Immunological and Chemical Identification of Intracellular Forms of Adenovirus Type 2 Terminal Protein

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Highly purified adenovirus type 2 terminal protein (TP) with an apparent M_r of 55,000 (55K) was prepared in quantities of 10 to 30 μ g from guanidine hydrochloride- or sodium dodecyl sulfate-disrupted virions (60 to 120 mg). Guinea pigs were immunized with 14 to 20 injections of TP in amounts of 1 to 2 μ g. Antiserum to TP was used to study the intracellular polypeptides related to adenovirus type 2 TP. By immunoprecipitation with anti-TP serum, we identified 80K and 76K polypeptides in the nucleoplasmic and cytoplasmic S100 fractions of [35 S]methionine-labeled cells early and late after infection with Ad2. By immunautoradiographic analysis which eliminates coprecipitation of unrelated proteins, we identified an 80K polypeptide (probably an 80K-76K doublet) in unlabeled, late infected cells, using anti-TP serum and 125 I-labeled staphylococcal protein A. About two- to threefold-higher levels of the 80K and 76K polypeptides were present in the nucleoplasm than in the S100 fraction, and two- to threefold-higher levels were found in late infected cells than in early infected cells (cycloheximide enhanced, arabinofuranosylcytosine treated). We did not detect the 80K or 76K polypeptide in uninfected cells, indicating that these polypeptides are virus coded. Tryptic peptide map analysis showed that the 80K and 76K polypeptides are very closely related and that they share peptides with the DNA-bound 55K TP. Our data provide the first direct demonstration of intracellular 80K and 76K forms of TP. The intracellular 80K and 76K polypeptides are closely related or identical to the 80K polypeptide that Challberg and co-workers (Proc. Natl. Acad. Sci. U.S.A. 77:5105-5109, 1980) detected at the termini of adenovirus DNA synthesized in vitro and to the 87K polypeptide that Stillman and co-workers (Cell 23:497-508, 1981) translated in vitro. We did not detect the 55K TP in early or late infected cells, consistent with the proposal by Challberg and co-workers that the 80K polypeptide is a precursor to the virion-bound TP and that the conversion of the 80K polypeptide to the 55K TP occurs during virus maturation. The 80K and 76K polypeptides have many more methionine-containing tryptic peptides than does the 55K TP, and most of the tryptic peptides unique to the 80K and 76K polypeptides are very hydrophobic. Thus, the conversion of the 80K and 76K polypeptides to the 55K TP may involve the removal of a specific hydrophobic protein region.

Evidence that human adenovirus DNA molecules are tightly associated with a protein began to accumulate almost two decades ago. Adenovirus type 2 (Ad2), Ad4, Ad12, and Ad18 DNAs were isolated as homogeneous DNA molecules sedimenting at 30 to 32S, but "treatment with papain was required to separate viral DNA from a viral protein component" (12). Adenovirus DNA, isolated by the disruption of virions with guanidine hydrochloride (without protease treatment), aggregated into circular molecules and oligomers (24, 25). Since these aggregates were converted to linear DNA molecules upon treatment with a protease, it was proposed that

a virion protein was tightly bound to the termini of adenovirus DNA (24, 25). Subsequent studies provided evidence that a protein was covalently bound to the 5' ends of adenovirus DNA (4, 5, 22, 23, 27). Rekosh et al. (23) reported that the Ad5 terminal protein (TP) has an apparent molecular weight of 55,000 (55K) on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Green et al. (10) showed that the TPs of five different human adenovirus groups, group A (Ad12), group B (Ad7), group C (Ad2), group D (Ad19), and group E (Ad4) (11), have similar apparent molecular weights (55K). Structurally, they possess closely related chymotryptic and tryptic peptide

maps, suggesting that the primary sequence of adenovirus TPs is conserved among the different adenovirus groups (11).

TP probably plays a role in adenovirus DNA replication, but its exact function is not known. A protein is tightly bound to the termini of adenovirus DNA replicated *in vivo* (7, 9, 18, 28, 29, 32, 33) and *in vitro* (5, 9, 30, 33). Challberg et al. (5) reported that an 80K polypeptide, and not the virion DNA-bound 55K TP, is found at the termini of nascent Ad2 DNA replicated *in vitro*; partial proteolysis experiments indicated that TP and the 80K polypeptide are related. An 80K protein (6) and an 87K protein (30) were found at the DNA termini of Ad2 *ts1* virions formed at the nonpermissive temperature (80K and 87K are molecular weight estimates from two different laboratories of probably the same protein using different molecular weight markers). Since this mutant probably codes for a defective protease that processes several viral structural proteins (2), it was proposed that the 80K polypeptide was cleaved to the 55K TP during virus maturation (6, 30).

