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Antibodies with Specificities against a Dispase-Produced 15-Kilodalton Hexon Fragment Neutralize Adenovirus Type 2 Infectivity

MIKAEL J. VARGA,[1]* TOMAS BERGMAN,[2] AND EINAR EVERITT[1]

Department of Microbiology, University of Lund, Lund,[1] and Department of Chemistry I, Karolinska Institute, Stockholm,[2] Sweden

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During the entrance of adenovirus type 2 into cells, it has been suggested that the virion undergoes a conformational change. In this investigation, we have further characterized the hypothetical conformational change, which the structural protein hexon undergoes in response to low pH. From pH 5.0 to pH 6.0, the proteolytic enzyme dispase cleaved the hexon into a few distinct fragments with a dominating low-molecular-weight fragment with a molecular weight of 15,000 (15K peptide), whereas between pH 6.5 and pH 8.0, the cleavage of the hexon was negligible. The degradation of the hexon with dispase at low pH was not due to an increased activity or alteration of the active site of dispase at low pH. The 15K fragment was identified as a segment of the N-terminal part of the hexon polypeptide beginning at amino acid residue 5. An immune serum produced in response to acid-treated and glutaraldehyde-fixed hexons contained a small amount of antibodies directed towards the 15K fragment, as judged by Western immunoblotting. An anti-15K antibody fraction was isolated by affinity chromatography by removing antibodies recognizing the hexon in the alkaline configuration. Such antibodies displayed a higher relative titer at pH 5.0 than at pH 7.5 in an enzyme-linked immunosorbent assay. The isolated antibodies showed a specific neutralizing capacity five times higher than that of the corresponding unfractionated polyclonal anti-hexon serum; however, the neutralizing ability was independent of pH. The neutralization of adenovirus type 2 infection by the isolated anti-15K antibodies implies that the N-terminal end of the hexon may play a critical role in the early steps of the virion-cell interaction.

The process of virus penetration into the host cell represents one of the initial steps in the infectious cycle. Information on the molecules contributing to penetration for a number of different virus-host systems is accumulating (30), although very little is known about the molecular mechanisms by which adenoviruses gain access to the cytoplasmic compartment of the cell. Early electron microscopic data suggest that adenoviruses enter by direct penetration (4, 21). More recently, experimental evidence for internalization by receptor-mediated endocytosis has accumulated (5, 9, 28). It has been shown that during this process the penton base plays a crucial role in the virus escape from endosomes. Hypothetically, the penton base undergoes a pH-dependent conformational change in response to the low pH in this cellular compartment and subsequently becomes hydrophobic and thereby interacts with and penetrates the endosomal membrane (3, 25–27). Recently, we have shown that the hexon protein of the viral capsid also has the potential for undergoing a conformational change at low pH, as judged by different patterns of protease sensitivity at pH 5.0 and pH 7.5. At pH 5.0, dispase-digested hexons show a limited number of cleavage products, whereas at pH 7.5 the degradation is negligible. The major low-molecular-weight cleavage product that is generated by dispase treatment at low pH has an estimated M_r of approximately 15,000 (15K peptide), on the basis of migration upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7).

In previous reports, it has been shown that adenovirus type 2 (Ad2) infectivity can be immunologically inactivated by the following two pathways: aggregation of virions or prevention of the release of virions from the endosomes into the cytoplasm (4, 31, 34). The latter effect can be obtained by using anti-hexon- or anti-penton-base antibodies which cause an entrapment of the virions within the endosomes. The neutralization mechanism used by these two antisera is probably either direct covering of the penton base or inhibition of the low-pH-induced conformational change of the virion. For anti-hexon antibodies, experimental neutralization values under steady-state conditions suggest a single-hit model indicating that only one antibody, binding to the virion, is needed for the neutralization of virus infectivity.

In this report, we further describe the low-pH-induced conformational change of the hexon antigen, and by isolating polyclonal monospecific antibodies against the 15K peptide, we have investigated the possible functional aspects of this fragment during the early steps of Ad2 infection.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in Eagle minimal essential medium (EMEM) (Biological Industries, Beth Haemek, Israel) with 3.5% fetal calf serum (Flow Laboratories, Irvine, Scotland) and 20 μg of gentamicin (Biological Industries) per ml. The cells were maintained at densities of 2.5×10^5 to 6×10^5 cells per ml in Spinner culture bottles and were routinely screened for *Mycoplasma* infections, as described by the manufacturer of *Mycoplasma* T-C II (Gen-Probe, San Diego, Calif.). Ad2 was propagated, radioisotope labeled ([^{35}S]methionine), and purified as described previously (6, 8).

