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Purification and Preliminary Immunological Characterization of the Type 5 Adenovirus, Nonstructural 100,000-Dalton Protein

ELIZABETH A. OOSTEROM-DRAGON AND HAROLD S. GINSBERG*

Department of Microbiology and Cancer Center, Columbia University College of Physicians and Surgeons, New York, New York 10032

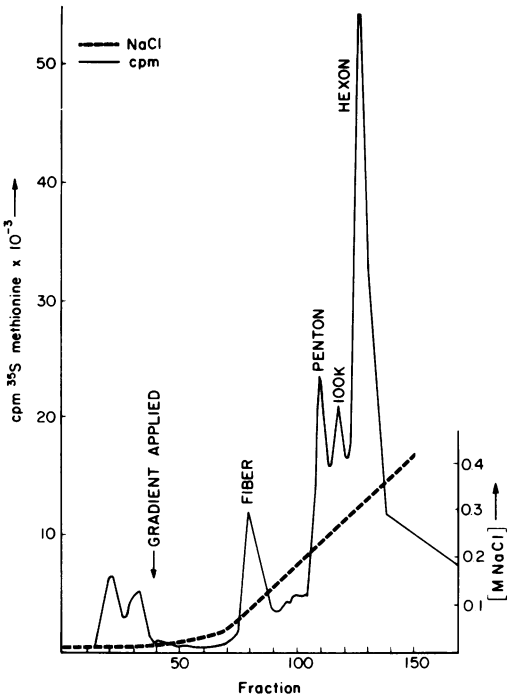
The nonstructural 100,000-dalton (100K) protein of type 5 adenovirus was isolated and purified from infected KB cells by a combination of ion-exchange and affinity chromatographies. Rabbit antiserum containing specific 100K protein antibodies was used for indirect immunofluorescence examination of cells infected with wild-type virus, 100K mutants, and hexon mutants. The 100K protein, which is synthesized as a late protein, was observed primarily in the cytoplasm of cells infected with wild-type and mutant viruses.

During the late phase of type 2 adenovirus and type 5 adenovirus (Ad5) multiplication, a phosphorylated 100,000-dalton protein (termed the 100K protein) is synthesized in large quantities (1, 17). However, unlike other late proteins, the 100K protein is not found in mature virions (1, 17). Although the function of this protein is unknown, an unique group of hexon-minus, temperature-sensitive (*ts*) mutants of Ad5 map in the region coding for the 100K protein rather than in the hexon gene (2, 5, 6, 19). These findings suggested that the 100K protein is essential for the production of hexons.

To investigate the function of the 100K protein, it appeared necessary to purify this protein and to determine its immunological characteristics. This note describes the isolation and purification of the 100K protein from Ad5-infected KB cells, the production of an antiserum directed against the "native 100K" protein, and the intracellular localization of the protein by indirect immunofluorescence