For some time, it was uncertain whether TP was of viral or cellular origin, since peptide mapping experiments showed that TP was unrelated to all known Ad2 early proteins and to virion structural proteins (15, 17). Very recently, an 87K polypeptide that shares peptides with TP was translated *in vitro*, using Ad2 mRNA as the template, thus proving that TP is virus coded (30).

The "intracellular" precursors to adenovirus TP have not been established. We describe here our studies which utilized antiserum against highly purified Ad2 TP (isolated from virions) to probe infected cells for intracellular forms of TP. We show by immunoprecipitation and immunautoradiography that 80K and 76K polypeptides are present in early and late infected cells, but not in uninfected cells. The 80K and 76K polypeptides are very closely related by tryptic peptide analysis and share peptides with the 55K TP. No 55K TP was detected in infected cells, consistent with the hypothesis that the 80K polypeptide bound to viral DNA is a precursor to mature virion TP.

MATERIALS AND METHODS

Cells and virus. Ad2 (strain 38-2) was grown in suspension cultures of KB cells in Eagle minimal essential medium containing 5% horse serum (8, 14). For the preparation of TP from virions, cells (10- to 20-liter quantities) were harvested at 36 h postinfection and suspended in 10 ml of 10 mM Tris-hydrochloride (pH 8.1)-1 mM phenylmethylsulfonyl fluoride per liter of cell suspension. The virus was purified by two density gradient bandings in CsCl solution (14) containing 1 mM phenylmethylsulfonyl fluoride.

Purification of preparative quantities of Ad2 TP. Purified Ad2 (60 to 120 mg of virus containing 8 to 16 mg of DNA) was dialyzed at 4°C against 100 volumes of TEM buffer (10 mM Tris-hydrochloride [pH 8.1], 1 mM EDTA, 1 mM 2-mercaptoethanol) containing 0.1 mM phenylmethylsulfonyl fluoride and mixed with an equal volume of 8 M guanidine hydrochloride in TEM buffer containing 0.05% sodium laurylsarcosinate. The Ad2 DNA-TP complex was purified by exclusion chromatography on a Sepharose C1-4B column (19 cm² by 55 cm) as described previously (10). Alternatively, with some preparations, dialyzed Ad2 was mixed with an equal volume of 4% SDS-0.3 M LiCl-20 mM Tris-hydrochloride (pH 7.9)-100 mM dithiothreitol-20% glycerol, heated at 80°C for 10 min, and purified on a Sepharose C1-4B column (19 cm² by 55 cm) as described by Harter et al. (17). With both procedures, after exclusion chromatography, the Ad2 DNA-TP complex was centrifuged to equilibrium in a CsCl gradient (10). The purified Ad2 DNA-TP complex was digested with micrococcal nuclease and electrophoresed on a 10 to 18% SDS-polyacrylamide gradient gel (0.75 mm thick, 10 cm long) (10). The band of Coomassie blue-stained Ad2 TP (see Fig. 1) was excised and used either for immunization of animals or for the experiments described below.

Preparation of antisera to Ad2 TP. Excised gel bands containing TP were washed for several hours with 10% methanol and lyophilized to dryness. The dried bands were ground with a minimal amount of distilled water with a mortar and pestle and homogenized with an equal volume of Freund complete adjuvant with a 5-ml glass syringe fitted with an 18-gauge needle. The final concentration of TP was 2 to 4 µg/ml. Five guinea pigs were inoculated with 0.8 to 2 µg of TP at 2- to 4-week intervals. The initial injection was in the footpads, and subsequent injections were subcutaneous in the subscapular or cervical regions. From 11 to 15 injections were necessary to induce detectable antibodies to TP. Animals were sacrificed by exsanguination after 14 to 20 injections, 2 weeks after the last injection. Blood was allowed to clot and retract at room temperature for several hours or overnight at 4°C, and sera were removed and stored at -70°C.

Two rabbits received 17 inoculations of about 5 µg of the SDS-polyacrylamide gradient gel-purified TP described above over a period of 15 months. One rabbit showed detectable antibody after 12 injections.