Purification of the hexon antigen. The soluble antigen accumulating late in the productive infection was recovered after virus isolation and separated by ion-exchange chromatography on DEAE-cellulose. The hexon antigen was further purified by exclusion chromatography on Sepharose CL-6B.

* Corresponding author.

This was done as described previously (7, 32), except that phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) in ethanol was added to all chromatography buffers to give final concentrations of 2.5 mM phenylmethylsulfonyl fluoride and 7.1% ethanol.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in gel slabs with 13% acrylamide essentially by the method of Maizel (19), by using a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.). Gels were stained, dried, and exposed to autoradiographic films as previously described (32).

Proteolytic digestion of the hexon antigen. Enzymatic treatments were performed as described previously (7). Briefly, hexons at a concentration ranging between 60 and 160 μg/ml were preincubated in $CaCl_2$-containing buffers (2.5 mM) (for details, see legends to Fig. 1 to 4) for 30 min at 37°C before the proteolytic enzymes (dispase and trypsin were from Sigma, and subtilisin was from Boehringer Mannheim Scandinavia AB, Bromma, Sweden) were added to give a final enzyme protein-over-hexon protein ratio of one to five. After incubation at 37°C for 15 min, the reactions were stopped either by adding the Laemmli SDS-disruption buffer (18) or by adding EDTA to a final concentration of 10 mM and then putting the samples on ice.

To quantify the dispase activity against the native hexon protein at different pHs, ^{35}S-hexons were cleaved as described above and subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue, and the bands located in the 120K and 15K regions were both excised. The gel slices were dissolved in 0.5 ml of 30% H_2O_2, supplemented with 5 μl of acetic acid, and incubated at 65°C for 10 h. Finally, 10 ml of Ready Safe (Beckman Instruments AB, Bromma, Sweden) was added and the radioactivity was measured by liquid scintillation spectrometry. Background values were obtained by cutting out protein-free gel slices. The degree of cleavage was calculated as follows: [counts per minute (cpm) in the 15K region/(cpm in the 15K region + cpm in the hexon)] × 100; the maximal enzymatic activity achieved was set at 100%.

To investigate whether dispase itself displayed any pH-dependent activity, ^{35}S-hexons were denatured by boiling for 1 min and then cleaved by dispase at different pHs. The degree of cleavage was monitored by assessment of relative trichloroacetic acid nonprecipitable radioactivity at each pH.

N-terminal amino acid sequence analysis of the 15K fragment. Hexons were cleaved by dispase as described above at a concentration of 1.0 mg/ml, and the 15K fragment was purified by SDS-PAGE followed by detection, excision, electroblotting, and sequence analysis by the methods of Bergman and Jörnvall (2).

Production of monospecific polyclonal antisera against the hexon antigen. Three different anti-hexon serum specimens were obtained by immunization of rabbits with three different preparations of hexons isolated from the soluble pool of structural proteins. Hexons at a concentration of 154 μg/ml were incubated in 0.01 M phosphate buffer (pH 7.5)–0.15 M NaCl (PBS) or in 0.015 M acetate buffer (pH 5.0)–0.15 M NaCl for 30 min at 37°C, and then glutaraldehyde was added to each sample to give a final concentration of 0.1%. The proteins were fixed for 10 min at 37°C. Remaining active aldehyde groups were quenched by adding glycine to a final concentration of 0.1 M, and the samples were subjected to further incubation for 20 min at 20°C followed by a buffer exchange via a PD-10 exclusion chromatography column equilibrated with 0.01 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5)–0.15 M NaCl and 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.0)–0.15 M NaCl for the alkaline- and acid-fixed hexons, respectively (both HEPES and MES were from Sigma). These immunogens were used for the production of antisera referred to as anti-(FB)hexon serum and anti-(FA)hexon serum, where FB and FA stand for fixed base and fixed acid, respectively. Native (N) hexons without any further treatment were used for raising an anti-(N)hexon serum.

For each of the three immunogens (FB, FA, and N), three immunizations, using 50 μg of hexon on each occasion, were performed within a month (15). Serum was collected 7 days after the last booster dose. Prior to use, antisera were heat inactivated at 56°C for 30 min, titrated by rocket immunoelectrophoresis (RIE) (33), and subsequently diluted with PBS to obtain the same concentration of anti-hexon antibodies as the anti-(N)hexon serum.