KB cells, at a density of 2×10^5 cells per ml in Spinner Eagle medium (GIBCO Laboratories) supplemented with 5% calf serum, were infected with Ad5 wild-type virus at a multiplicity of 50 PFU/cell (9, 12, 18). At approximately 13 h postinfection, the cells were sedimented (800 × *g*, 15 min) and suspended at a density of 4×10^5 cells per ml in Spinner Eagle medium containing 10% of the normal methionine concentration and supplemented with 5% calf serum. [^{35}S]methionine (Amersham Corp.; 800 to 1,200 Ci/mmol) was added at a concentration of 2 μCi/ml, and viral replication continued at 37°C. The labeled, infected cells were harvested 42 h postinfection, washed twice with 0.2 M phosphate-buffered saline (pH 7.2), resuspended at a concentration of 10^7 cells per ml in a low-salt (L) buffer (0.01 M Tris-hydrochloride [pH 7.6], 0.01 M NaCl, 0.0015 M $MgCl_2$, 0.001 M DL-dithiothreitol), and sonicated (MSE sonicator) four times for 30 s on ice at maximum frequency (8). The lysate was centrifuged at 27,000 rpm for 2 h at 4°C in a Spinco SW50.1 rotor, and the pellet was suspended to one-third the original volume in a high-salt (H) buffer (0.01 M Tris-hydrochloride [pH 7.6], 1.7 M NaCl, 0.0015 M $MgCl_2$, 0.001 M DL-dithiothreitol) by sonicating twice for 20 s on ice and was then kept overnight at 4°C. The suspension was centrifuged at 30,000 rpm for 2 h in the SW50.1 rotor. Seventy to ninety percent of the 100K protein was recovered in the supernatant. The supernatant was briefly dialyzed against L buffer containing 0.1% Triton X-100 to facilitate the removal of excess salt before ion-exchange chromatography. The white flocculent precipitate which formed during dialysis was sedimented at 2,000 rpm for 5 min. (Since this precipitate contained several proteins, including some 100K protein, it was necessary to minimize dialysis to prevent excessive loss of the 100K protein.) The supernatant, which contained the majority of the 100K protein, was applied to a DEAE-cellulose column equilibrated with L buffer containing 1.0% Triton X-100, after which the column was washed with L buffer containing 0.1% Triton X-100. The protein was eluted with increasing concentrations of NaCl developed in a four-chamber gradient mixer; each chamber contained 40 ml of 0.01 M Tris-hydrochloride (pH 7.6), 0.0015 M $MgCl_2$, 0.001 M DL-dithiothreitol, 0.1% Triton X-100, and increasing concentrations of NaCl (0.01, 0.1, 0.25, and 0.5 M, respectively).

The 100K protein eluted from the DEAE-cellulose column between the penton and hexon peaks (Fig. 1). The polypeptide content of the



column fractions was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11). Fractions in which the 100K protein was the major component were pooled and applied to a column of Sepharose 4B to which antibodies to Ad5 virion proteins were conjugated (4). The contaminating hexon and penton molecules bound to the Sepharose-virion antibody complex, whereas the 100K protein appeared in the void volume. The material in the void volume was pooled and assayed for purity by two-dimensional gel electrophoresis (14) (Fig. 2). The procedure described gave an average yield of 800 μg of purified 100K protein per liter of infected cell culture. Purification of

FIG. 1. *DEAE-cellulose chromatography of dialyzed H-soluble material labeled with [^{35}S]methionine. Material was applied to the column and eluted with an increasing NaCl concentration of 0.01 M Tris-hydrochloride [pH 7.6]–0.001 M* DL*-dithiothreitol–0.0015 M $MgCl_2$–0.01% Triton X-100 in a four-chamber gradient device. The concentration of NaCl in the chambers was 0.01, 0.1, 0.25, and 0.5 M. Fractions (1 ml each) were collected, and 0.05-ml samples were spotted on paper disks and counted in a Packard liquid scintillation counter.*

