Characterization of a Major Histocompatibility Complex Class I Antigen-Binding Glycoprotein from Adenovirus Type 35, a Type Associated with Immunocompromised Hosts

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Adenovirus type 35 (Ad35) is a group B adenovirus that has been isolated primarily from patients with acquired immunodeficiency syndrome and other immunodeficiency disorders. We have studied the interaction of this unique adenovirus with the immune system by analyzing Ad35 early viral proteins in infected HeLa cells. We have identified a 29,000-Mr Ad35 early glycoprotein, E29, which associates with class I antigens of the major histocompatibility complex (MHC) in the endoplasmic reticulum. Ad35 E29 is analogous to the group C Ad2 early glycoprotein E3-19K (E19), which has been shown to interfere with the expression of class I antigens on the cell surface (H. Burgert and S. Kvist, Cell 41:987-997, 1985). In contrast to the Ad2 glycoprotein, Ad35 E29 was synthesized in much smaller amounts, was more extensively glycosylated, and did not cross-react with polyclonal antibody against the Ad2 protein. As a control, a class I antigen-binding glycoprotein from another group B adenovirus, Ad7, was also characterized and was found to have properties similar to those of Ad35 E29. Therefore, the differences in the glycosylation and quantity of class I antigen-binding glycoproteins between Ad35 and Ad2 are group related. Inhibition of the expression of MHC class I antigens, which are needed for cytotoxic-T-lymphocyte recognition of virus-infected cells, appears to play a vital role in the adenovirus life cycle in vivo. Our data indicate that this function has been conserved despite significant differences in the MHC class I antigen-binding glycoprotein and in the pathogenicity between serotypes.

Adenoviruses are lytic DNA viruses which cause a variety of acute diseases, including respiratory, gastrointestinal, urinary, and ophthalmologic infections (12). There are 41 known human serotypes, which have been subdivided into seven groups (A to G) based on hemagglutination patterns, oncogenicity in rodents, and DNA homology (27). In addition to acute pathology, adenoviruses are persistently shed in feces for years postinfection in normal individuals (7). Adenoviruses can also exist in a latent state and cause disseminated disease in immunocompromised patients due to reactivation (22, 28). The mechanism(s) by which adenoviruses establish persistent and latent infections is unknown.

We have been studying adenovirus type 35 (Ad35) and the closely related Ad34, which are group B adenoviruses that have been isolated primarily from immunocompromised hosts (11, 24). We have reported previously that over 10% of patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex examined at our institution had urine cultures positive for Ad35, though it was not isolated from other body sites (13). Most of the patients in our survey remained free of symptoms characteristically attributed to adenovirus infections despite persistent Ad35 viruria and did not produce specific antibody to Ad35 (6). It is unclear whether the frequent isolation of Ad35 in AIDS patients is due to reactivation or represents primary infection. Presumably, Ad35 can remain latent in the kidney, as reflected by the transmission of Ad35 infection via renal transplantation, but this is an unusual event. Ad35 infection appears to be uncommon in the general population because this serotype was rarely isolated during our survey of normal individuals with febrile illnesses and because Ad35-specific antibody was present at a very low titer in pooled immunoglobulin.

We have proposed that Ad35 may be unique from other adenoviruses in its interaction with the immune system. Unfortunately, there is no well-established animal model using human adenovirus strains that can be used to study this hypothesis. However, intriguing in vitro studies have implicated several adenovirus early proteins as modulators of the immune response, specifically via interference with the expression of major histocompatibility complex (MHC) class I antigens. In cells transformed by Ad12 (group A), a serotype which is highly oncogenic in rodents, the adenovirus early region E1A has been shown to specifically inhibit the posttranscriptional processing of class I mRNA (26). In contrast, in the group C Ad2-infected cells and in cells transfected with the Ad2 early region 3 (E3), the E3-19K glycoprotein binds to class I antigens in the endoplasmic reticulum (1, 3). This protein-protein interaction inhibits the normal processing and transport of the MHC antigens, thereby reducing expression on the cell surface. Since class I antigens are necessary for cytotoxic-T-lymphocyte recognition of virus-infected cells, a reduction in the level of cell surface MHC antigens may modulate acute pathogenicity (4). In addition, this function may be important in the establishment of viral persistence and latency.

