virions (Fig. 9B) migrates precisely with the major viral protein which has an estimated molecular weight of $\sim 60,000$.

Comparison of the smaller nucleocapsid subunits of the three viruses. Not only are the larger nucleocapsid proteins found in the SV5, NDV, and Sendai virions similar in size, but the smaller subunits which are formed by proteolytic cleavage of the larger subunits are also very similar in size. This was shown by coelectrophoresis of various combinations of the smaller nucleocapsid proteins of the different viruses (Fig. 10). The migration of the nucleocapsid proteins confirmed their overall similarity in molecular weight and also that the NDV and Sendai virus proteins are slightly larger than that of SV5.

DISCUSSION

Two forms of helical nucleocapsids of paramyxoviruses have been found; one contains a protein subunit which is larger than that of the other form. The larger protein subunit, found in the virion or in cells not exposed to trypsin, has an estimated molecular weight of 61,000, 60,000, or 56,000 in SV5, Sendai, and NDV, respectively; the smaller subunit, isolated from infected cells dispersed by trypsin, has a molecular weight of 43,000, 46,000, and 47,000, respectively, in these same viruses. The smaller subunit is apparently the result of proteolytic cleavage of the larger subunit. That this cleavage is highly specific is

FIG. 6. Conversion of SV5 nucleocapsid protein from the large form to the small form by trypsin dispersion of infected cells. Replicate monolayers from the same passage of BHK21-F cells were infected with SV5 in the presence of ^3H -leucine and after 21 hr were divided into two lots and dispersed with EDTA alone (A), or dispersed with EDTA and trypsin (B). The ^3H -labeled SV5 nucleocapsid was then isolated from each lot of cells and subjected to coelectrophoresis with a marker preparation of the small form of nucleocapsid protein labeled with ^{14}C -amino acids. Symbols: $\bullet$, ^3H -labeled nucleocapsids; $\circ$, ^{14}C -labeled nucleocapsid.

suggested by several findings. (i) Not only can a helical nucleocapsid be composed of the smaller subunits, but the helix appears to be a more tightly bonded and stable structure than the nucleocapsid formed by the larger subunit which is found in the virion. (ii) The cleaved form of nucleocapsid subunits of the three viruses are very similar in size. (iii) With all three viruses,

FIG. 7. Conversion of SV5 nucleocapsid protein isolated from virions to smaller molecules by treatment with trypsin in vitro. (A) Coelectrophoresis of nucleocapsid protein labeled with ^3H -leucine isolated from virions with a marker preparation of the small form of nucleocapsid labeled with ^{14}C -amino acids. (B) Coelectrophoresis of the same materials after treatment of the ^3H -labeled virion nucleocapsid with crystalline trypsin (500 $\mu\text{g}/\text{ml}$) at 37 C for 30 min and repurification in a CsCl gradient.

brief exposure of infected cells to trypsin is sufficient to convert all of the subunits of all the nucleocapsids to a smaller form. The relatively broader distribution of the smaller subunits in some of the gels (Fig. 1, 2, 6A) could indicate a slight heterogeneity in size; however, this material always migrates as a single peak which is frequently quite sharp (Fig. 6B, 9, 10). Thus, the smaller subunit molecules appear to be closely similar, if not identical, in size.

The enzyme(s) involved and the site at which the cleavage of the paramyxovirus nucleocapsid subunits occurs are not yet clear. It is possible that trypsin enters the cells during their brief

FIG. 8. Coelectrophoresis of NDV virion proteins with NDV nucleocapsid isolated from trypsinized infected cells or with NDV nucleocapsid isolated from mature virions. (A) Virion proteins labeled with ^3H -leucine and nucleocapsid protein from trypsinized cells labeled with ^{14}C -amino acids. (B) Virion proteins labeled with ^{14}C -amino acids and nucleocapsid protein from virions labeled with ^3H -leucine. Symbols: $\bullet$, nucleocapsid protein; $\circ$, virion proteins.