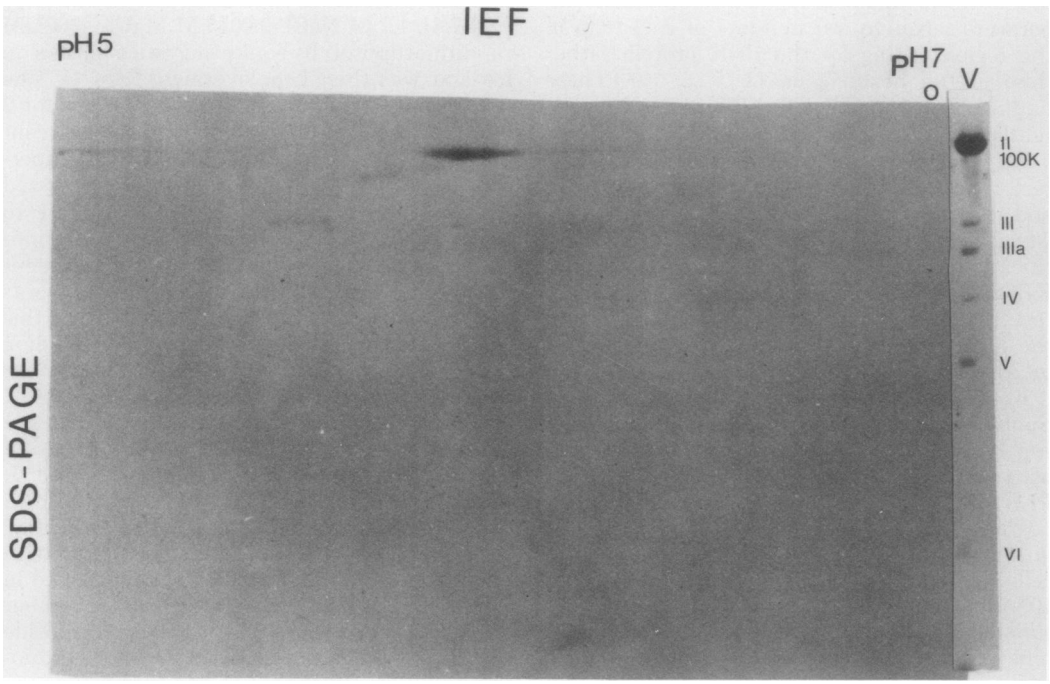


FIG. 2. *Autoradiogram of two-dimensional PAGE of purified 100K protein. Purified 100K protein was lyophilized, suspended in O'Farrell lysis A buffer (14), and electrophoresed on a pH 5 to 7 isoelectric focusing gel (IEF). After the isoelectric focusing run, the gel was equilibrated with SDS buffer (14) and electrophoresed in a 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE). The gel was examined by autoradiography (9). O is the origin of isoelectric focusing. The viral marker gel was electrophoresed separately and aligned according to the positions of proteins II and III (hexon and penton base polypeptides, respectively) seen in accompanying two-dimensional PAGE of the preparation after DEAE-chromatography (data not shown).*

the 100K protein from an artificial mixture of extracts of [^{35}S]methionine-labeled Ad5-infected KB cells and ^{3}H-amino acid-labeled uninfected KB cells (6×10^6 cpm with a specific activity of 130 cpm/μg) demonstrated host protein contamination of the 100K protein from the affinity chromatography fraction to be less than 0.01%.

To obtain specific 100K protein antibodies, purified 100K protein (100 to 200 μg) was mixed with an equal volume of Freund adjuvant, mixed by sonication to form an emulsion, and injected intramuscularly into a rabbit. The rabbit was bled after 2 weeks, reinjected twice (200 μg of protein) at 14-day intervals, and bled 7 days after each injection.

The 100K antiserum was assayed for specificity by double immune agar gel diffusion against 100K protein, purified hexons, and total infected cell lysate. Only a homologous 100K-antibody precipitin line was visible. When purified hexon antiserum was also used, the 100K precipitin line showed nonidentity with the hexon-antihexon precipitin line (data not shown).

Immunoprecipitation (10, 15) followed by SDS-PAGE demonstrated that only the 100K protein was precipitated when the pooled material from the affinity column was used as antigen. However, when an infected whole-cell lysate was used, two to four proteins were precipitated: hexon and 100K polypeptide in roughly equimolar amounts and lesser quantities of 95K and sometimes 37K polypeptides.

To remove the contaminating antihexon activity, in order to study the relationship between hexon and 100K protein, purified hexon (12) was conjugated to carboxymethyl-Bio-Gel A by EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] (Bio-Rad Laboratories) and the anti-100K serum was adsorbed batchwise or by passage through a column. This adsorbed antiserum immunoprecipitated less purified hexon than did normal rabbit serum, whereas purified 100K protein was quantitatively precipitated. However, when infected cell lysates were used, small amounts of hexon and 95K, as well as large amounts of 100K protein, were still immunoprecipitated (Fig. 3).

Since hexon antibodies are the major adenovirus-neutralizing antibodies (18), neutralization studies were done to determine whether the adsorbed 100K serum contained a significant amount of hexon antibodies. Table 1 shows that neither the impure nor the adsorbed 100K antiserum contained neutralizing antibodies. Thus, the precipitation of hexon by the 100K antiserum was due either to specific interaction between 100K and hexon polypeptides or to nonspecific aggregation.