As an initial attempt to study the biology of Ad35, we have analyzed Ad35 early proteins in infected HeLa cells. We have identified a 29,000-Mr Ad35 early viral glycoprotein which is analogous to Ad2 E19 and can be immunoprecipi-
tated with MHC class I antigens. We characterize here Ad35 E29 and compare it with the Ad2 glycoprotein.

**MATERIALS AND METHODS**

Cells and viruses. HeLa cells were grown in suspension cultures in Joklik modified Eagle suspension medium (SM) supplemented with 7% calf serum. Ad35 Holden, the Ad35 prototype, was kindly provided by Michael Oxman, University of California at La Jolla. Ad35 Holden was used for all Ad35 infections unless otherwise indicated. Due to the poor replication of Ad35 Holden in HeLa cells, however, the closely related Ad34 Rod was used later in our studies. Ad34 Rod, a urine isolate obtained in 1979 from a bone marrow transplant patient, was typed as Ad34 by hemagglutination inhibition and was provided by J. Gold and D. Armstrong, Memorial Sloan-Kettering Hospital, New York, N.Y. Genomic analysis with the restriction endonucleases Smal, HpaI, and BamH1 revealed that Ad34 Rod shared 23 of 24 restriction sites with Ad35 Holden (data not shown). Ad7 Gomen was obtained from the American Type Culture Collection, Rockville, Md. Each adenovirus serotype was purified from infected HeLa cells by isopycnic banding twice on CsCl gradients, and the number of particles was measured by determining the optical density at 260 nm as previously described for Ad2 (18).

**Radiolabeling of early viral proteins.** Titration of each adenovirus stock was performed to optimize the early phase of infection in HeLa cells. All infections were designed to produce an amount of the early viral DNA-binding protein (DBP) comparable to that in cells infected with Ad2 at 4 \times 10^3 particles per cell (approximately 200 PFU per cell). Ad35 Rod infections were performed with 4 \times 10^4 particles per cell, whereas for both Ad35 Holden and Ad7 it was necessary to use 4 \times 10^5 particles per cell. Virus was adsorbed onto HeLa cells at a concentration of 5 \times 10^6 cells per ml for 30 min at 37°C in SM without serum. At time zero, cells were dilute to 10^6 cells per ml in SM with 7% calf serum. At 2 h postinfection cycloheximide (25 \mu g/ml) was added and was removed at 5 h by washing the cells in phosphate-buffered saline. Cells were then suspended in SM containing 7% calf serum, 1/20 the normal concentration of methionine, cyto-
sine arabinoside (4 \mu g/ml), and 20 Ci of L-[35S]methionine per ml (600 Ci/mmole). At 9 h postinfection, cells were washed once in cold phosphate-buffered saline, suspended at 10^7 cells per ml in 0.02 M Tris hydrochloride (pH 8) containing 0.001 M NaCl, and sonicated twice for 20 s on ice. By using the same protocol, cells were also labeled either with 40 Ci of L-[4,5-3H]leucine per ml (120 Ci/mmole) in SM containing 1/20 the normal concentration of leucine or with 40 Ci of a 3H-labeled amino acid mixture per ml (containing 15 amino acids at 10 to 80 Ci/mmole) in SM with 1/20 the normal levels of each amino acid in the mixture. For radioactive carbohydrases, cells were labeled for 1 h with 50 Ci of d-[2-3H]mannose (20 Ci/mmole) or d-[6-3H]glucosamine hydrochloride (40 Ci/mmole) per ml in SM with 1/10 the normal level of glucose. Cells were then suspended in regular SM with excess unlabeled mannose (0.005 M) and incubated for another 3 h before being harvested. All radioactive compounds were purchased from Amersham Corp., Arlington Heights, Ill.