¹²⁵I labeling of purified TP for immunoprecipitation analysis. Dried gel bands of excised TP were rehydrated with 1 ml of 50 mM sodium phosphate (pH 7.5)-0.1% SDS and incubated at 37°C overnight. Supernatant fluids were clarified by centrifugation and used directly for ¹²⁵I labeling by either the Iodogen (21) or the chloramine-T method. The concentration of TP was about 10 µg/ml. For the Iodogen method, a glass test tube (13 by 100 mm) was coated with 1.25 µg of 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril (Iodogen, Pierce Chemical) dissolved in 200 µl of methylene chloride. The test tube was dried in a stream of nitrogen and rinsed with 50 mM sodium phosphate (pH 7.5) just before use. A 1-mCi amount of Na¹²⁵I (New England Nuclear Corp.; 17 Ci/mg in 0.1 N NaOH) in 2.1 µl was added, and the pH was neutralized

with 2.1 μ l of 0.1 N HCl. After 200 μ l of TP was added, the mixture was incubated at room temperature for 1.5 h. 125 I-labeled TP was purified by exclusion chromatography on a Bio-Gel P-10 column (10-ml volume) equilibrated with 50 mM sodium phosphate (pH 7.5)–0.1% SDS. Bovine serum albumin (final concentration, 0.01%) was added to freshly prepared 125 I-labeled TP, and the solution was stored at 4°C. For the chloramine-T method, 1 mCi of Na 125 I was neutralized in a glass test tube (13 by 100 mm) with 0.1 N HCl, and 200 μ l of TP was added, followed by 6 μ l of chloramine-T (10 μ g). After incubation at room temperature for 90 s, the reaction was stopped by adding 10 μ l of sodium metabisulfite (50 μ g). 125 I-labeled TP was purified as described above.

Preparation of nucleoplasmic and S100 fractions of [35 S]methionine-labeled and unlabeled cells infected early and late with Ad2. For the preparation of Ad2 early nucleoplasmic and S100 fractions, 200 ml of KB cells at about 4×10^5 cells per ml was centrifuged at 800 rpm for 20 min in an International PR-2 centrifuge at room temperature, suspended in 10 ml of minimal essential medium without serum, and infected with 500 PFU of CsCl density gradient-purified Ad2 per cell (3). After 1 h of adsorption at 37°C, cells were diluted to 200 ml with minimal essential medium containing 5% horse serum and 25 μ g of cycloheximide per ml and further incubated at 37°C. At 4.5 h postinfection, 1- β -D-arabinofuranosylcytosine was added to 20 μ g/ml, and the cells were centrifuged at room temperature and washed twice with warm, methionine-free minimal essential medium containing 5% horse serum and 20 μ g of 1- β -D-arabinofuranosylcytosine per ml. The cells were resuspended in 80 ml of methionine-free minimal essential medium containing 1- β -D-arabinofuranosylcytosine and 5% horse serum (final cell density, about 1.0×10^6 cells per ml) and labeled with [35 S]methionine (10 μ Ci/ml, 1,044 Ci/mmol) from about 5.5 to 15.5 h postinfection. Cells were harvested by centrifugation at 1,200 rpm for 10 min at 4°C and washed twice with cold phosphate-buffered saline lacking Ca $^{2+}$ and Mg $^{2+}$ and containing 1 mM phenylmethylsulfonyl fluoride. The cell pellet was suspended in 4 ml of buffer A (10 mM Tris-hydrochloride [pH 8.5], 250 mM sucrose, 3.7 mM CaCl $_2$, 12 mM MgCl $_2$, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40) and incubated at 4°C for 20 min with occasional mixing. After examination by phase-contrast microscopy to ensure complete cell lysis, nuclei were centrifuged at 2,200 rpm for 10 min and washed with 2 ml of buffer A. The supernatant fluids were combined (cytoplasmic fraction), adjusted to 0.4 M KCl, and incubated for 5 min at 4°C. After the addition of sodium deoxycholate to 1%, the cytoplasmic fraction was centrifuged at 40,000 rpm in a Beckman 50 Ti rotor for 1 h at 4°C. The supernatant fluid (S100 fraction) was stored at –70°C.

The nuclear pellet was sonicated in 2 ml of 10 mM Tris-hydrochloride (pH 7.4)–5 mM MgCl $_2$ –1 mM phenylmethylsulfonyl fluoride at full power in a Raytheon sonic oscillator. One half of each preparation was treated with 50 μ g of pancreatic DNase per ml at 4°C for 30 min. Nonidet P-40 and sodium deoxycholate were added to both preparations to final concentrations of 1% each. After centrifugation at 40,000 rpm in

the Beckman 50 Ti rotor for 1 h at 4°C, the supernatant fluid (nucleoplasmic fraction) was stored at –70°C.

For the preparation of [35 S]methionine-labeled S100 and nucleoplasmic fractions from Ad2 late infected cells, 200 ml of infected cells (without drug treatment) was labeled from 10 to 20 h postinfection. Uninfected cells were similarly labeled for 10 h. Unlabeled early and late infected cell preparations were fractionated in a similar manner with cells infected in complete (methionine-containing) minimal essential medium.