To begin characterization of the 100K protein, immunofluorescence studies were done to determine its cellular localization. HeLa cell monolayers on glass cover slips were infected at 32 and 39.5°C either with wild-type virus or with various *ts* mutants with defects in the 100K (H5*ts*116, H5*ts*115, H5*ts*122, H5*ts*132, H5*ts*17,

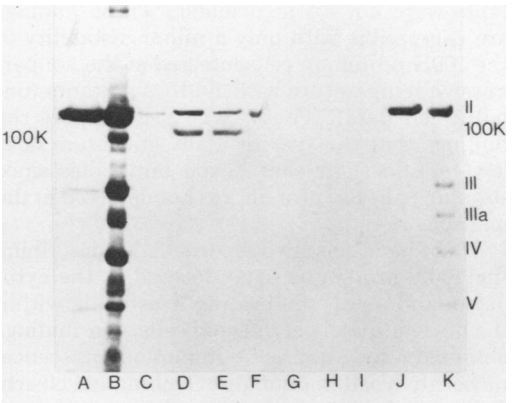


FIG. 3. *Autoradiogram of immunoprecipitated [^{35}S]methionine-labeled polypeptides. Infected whole-cell lysate or purified hexon were immunoprecipitated with normal rabbit serum, anti-100K, adsorbed anti-100K, or antihexon. Immune complexes were precipitated with Staphylococcus aureus, dissociated with 2% SDS, and electrophoresed in a 10% polyacrylamide gel containing 0.1% SDS. Slots A and C through E are infected whole-cell lysates immunoprecipitated with: (A) antihexon; (C) normal rabbit serum; (D) anti-100K; (E) adsorbed anti-100K. Slots G through J are purified hexons immunoprecipitated with: (G) normal rabbit serum (H) anti-100K; (I) adsorbed anti-100K; (J) antihexon. Slot B is infected whole-cell lysate, and slots F and K are Ad5 virion markers. Slot F contains 1/10 of the amount of virion as in slot K.*

TABLE 1. *Neutralization of Ad5 wild-type virus by anti-100K serum and antihexon serum*[a]

Time of reaction (min)	No. of plaques[b] after reaction with:				
	Anti-hexon	Anti-100K	Adsorbed anti-100K[c]	NRS	PBS
0	3	34	33	40	24
10	0	34	30	31	37
30	0	34	35	26	41
60	1	34	34	25	42

[a] At the start of the reaction, equal volumes of virus (approximately 10^5 PFU/ml) and diluted antiserum, normal rabbit serum (NRS), or phosphate-buffered saline (PBS) were mixed and incubated at 37°C. After incubation for the times stated, 0.1 ml was removed from each mixture and added to 10 ml of phosphate-buffered saline, and a plaque assay was done on HeLa cell monolayers.

[b] The average of three dishes per sample.

[c] Adsorbed by passage through a column of carboxymethyl-Bio-Gel A coupled with hexon.

and H5*ts*20) or hexon (H5*ts*147 and H5*ts*138) genes. In all cases, 100K antibodies produced intense cytoplasmic staining with rare nuclear speckling (Fig. 4). It is particularly noteworthy that even at the nonpermissive temperature the staining characteristics of cells infected with wild-type virus, 100K mutants, and hexon mutants were not distinguishable. These findings are compatible with only a minor instability of the 100K protein in cells infected at the nonpermissive temperature with 100K *ts* mutants (unpublished data). These data also support the findings that the specific 100K antiserum does not contain significant hexon antibodies since staining of hexon protein was not detected in the nucleus.

There have been two reports (3, 13) describing the 100K protein as being located in the cytoplasm and one (7) indicating it as being within the nuclear matrix of infected cells. Our findings obtained by indirect immunofluorescence, rather than cell fractionation techniques, clearly demonstrate that the 100K protein is present in the cytoplasm of infected cells at all times during the late period of viral replication.

Complementation and recombination analyses indicate that mutations in two widely separated genes yield *ts* mutants that are phenotypically expressed as hexon minus (i.e., immunologically reactive, trimeric hexons are not detectable at the nonpermissive temperature [9, 16]). One set of mutants (e.g., H5*ts*128 and H5*ts*135) affect the hexon gene (P. A. Luciw and H. S. Ginsberg, manuscript in preparation), and marker rescue experiments demonstrate that H5*ts*115 and H5*ts*116 express mutations in the gene for the 100K protein (Oosterom-Dragon and Ginsberg, manuscript in preparation). These data suggest that the 100K protein is required for the assembly of hexons.