**SDS-PAGE.** The procedure for sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was as previously described (17). Whole-cell sonicates were solubilized in gel sample buffer and analyzed in 10 or 13% polyacryl-

**RESULTS**

**Identification of Ad35 early proteins.** Ad35 early proteins from infected HeLa cells radiolabeled with [35S]methionine were analyzed. Uninfected cells, as well as cells infected with Ad2 (group C) and Ad7 (another group B adenovirus),
were also included in the study as controls. As described above, cells were infected with each purified virus at a high multiplicity of infection in order to produce a synchronized early infection. Cycloheximide (25 μg/ml) was added at 2 h postinfection to enhance the relative abundance of early viral RNA (6). At 5 h, the cycloheximide was removed, and the cells were labeled with [35S]methionine for 4 h in the presence of cytosine arabinoside (4 μg/ml) to prevent both viral DNA synthesis and the subsequent synthesis of late viral proteins. Whole-cell extracts were analyzed by SDS-PAGE (Fig. 1). In the Ad2-infected cell lysate, polypeptides representing the early-region E2-72K (DBP), E3-19K, E1B-15K, E3-14K, and E4-11K were clearly visualized, as indicated in the figure, above the cellular protein background in the uninfected control (20). In contrast, the viral polypeptide patterns seen in the Ad35- and Ad7-infected cell lysates differed from those of lysates infected with Ad2 but were identical to each other. The Ad35 and Ad7 60K polypeptides presumably represented DBPs, and the three smaller viral polypeptides in the 10,000- to 15,000-Mr range also appeared to be similar to the smaller early Ad2 proteins. However, no protein analogous to Ad2 E19 was specifically identified.

**Ad35 produces a 29,000-Mr, early glycoprotein.** Glycoproteins from Ad35-infected cells and from controls were specifically labeled with [3H]mannose during early infection, as described above. Cells were labeled for 1 h in SM containing 1/10 the normal level of glucose, followed by a 3-h cold chase with excess unlabeled mannose; this was done in order to avoid possible aberrant glycosylation due to prolonged carbohydrate starvation. Labeled glycoproteins were analyzed on a 10% polyacrylamide gel (Fig. 2). In Ad2-infected cells, a single major 25,000-Mr band was visualized, which corresponded to the E19 glycoprotein. It should be noted that Ad2 E19, which has a predicted Mr of 19,000 based on its DNA sequence, migrated with a mobility of 25,000 Mr in SDS-PAGE due to the presence of two carbohydrate residues per molecule, as previously reported (16). In Ad35- and Ad7-infected cells, analogous major glycoprotein bands were visualized near the 31,000-molecular-weight marker. The molecular weights of both the Ad7 and Ad35 glycoproteins were determined to be 29,000 based on their mobilities in 13% polyacrylamide gels (see Fig. 5).

The relative amounts of the Ad2 and Ad35 glycoproteins were determined. Even after prolonged exposure of the [35S]methionine-labeled lysates shown in Fig. 1, the Ad35 29K protein (E29) was not visualized above the cellular protein background. The experiment was thus repeated using amino acid labels other than [35S]methionine in order to rule out the possibility that the methionine content in the Ad35 glycoprotein was unusually low. It was possible to detect Ad35 E29 in Ad35-infected-cell lysates when labeled with a combination of [3H]leucine and [35S]methionine. The 29K band present in the Ad35 lysate was more intense than that present in either the uninfected or the Ad2-infected lysates (Fig. 3). This band presumably represents the Ad35

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**FIG. 1.** [35S]methionine-labeled early adenovirus proteins in HeLa cells infected with Ad2, Ad7, or Ad35. HeLa cells were labeled for 4 h with [35S]methionine during early-adenovirus infection, along with an uninfected control, as described in the text. Equal amounts of radioactivity (2 × 10^6 cpm) from each lysate (2 × 10^4 to 4 × 10^5 cells) were analyzed on a 10% polyacrylamide gel. Ad2 virion proteins were used as markers, indicated as II (hexon) and the two core proteins V and VII, whose mobilities relative to standard M, markers are shown in Fig. 3.