Immunoprecipitation analysis. The [35 S]methionine-labeled S100 and nucleoplasmic fractions were analyzed by immunoprecipitation with guinea pig antiserum to TP by slight modifications of procedures described previously (16). Samples of the [35 S]methionine-labeled nucleoplasmic and S100 fractions (0.8×10^7 to 1.4×10^7 cpm) were incubated overnight at 4°C with 20 μ l of guinea pig antiserum to TP in a 1.0-ml reaction mixture containing 150 mM NaCl, 50 mM Tris-hydrochloride (pH 7.4), 5 mM EDTA, 0.05% Nonidet P-40, 0.02% NaN $_3$, and 0.1% bovine serum albumin. After incubation with 200 μ l of a Formalin-fixed 10% suspension of *Staphylococcus aureus* for 2 h at 4°C, the immunoprecipitate was centrifuged for 1 min in a Beckman model B centrifuge and washed five times with cold 500 mM NaCl–10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA–0.5% Nonidet P-40–1% sodium deoxycholate by vigorous resuspension with a Vortex mixer and recentrifugation. Immunoprecipitated polypeptides were eluted with 40 μ l of 50 mM Tris-hydrochloride (pH 6.8)–2% SDS–10% glycerol–0.02% bromphenol blue at 37°C for 10 min and electrophoresed on a linear 10 to 18% SDS-polyacrylamide gradient slab gel (0.15 cm thick; 10, 14, or 27 cm long). Gels were dried and autoradiographed with Kodak X-Omat R film.

In most immunoprecipitation experiments with late extracts, nonspecific precipitation of [35 S]methionine-labeled viral structural proteins (which are present at high levels at late times postinfection) with both anti-TP and normal sera caused serious background difficulties. This problem was minimized in later experiments by pretreatment of each immunoprecipitation reaction with 20 μ l of normal serum for 1 h at 4°C, followed by incubation with 200 μ l of *S. aureus* for 1 h at 4°C and centrifugation. The supernatant was then used for immunoprecipitation with anti-TP serum.

125 I-labeled TP (2,000 to 10,000 cpm) was immunoprecipitated with 0.5- to 20- μ l amounts of guinea pig antiserum by the procedures described above.

Immunoblotting analysis. Immunoblotting analysis of unlabeled TP, using guinea pig antisera and 125 I-labeled staphylococcal protein A, was performed as recently described by Symington et al. (31). To avoid overloading gels with DNase-treated nuclear extracts and to increase the sensitivity of detection of TP, we developed a two-stage procedure, as follows. Unlabeled nucleoplasmic and S100 fractions of late infected and uninfected cells were prepared and immunoprecipitated as described above. The immunoprecipitates were electrophoresed on 5 to 18% SDS-polyacrylamide gels and transferred to diazobenzoyloxymethyl (DBM) paper as described previously (31). The DBM paper was then incubated se-

quentially with anti-TP serum and ^{125}I -labeled staphylococcal protein A and autoradiographed (31).

Two-dimensional tryptic peptide maps of [^{35}S]methionine-labeled polypeptides. Bands of [^{35}S]methionine-labeled polypeptides identified on dried gels by autoradiography were excised, treated with trypsin, and analyzed by two-dimensional electrophoresis (pH 1.7)–thin-layer chromatography as described previously (16).

HPLC of tryptic fragments of [^{35}S]methionine-labeled polypeptides. Tryptic peptides prepared from [^{35}S]methionine-labeled polypeptides on dried gel bands (as described above) were analyzed by high-performance liquid chromatography (HPLC) as described previously (19, 26), except that a 0 to 40%, rather than a 0 to 20%, 1-propanol gradient was used. The tryptic digests were mixed with 50 μg of a limit tryptic digest of apomyoglobin, which provided an internal control for the resolution of tryptic peptides (monitored by an automatic fluorescence detection system [26]), and made up to 500 μl with 0.5 M pyridine formate (pH 4.1). The digests were subjected to HPLC on a C_{18} reverse-phase column (Altex ultrasphere-ODS, 4.6 by 250 mm). The column was eluted with a linear gradient of 0 to 40% 1-propanol in 0.5 M pyridine formate (pH 4.1) for 110 min at a flow rate of 0.66 ml/min. Fractions (1 ml each) were collected, and 0.8-ml amounts were counted in 5 ml of Aquasol (New England Nuclear Corp.) with a scintillation counter.