FIG. 9. Coelectrophoresis of Sendai virion proteins with Sendai nucleocapsid isolated from trypsinized infected cells or with Sendai nucleocapsid isolated from mature virions. (A) Virion proteins labeled with ^{14}C -amino acids and nucleocapsid protein from trypsinized cells labeled with ^3H -leucine. (B) Virion proteins labeled with ^{14}C -amino acids and nucleocapsid protein from virions labeled with ^3H -leucine. Symbols: $\bullet$, nucleocapsid protein; $\circ$, virion proteins.

exposure to this enzyme and accomplishes the quantitative cleavage of the larger subunits to the smaller ones, or that a sufficient amount of trypsin might remain after harvesting of cells and act on the isolated nucleocapsid after disruption of the cells. This seems unlikely because, although trypsin does cleave the isolated nucleocapsid *in vitro*, this treatment has never yielded a preparation which migrated as the single peak that is consistently obtained from trypsinized cells (Fig. 6, 7). Another explanation, and the one we currently favor, is that trypsinization of the cells somehow triggers a cellular enzymatic mechanism which accomplishes with remarkable efficiency a specific cleavage. This interpretation is supported

by the fact that we have observed in several experiments that SV 5-infected MDBK cells dispersed by EDTA without trypsin contain nucleocapsids with subunits intermediate in size between the large and small forms. Therefore, it is possible that intracellular enzymes could be responsible for the cleavage and that the enzymatic mechanism may be activated very efficiently by exposure of cells to trypsin, but inconsistently by dispersal of cells with EDTA alone.

If such a mechanism exists in cells infected with paramyxoviruses, it is possible that intracellular cleavage of nucleocapsid could occur without treatment of the cells. Such alteration of the nucleocapsid might be involved in the accumulation

FIG. 10. Comparison of the small forms of nucleocapsid proteins of three paramyxoviruses isolated from trypsinized infected cells. Cells were infected with SV5, NDV, or Sendai virus in the presence of ^{14}C - or ^3H -amino acids. The nucleocapsids were isolated, identified by electron microscopy, mixed in various combinations, and subjected to coelectrophoresis. In each panel, the left hand ordinate represents ^3H disintegrations/minute, and the right hand represents ^{14}C disintegrations/minute.

of large amounts of nucleocapsid in infected cells and play a role in the persistent infections which occur with paramyxoviruses (6, 18, 26). The cleaved nucleocapsid might be unsuitable for incorporation into virions, either because of its decreased flexibility or because of the loss of a portion of the subunit which is necessary for proper interaction with the cell membrane during virus assembly. In those paramyxovirus infections in which large amounts of nucleocapsid accumulate intracellularly, the yield of infective virus is usually low (4, 11, 12, 21). It is possible that such a mechanism might play a role in persistent infections not only in cell culture but also in such disease states as subacute sclerosing panencephalitis, a disease of the human central nervous system associated with chronic infection with measles virus and characterized by large intracellular inclusions of nucleocapsid (22, 23, 25). This hypothesis can be tested by determination of the nature of the nucleocapsid protein subunits in persistently infected cells.

The present results provide an explanation for a number of observations and apparent discrepancies in the literature. Hosaka (19) noted that trypsin treatment of Sendai nucleocapsids isolated from virions results in nucleocapsids with less flexibility and more constant diameter. NDV nucleocapsids obtained from virions by Kingsbury and Darlington (20) were more loosely coiled and consequently of greater average length than the NDV or SV5 nucleocapsids isolated from trypsinized infected cells in this laboratory (8, 9). NDV nucleocapsid protein with a molecular weight of 54,000 to 62,000 was isolated by

two groups from virions (3, 15) and by a third from infected cells never exposed to trypsin (17). Further, Bikel and Duesberg (3) reported that treatment of NDV nucleocapsid with Pronase yielded a protein with faster electrophoretic mobility than the native subunit. Finally, the erroneous designation of the smallest protein in the SV5 virion as the nucleocapsid protein (5) was due to the very close similarity in size of that virion protein to the small form of SV5 nucleocapsid protein, i.e., ~43,000 daltons. It is now clear that the subunit of the SV5 nucleocapsid found in the virion has a molecular weight of ~61,000.

It is interesting from the point of view of virus structure, self-assembly, and bonding between protein subunits that a helical structure can be composed of a subunit which is a portion of a larger subunit which forms the helical nucleocapsid in the virion. It is not known whether the smaller subunits can aggregate to form the helix, since the cleavage may always involve a preformed nucleocapsid composed of the larger subunits; however, it is clear that the smaller subunits do maintain a very stable, relatively rigid helical structure. In considering the evolution of paramyxoviruses, it is apparent that there is an advantage in flexibility of the nucleocapsid which would facilitate its incorporation into the budding virion.

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grant AI-05600 from the National Institute of Allergy and Infectious Diseases and by contract AT(30-1)-3983 from the U. S. Atomic Energy Commission. The electron microscope was ob-

tained through equipment grant GB-7681 from the National Science Foundation.

We thank Cathleen O'Connell, Ann Erickson, and Toyoko Kikuchi for excellent technical assistance.

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