**FIG. 2.** [3H]mannose-labeled early-adenovirus glycoproteins in HeLa cells infected either with Ad2, Ad7, or Ad35, in comparison to an uninfected control. Cells were pulse-labeled with [3H]mannose for 1 h, followed by a 3-h chase with unlabeled mannose, during early-adenovirus infection (as described in Materials and Methods). Whole-cell lysates (3 × 10^6 cpm; 1 × 10^6 to 2 × 10^6 cells each) were analyzed by SDS-PAGE on a 10% gel. Molecular weight standards utilized were phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) (all from Bio-Rad).
Purified Ad35 E29 carbohydrate was treated with endo H and analyzed by gel filtration on a Bio-Gel P4 column (Fig. 4). The [\(^1^H\)mannose-labeled Ad35 carbohydrate was purified by elution from the 29K polyacrylamide gel band and removal of the protein component by prolonged digestion with pronase, as described in Materials and Methods. Purified, endo H-treated, \([^{14}C]\)mannose-labeled Sindbis virus carbohydrates were used as oligosaccharide markers and have been characterized in detail (8, 25). Endo H specifically cleaves asparagine-linked (N-linked) high-mannose oligosaccharides such as Man\(_{6,9}\) GlcNAc\(_2\) (Man, mannose; GlcNAc, N-acetylglucosamine) between the first and second GlcNAc. In contrast, N-linked complex oligosaccharides, in which the distal mannose residues have been replaced by other sugars such as galactose and sialic acid, are resistant to endo H. As shown in Fig. 4, the \([^{1}H]\)mannose-labeled Ad35 carbohydrate eluted much later than the complex Sindbis glycopeptides (\(S_0, S_1, S_2,\) and \(S_3\)), which appeared near the void volume. This indicated that the Ad35 carbohydrate was completely sensitive to endo H. Moreover, a single major peak of \([^{1}H]\)mannose activity was present at the position corresponding to the \(\text{Man}_9\text{GlcNAc} (\text{M}_9)\) oligosaccharide marker. This high-mannose carbohydrate structure is a characteristic oligosaccharide found in glycoproteins located in the endoplasmic reticulum, whose carbohydrates are not further processed in the Golgi apparatus. \(\text{M}_8\) is also the major carbohydrate moiety found in the Ad2 endoplasmic reticulum protein, E19 (16).

Since the Ad35 29K carbohydrate moiety is the same as that of Ad2, we concluded that the Ad35 glycoprotein must contain a greater number of oligosaccharides per molecule to account for the intensity of the \([^{1}H]\)mannose band. The exact number of carbohydrate residues on the Ad35 glycoprotein was determined. By titration of endo H on \([^{1}H]\)glucosamine-labeled Ad34 Rod-infected lysates, a series of partially deglycosylated products was obtained (Fig. 5). \([^{1}H]\)glucosamine was used to label the viral protein to ensure...
visualization of the end product completely deglycosylated of mannose, because endo H treatment leaves a single GlcNAc molecule attached to Asn per moiety. The resulting four-component 'ladder' indicated that there were four carbohydrate moieties per molecule, which represents twice the number present in the Ad2 glycoprotein. This difference is consistent with the size disparity of 4,000 Mr between the Ad2 and Ad35 glycoproteins. Upon complete endo H digestion, the Ad35 protein had a mobility of approximately 19,000 Mr. Notably, this deglycosylated Ad35 protein was similar in mobility to the unglycosylated Ad2 E19 (Fig. 5, last lane) from Ad2-infected cells which were treated with tunicamycin to inhibit glycosylation.

Immunoprecipitation of Ad35 E29. For further comparison of the Ad2 and Ad35 glycoproteins, the antigenicity was evaluated by immunoprecipitation of the [1H]mannose-labeled cell lysates. A polyclonal antiserum against purified, denatured Ad2 E19 was produced in a rabbit, as described in Materials and Methods. Cell lysates were solubilized in 1% SDS to dissociate the viral proteins, and the denatured lysates were diluted to 0.1% SDS before immunoprecipitation. This antiserum specifically precipitated the E19 glycoprotein from the Ad2-infected cell lysate (Fig. 6). In contrast, neither the Ad35 nor the Ad7 glycoprotein was precipitated by this Ad2 antiserum. Thus, the Ad35 and Ad7 glycoproteins do not appear to share antigenic determinants with the Ad2 glycoprotein.