RESULTS

Preparative isolation of TP from Ad2 virions. To prepare monospecific antiserum to Ad2 TP, it was necessary to prepare the TP in quantity and in a highly purified form. Large quantities were needed because the induction of detectable levels of antibody to TP required from 11 to 15 injections of 0.8 to 2 μg of gel-purified TP for each guinea pig. Highly purified TP was required because TP is a minor component of Ad2 virions; i.e., two molecules of TP (M_r , ~55K) represent only 0.06% of the total virion (M_r , 175×10^6 [13]). Thus, TP is readily contaminated by other virion proteins which would also elicit antibodies and limit the use of anti-TP serum for immunological studies. The DNA-TP complex was purified by exclusion chromatography of guanidine hydrochloride- or SDS-disrupted virions on a Sepharose C1-4B column, followed by equilibrium centrifugation in a CsCl density gradient. After digestion of the DNA-TP complex with micrococcal nuclease, TP was further purified by SDS-polyacrylamide gel electrophoresis. A typical gel electropherogram of Coomassie blue-stained TP is shown in Fig. 1. Lanes 1 to 6 each contain ~3 μg of TP plus 3 μg of nuclease, and lane 7 contains 3 μg of nuclease alone. The need for the gel electrophoresis step is evident from Fig. 1; i.e., minor quantities of virion proteins, in particular hexon, still contaminate the TP even after exclusion chromatogra-

FIG. 1. SDS-polyacrylamide gel electropherogram of purified Ad2 DNA-TP complex digested with micrococcal nuclease. A typical preparation of Ad2 DNA-TP complex purified by gel filtration and density gradient centrifugation was digested with micrococcal nuclease (4 μg of nuclease per mg of DNA-TP at 37°C overnight), electrophoresed on a 10 to 18% SDS-polyacrylamide gel, and stained with Coomassie blue as described in the text. Lanes 1 to 6 each contain about 3 μg of TP and 3 μg of micrococcal nuclease; lane 7 contains nuclease only. M, markers of bovine serum albumin (68K) and ovalbumin (43K) were run on a parallel lane.

phy and density gradient centrifugation of the Ad2 DNA-TP complex. The yields of complex in 13 different preparations, starting with 60 to 120 mg of purified virus, ranged from 45 to 70% after the equilibrium centrifugation step. The recovery after gel electrophoresis was 1 to 2 μg of TP per mg of viral DNA, 25 to 50% of the theoretical yield.

Preparation and characterization of guinea pig antiserum to Ad2 TP. The experiments described below were performed with guinea pig antiserum which is referred to as "anti-TP." Sera were tested for anti-TP activity by immunoprecipitation of ^{125}I -labeled TP or by immunautoradiography of unlabeled TP. TP eluted from SDS-polyacrylamide gels and radio-labeled with ^{125}I by the Iodogen or chloramine-T method migrated on SDS-polyacrylamide gels mainly as a single component with an approximate molecular weight of 55K (Fig. 2A). The immunoprecipitation of ^{125}I -labeled TP with increasing levels of anti-TP is shown in Fig. 3. Maximum precipitation occurred with about 4 to 10 μl of serum in a 1.0-ml immunoprecipitation reaction; from 20 to 50% of the ^{125}I -labeled TP counts per minute were immunoprecipitated, depending upon the age of the ^{125}I -labeled TP preparation. The immunoprecipitate was electrophoresed on an SDS-polyacrylamide gel and transferred to DBM paper. As shown in the autoradiograph (Fig. 2C), ^{125}I -labeled TP was immunoprecipitated by anti-TP, but not by normal serum.

FIG. 2. Autoradiographs and immunoautoradiographs of Ad2 TP. (A) Autoradiograph of Ad2 TP labeled with ^{125}I by the Iodogen and chloramine-T methods and electrophoresed on a 5 to 18% SDS-polyacrylamide slab gel. (B) Immunoautoradiograph of Ad2 TP. Purified, unlabeled TP (50 ng) was electrophoresed on a 5 to 18% SDS-polyacrylamide gel, transferred to DBM paper, and detected by the use of anti-TP serum and ^{125}I -labeled staphylococcal protein A. (C) Autoradiograph of ^{125}I -labeled Ad2 TP after immunoprecipitation, electrophoresis on a 5 to 18% SDS-polyacrylamide gel, and transfer to DBM paper. Exposures were for 2 to 4 h for the ^{125}I lanes and for 1 day for the ^{14}C -labeled marker proteins (New England Nuclear Corp.): phosphorylase b (92.5K), bovine serum albumin (69K), ovalbumin (46K), and carbonic anhydrase (30K).

Unlabeled TP was readily detected by immunoautoradiography. The TP (50 ng) was electrophoresed on an SDS-polyacrylamide gel, electrophoretically transferred to DBM paper, and treated sequentially with anti-TP and ^{125}I -labeled staphylococcal protein A. The immunoautoradiograph shows a single sharp band of TP (Fig. 2B).