The findings that antinative trimeric (12S) hexon did not immunoprecipitate the 100K polypeptide but that anti-100K precipitated the 100K and hexon polypeptides are consistent with the following hypotheses. The co-immunoprecipitation of the 100K and hexon proteins is nonspecific and occurs because the 100K protein inadvertently aggregates with hexon. Although there are no data available to eliminate this hypothesis, it seems unsatisfactory, however, since the penton and fiber proteins were not also precipitated. Alternatively, the hexon

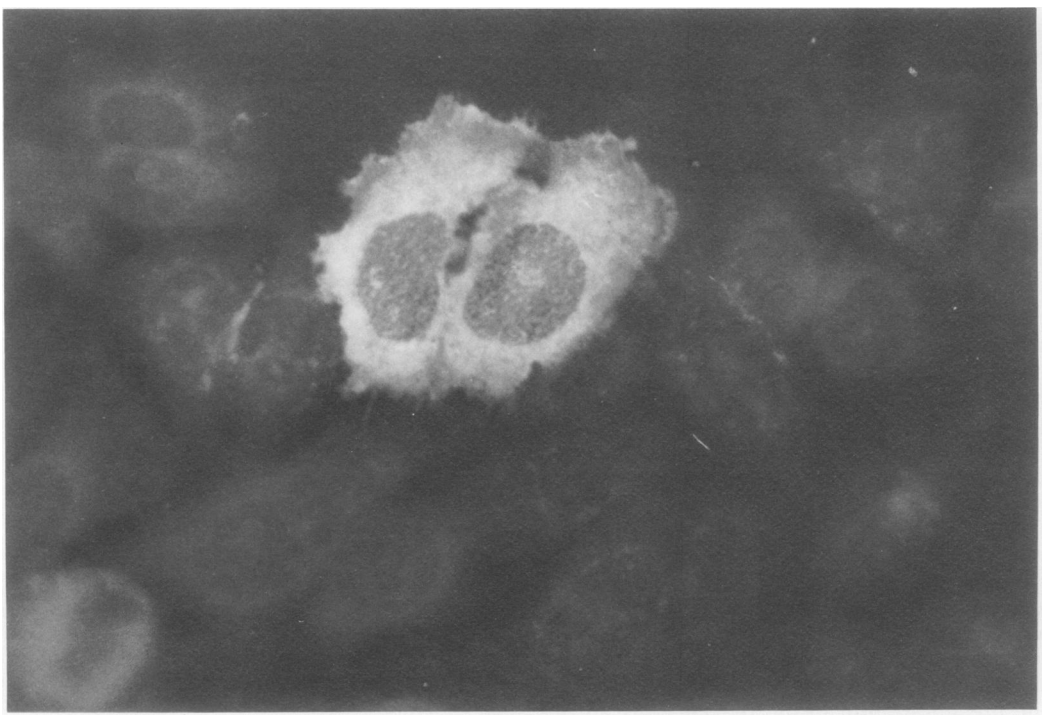


FIG. 4. *Indirect immunofluorescence of Ad5 wild type-infected HeLa cells stained with anti-100K serum. Monolayers of HeLa cells were infected with approximately 0.1 PFU of Ad5 wild-type virus per cell; at 20 h postinfection, the cells were fixed with acetone, reacted with the anti-100K serum, stained with fluorescein-conjugated goat anti-rabbit immunoglobulin G, and viewed with a UV light microscope.*

and 100K polypeptides functionally interact during the development of hexons. It should be emphasized that the anti-100K serum does not contain antihexon antibodies and that the anti-100K serum cannot immunoprecipitate 12S hexons in the absence of 100K, suggesting that an association between the two proteins is necessary for co-precipitation.

(The data in this paper are from a thesis to be submitted by E.A.O.-D. in partial fulfillment of the requirements for the Ph.D. degree in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University, New York.)

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