As a measure of functional activity, the Ad35 29K glycoprotein was then analyzed for the ability to bind to MHC class I antigens by immunoprecipitation with a monoclonal antibody (W6/32) against HLA class I heavy-chain molecules. The 45K band (Fig. 7) which represents HLA class I heavy chains was precipitated by this antibody from the uninfected-cell-lysate control. In the Ad2 cell lysate, a 25,000-Mr band corresponding to Ad2 E19 was coprecipitated with heavy chain. In addition, immunoprecipitation of the Ad2 lysate with antibody to Ad2 E19 coprecipitated HLA class I heavy chains. As shown previously, this upper band may also represent E19 dimers which are present in vivo (21). In the Ad35 cell lysate, the antibody against HLA class I antigens coprecipitated the 29K viral glycoprotein band with heavy chain. It should be noted that the class I heavy chain in the immunoprecipitates from both the Ad2- and the Ad35-infected cell lysates migrated slightly faster than the heavy chain from uninfected cells. This can be attributed to the inhibition of terminal glycosylation of the heavy chain as a result of its association with either viral glycoprotein, as described previously with Ad2-infected cells (3). We conclude that Ad35 E29 was associated with class I antigens, a finding analogous to that with the Ad2 glycoprotein. Similarly, the Ad7 glycoprotein was also coprecipitated with antibody to HLA class I heavy chain (data not shown).

**FIG. 6.** Immunoprecipitation of [1H]mannose-labeled Ad2-, Ad7-, and Ad35-infected HeLa cell lysates with a polyclonal rabbit antibody against purified Ad2 E19 (P) and preimmune control sera (I). Portions (2 x 10^6 cpm) of each lysate were solubilized in 100 μl 1% SDS–0.001 M EDTA–0.01 M Tris hydrochloride (pH 8) for 15 min. The denatured lysates were diluted to 0.1% SDS with a 2% Triton X-100 solution, and the debris was centrifuged (as described in the text). The antibody, prepared against purified Ad2 E19, as described in Materials and Methods, was incubated with the lysates overnight, and immune complexes were precipitated with Staph A. Ad2 virion proteins are shown in the lane on the left with three viral proteins designated as in Fig. 1.

**DISCUSSION**

We have identified a 29,000-Mr, Ad35 early glycoprotein, E29, which was associated with MHC class I antigens in infected HeLa cells. Ad35 E29 contained N-linked high-mannose oligosaccharides, a feature characteristic of a glycoprotein located in the endoplasmic reticulum. Our data indicate that the group B Ad35 E29 is analogous to the
adenovirus glycoproteins of the characteristic quantity product that can and novirus, glycoprotein antigen-binding did as although E19, revealed several differences. There is comparison further cells characterized in this paper.

The E3 transcription unit, which encodes the Ad2 E19 glycoprotein, is a unique region which is not essential for viral replication in tissue culture (15). Besides the coding region for the E19 protein, the Ad2 E3 region contains eight open reading frames (ORFs) which could code for proteins, some of which contain typical transmembrane regions (9, 10). Although the E3 transcription units from the closely related group C viruses (Ad2 and Ad5) are highly homologous, the group B Ad3 E3 region has more limited DNA homology but a similar organization of its ORFs (23). An Ad3 ORF was identified which shared 54% DNA homology and 36% amino acid homology with Ad2 E19 and contained four putative N-linked glycosylation sites (Asn-X-Ser/Thr). We are currently sequencing the Ad35 E3 region to assist us in confirming the origin of the 29K glycoprotein characterized in this report. This data will further our understanding of the other E3 proteins which may also play a role in virus-host interaction. For example, preliminary analysis of the Ad35 E3 DNA sequence indicates that one of the other E3 ORFs is not conserved.

In conclusion, Ad35, like other adenoviruses, can interact with the immune system by the binding of its early viral glycoprotein to the MHC class I antigens. It is possible, however, that the unusual epidemiology of Ad35 is related to other E3 proteins. At this time, the 12% prevalence of the uncommon Ad35 in AIDS patients remains unexplained. Studies in our laboratory have shown that Ad35 and the human immunodeficiency virus can coinfect the human T4-lymphocyte line H9 (D. Hassin and M. S. Horwitz, unpublished observations). Perhaps coinfection of lymphocytes with Ad35 and human immunodeficiency virus takes place in vivo. Additional information about the biology of Ad35 is needed in order to understand its unique association with patients with AIDS and other immune disorders.

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