Identification of intracellular polypeptides related to Ad2 TP in the nucleoplasmic and cytoplasmic S100 fractions of cells labeled with [^{35}S]methionine at early and late times after infection. Nucleoplasmic and S100 fractions were prepared from [^{35}S]methionine-labeled uninfected KB cells and from infected KB cells at early and late times postinfection. Early infected cells were treated with cycloheximide from 1 to 4.5 h postinfection and labeled with [^{35}S]methionine from 5.5 to 15.5 h postinfection in the presence of 1- β -D-arabinofuranosylcytosine to block the late stages of infection. Late infected cells were labeled from 10 to 20 h postinfection. Uninfected KB cells were labeled for 10 h. Portions of each nucleoplasm preparation were treated with pancreatic DNase to liberate TP bound to viral DNA.

[^{35}S]methionine-labeled nucleoplasmic and

S100 preparations were immunoprecipitated with anti-TP, and the immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel and autoradiographed. As shown in Fig. 4a, distinct 80K and 76K polypeptides were immunoprecipitated from the nucleoplasmic and S100 fractions of Ad2 early and late infected cells by anti-TP (lanes designated TP), but not by normal guinea pig serum (lanes designated N). Treatment of the nucleoplasm with DNase increased the levels of the 80K and 76K polypeptides immunoprecipitated by anti-TP (see below for quantitation), suggesting that some of the 80K and 76K polypeptides may be bound to viral DNA. No 80K or 76K polypeptide was detected in the nucleoplasmic and S100 fractions of uninfected KB cells (Fig. 4a). In addition, no 55K TP was detected in early or late infected cells, suggesting the possibility that the 80K and 76K polypeptides, which we show here as immunologically and chemically related to the 55K TP, may be precursors to the virion-bound 55K TP. Recent reports by Challberg et al. (5, 6) and by Stillman et al. (30) support this possibility.

To obtain a quantitative estimate of the relative amounts of the 80K and 76K polypeptides immunoprecipitated from the nucleoplasmic and S100 fractions of early and late infected cells, densitometric tracings of each gel autoradiogram in Fig. 4a were obtained (Fig. 4b). The areas under the peaks of the 80K and 76K polypeptides were measured. The results of this analysis of Fig. 4a showed that there was two to three times as much 76K polypeptide immunoprecipitated as 80K polypeptide, except in the late S100 fraction, in which about equal amounts of each form were found. In the early extract, approximately equal counts per minute of the 80K and 76K polypeptides were immunoprecip-

FIG. 3. Immunoprecipitation of ^{125}I -labeled Ad2 TP by guinea pig antiserum. Approximately 500-ng amounts of ^{125}I -labeled TP were immunoprecipitated with various amounts of anti-TP serum in a final volume of 1.0 ml. About 45% of the input counts per minute were precipitated with 4 to 10 μl of serum.

FIG. 4. (a) Autoradiogram of a polyacrylamide gel of [35 S]methionine-labeled polypeptides immunoprecipitated from cells early, late, and mock infected with Ad2 by guinea pig antiserum to the Ad2 TP. [35 S]methionine-labeled nucleoplasmic and S100 fractions of infected and uninfected cells were prepared and immunoprecipitated with anti-TP as described in the text; a preclearing with normal serum was not included in these experiments. M. markers are methyl- 14 C-labeled phosphorylase b (92.5K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K), lactoglobulin A (18.4K), and cytochrome c (12.3K). (b) Densitometric tracings of 80K and 76K polypeptides. The autoradiograms in (a) were traced with a Joyce-Loebl microdensitometer.

itated from the untreated nucleoplasm and the S100 fraction. Treatment of early nucleoplasm with DNase increased the amount of the 80K and 76K polypeptides about twofold. In late extracts, the amount of the 80K and 76K polypeptides immunoprecipitated from untreated nucleoplasm was two to three times that immunoprecipitated from the S100 fraction. The amount in the nucleoplasm was increased two to three times by treatment with DNase. Finally, about twice as much of the 80K and 76K polypeptides was immunoprecipitated from the nucleoplasm of late infected cells as was immunoprecipitated from that of early infected cells.

In addition to the 80K and 76K polypeptides which are precipitated only by anti-TP, the gels in Fig. 4a contain a background of virion structural proteins which are present at high levels late after infection and are nonspecifically precipitated by both anti-TP and normal guinea pig serum. This background was minimized by including a preclearing step with normal guinea pig serum (see Fig. 6 for an example) as described above. We also were able to reduce background by including unlabeled Ad2-disrupted virions in the immunoprecipitation reaction (data not shown). To be assured that the 80K and 76K polypeptides specifically react with anti-TP, we analyzed unlabeled extracts by immunautoradiography, as described below.

Immunautoradiographic detection of unlabeled 80K polypeptide in the nucleoplasmic and S100 fractions of Ad2 late infected cells. We have recently described a sensitive immunautoradiographic procedure to identify unlabeled proteins (31). Proteins are resolved by one- or two-dimensional SDS-polyacrylamide gel electrophoresis, transferred to DBM paper, and detected by a sequential reaction with specific antiserum and 125 I-labeled staphylococcal protein A. By this procedure, we can detect as little as 1 ng of protein present at a concentration of 1 part in 100,000 of cell protein. The major advantage of this method is that a single band of protein can be identified unobscured by extraneous protein bands that often contaminate immunoprecipitates of radiolabeled extracts. We have developed a two-step modification of this procedure which possesses increased sensitivity and avoids the problem of overloading gels with DNase-treated protein extracts. Unlabeled nucleoplasmic (DNase-treated) and S100 fractions prepared from 0.2×10^7 and 1×10^7 late infected cells were first immunoprecipitated with anti-TP. The immunoprecipitate was then electrophoresed on an SDS-polyacrylamide gel, transferred to DBM paper, and treated with anti-TP and 125 I-labeled staphylococcal protein A. A well-defined 80K

polypeptide band was detected in the nucleoplasm from 0.2×10^7 and 1×10^7 late infected cells (Fig. 5, lanes designated I). The 80K polypeptide was readily detected in the cytoplasm (S100 fraction) from 10^7 cells (Fig. 5). Erasure of this immunautoradiograph and analysis with antiserum to the Ad2 73K DNA-binding protein and to the Ad2 penton base protein showed no labeling, confirming that these proteins of similar molecular weight were not responsible for the band detected (data not shown). Our inability to resolve the 80K and 76K polypeptides in this experiment was probably due to the use of a short gel (14 versus 27 cm in Fig. 4). The 80K polypeptide was not detected in mock-infected cells (Fig. 5, lanes designated M). In agreement with the immunoprecipitation analysis (Fig. 4a), the 55K TP was not found in the nucleus or the cytoplasm of late infected cells.

Comparison of tryptic peptides of the 80K and 76K polypeptides and of virion-bound 55K TP by two-dimensional electrophoresis-chromatography and by HPLC. To establish the relationship between the intracellular 80K and 76K polypeptides and Ad2 TP, we mapped these polypeptides. Tryptic peptide maps of the [35 S]methionine-labeled 80K and 76K proteins resolved in the SDS-polyacrylamide gel electropherogram of Fig. 6 were analyzed (two-dimensional paper electrophoresis-thin-layer chromatography [16]) and compared with that of the 55K TP isolated from [35 S]-

FIG. 5. Immunautoradiographic detection of 80K polypeptide in Ad2 late infected cells. Extracts of unlabeled nucleoplasmic and S100 fractions corresponding to 0.2×10^7 or 1.0×10^7 Ad2 late infected cells were immunoprecipitated with 20 μ l of anti-TP serum. The immunoprecipitate was electrophoresed on a 5 to 18% SDS-polyacrylamide gel, transferred to DBM paper, and treated sequentially with anti-TP serum and 125 I-labeled staphylococcal protein A. I, Ad2 infected; M, mock infected; 14 C-labeled marker proteins are as described in the legend to Fig. 4a.

FIG. 6. Polyacrylamide gel electropherogram of the 80K and 76K polypeptides immunoprecipitated from the nucleoplasm of Ad2 late infected cells by guinea pig antiserum to TP and tryptic peptide maps of the 80K and 76K polypeptides and of the 55K TP isolated from Ad2 virions. A sample of nucleoplasm from [35 S]-methionine-labeled Ad2 late infected cells containing 2×10^7 cpm was immunoprecipitated with 20 μ l of anti-TP serum, and the immunoprecipitate was electrophoresed on an SDS-polyacrylamide gel as described in the text; preclearing with normal serum was included in these immunoprecipitation reactions. The bands of the 80K and 76K polypeptides were excised, and peptides were mapped. [35 S]-methionine-labeled TP for peptide mapping was isolated from Ad2 virions (15).

methionine-labeled Ad2 virions (Fig. 6). Seven methionine-containing peptides were identified in the Ad2 TP. Most, if not all, of these peptides are present in the maps of the 80K and 76K polypeptides. The 80K and 76K polypeptides are closely related and contain many more methionine-containing tryptic peptides than does the TP.

To confirm the peptide maps of Fig. 6, the tryptic peptides of the 80K and 76K polypeptides and of the 55K TP were analyzed also by reverse-phase HPLC. A total of 16 to 17 methionine-containing tryptic peptides could be identified in both the 76K and 80K polypeptides (Fig. 7). All but perhaps one peptide appear to be shared by the 80K and 76K polypeptides. The 55K TP peptide profile contains about seven methionine-labeled tryptic peptides, all of which appear to be shared with the 76K and 80K polypeptides. Most of the more hydrophobic peptides, which elute in the second half of the gradient (the gradient consists of 0 to 40% 1-

propanol in 0.5 M pyridine formate, pH 4.1), appear to be absent from the TP. Because of the relatively low amounts of [35 S]-methionine incorporated into the TP isolated from virions, it was difficult to obtain a more definitive profile of the hydrophobic peptide region. If the 80K and 76K polypeptides are precursors to the TP, these data suggest that a very hydrophobic region is removed during the maturation of the virion-bound 55K TP.

DISCUSSION

We have shown that guinea pig antiserum to highly purified Ad2 55K TP specifically immunoprecipitates an 80K and a 76K polypeptide from the nucleoplasmic and S100 fractions of [35 S]-methionine-labeled Ad2 early and late infected cells. We also demonstrated the presence of the 80K polypeptide (probably a doublet) in the nucleus and cytoplasm of unlabeled late infected cells by a two-step immunoautoradiographic procedure that used anti-TP serum and

FIG. 7. Reverse-phase HPLC of tryptic fragments from [35 S]methionine-labeled 80K and 76K polypeptides immunoprecipitated from the nucleoplasm of late infected cells and of Ad2 55K TP isolated from virions. See the legend to Fig. 6 for the isolation of the 80K and 76K polypeptides and TP. HPLC was run in 0.5 M pyridine formate (pH 4.1) on a 0 to 40% linear gradient of 1-propanol as described in the text.

125 I-labeled staphylococcal protein A. The 80K and 76K polypeptides are closely related to each other and to the Ad2 55K TP, as shown by comparison of [35 S]methionine tryptic maps. We did not detect the 55K TP in early or late infected cells, nor did we detect the 80K, 76K or 55K protein in uninfected cells by immunopre-

cipitation of labeled extracts or by immunoprecipitation of unlabeled extracts.

The intracellular 80K and 76K polypeptides that we have identified are most likely the same protein that was detected at the termini of in vitro-replicated Ad2 DNA (5) and that was translated recently in vitro with Ad2 mRNA as the template (30). Challberg et al. (5) proposed that the 80K polypeptide is a precursor to virion-bound TP. This suggestion is supported by recent findings that the 80K polypeptide, rather than the 55K TP, is bound to the DNA of Ad2ts1 virions grown at the nonpermissive temperature (6, 30). Ad2ts1 is defective in a protease that facilitates the post-translational cleavage of viral structural proteins during virus assembly (2). Presumably, this protease cleaves the adenovirus DNA-bound 80K protein to the 55K TP. Our inability to detect the 55K TP in infected cells is consistent with a precursor role for the 80K polypeptide and with the conversion of the 80K protein to the 55K protein within mature virions.

Recently Stillman et al. (30) translated in vitro an 87K polypeptide from Ad2 *l*-strand mRNA mapping between coordinates 11.2 and 31.5. The 87K polypeptide is related to the 55K TP and to the Ad2ts1 virion-bound 87K protein. Thus, it is probably the same protein as the 80K polypeptide observed by Challberg et al. (5, 6) on in vitro-replicated DNA and the intracellular 80K protein that we describe here. The difference in molecular weight estimates probably reflects a difference in molecular weight markers. The additional 76K polypeptide which we describe is not resolved on short gels (14 cm or less) in which an 80K doublet is observed. However, on 27-cm gels the 80K and 76K polypeptides are seen as discrete bands.

The 80K and 76K polypeptides probably represent closely related forms of the same protein; e.g., one species may be phosphorylated or may contain a deoxycytidylate residue (20). The 80K and 76K polypeptides contain many more methionine-containing tryptic peptides than does the 55K TP. Several of the tryptic peptides unique to the 80K and 76K polypeptides are very hydrophobic, since they eluted from a reverse-phase C_{18} HPLC column between 20 and 40% 1-propanol (Fig. 7). Thus, the 80K and 76K polypeptides are very hydrophobic proteins, and their conversion to the 55K TP involves the loss of a hydrophobic region. Of interest, the poliovirus terminal protein also has properties that typify hydrophobic proteins (1).

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