Sequential degradation of dispase-treated virions. Virions were treated with dispase as previously described (7) and then disrupted by pyridine. The released virion components were separated by sucrose gradient centrifugation (23). The polypeptide profiles of the different fractions were analyzed by SDS-PAGE.

Isolation of immunoglobulins against the 15K hexon fragment. Immunoglobulins against the 15K dispase cleavage product were isolated by negative-affinity chromatography on immobilized alkaline-fixed hexons. Hexons were subjected to fixing with 0.1% glutaraldehyde in PBS for 5 min at 37°C followed by quenching of the remaining active aldehyde groups with 0.1 M glycine for 20 min at 20°C. The column was prepared with 8 mg of alkaline aldehyde-treated hexons which, prior to coupling to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden), were dialyzed against the coupling buffer.

Portions [equivalent to 200 μl of the anti-(N)hexon serum] of the anti-(FA)- and the anti-(FB)hexon sera in PBS were subjected to passage through the column followed by one column wash of 0.5 M NaCl in phosphate buffer, pH 7.3. The retained material was eluted in 0.1 M Tris glycine, pH 11.5, containing 0.5 M NaCl. All UV-absorbing material above 0.01 at 280 nm was collected and dialyzed against double-distilled water. After lyophilization, the fractionated material was resuspended in PBS to give twice the original volume.

Epitope mapping by Western immunoblotting analysis. Purified hexons were enzymatically digested, resolved by SDS-PAGE, and then electrophoretically transferred to Zeta-Probe blotting membranes (Bio-Rad). After blocking of the filters, the hexon fragments were detected with the various rabbit anti-hexon sera followed by a ^{125}I-labeled immunoglobulin G (IgG) fraction of a goat anti-rabbit IgG serum (Organon Teknika, Malvern, Pa.) (11). The specific radioactivity of the secondary antibody was 3.6×10^5 cpm/μg of IgG, and about 14 μg of IgG was added to each immunoblotting membrane.

Immunoaggregation of virions and hexons by different anti-hexon sera. Samples (10 μl) of [^{35}S]methionine-labeled virions and hexons (4.1×10^8 virions and 0.23 μg of hexons) were incubated for 30 min at 37°C in PBS or in 10 mM MES (pH 5.0)–0.15 M NaCl; both buffers contained 1% bovine serum albumin (BSA). An equal volume of the appropriate antiserum was subsequently added. Antisera were diluted in the buffers described above to yield final serum dilutions ranging from 1/2 to 1/1,000. After incubation with antiserum

for 30 min at 37°C, 300 μl of 50 mM Tris hydrochloride (pH 7.5) containing 0.2% Triton X-100 was added, and after thorough mixing, the samples were centrifuged for 10 min at 8,000 × *g* (average). The pellets were dissolved in 100 μl of 0.1 M NaOH for 1 h at 65°C, transferred to vials with one wash of 200 μl of 1% acetic acid and one wash of 100 μl of 50 mM Tris hydrochloride (pH 7.5) containing 0.15 M NaCl, and subsequently assayed for radioactivity. The relative virion aggregation was calculated. Immunoaggregation of anti-hexon-treated virions with the IgG fraction of a goat anti-rabbit serum (Organon Teknika) as a secondary antibody was performed as described above with the addition, after the incubation with the rabbit anti-hexon serum, of 10 μl of a goat anti-rabbit serum in 0.3 M Tris hydrochloride (pH 7.5); the samples were further incubated for 30 min at 37°C. Equilibrium concentrations of the secondary antibody were used in all instances.

Spontaneous aggregation of virions and hexons was always monitored by replacing the anti-hexon serum with buffers or a preimmune serum.

Virus neutralization assay. Virus neutralizations were quantified by a progeny virus immunotitration method modified to assay the production of hexon antigen instead of progeny virus, since it has been shown that there is always a true correlation between the amount of progeny virus and the amount of accumulated hexons in infected HeLa cells (33). Virions were preincubated at a concentration of 4.1×10^{10} particles per ml in PBS with 1% BSA or 10 mM MES (pH 5.0) containing 0.15 M NaCl and 1% BSA at 37°C for 30 min. Volumes of 15 μl were withdrawn and added to equal volumes of antisera or suitable immunoglobulin fractions which, prior to use, were diluted with the buffers mentioned above to give final dilutions ranging from 1/2 to 1/1,000. After incubation at 37°C for 30 min, PBS containing 1% BSA was added to each sample to give a final sample volume of 150 μl. Samples of 119 μl were removed from such virus-antibody mixtures and added to 1.52×10^7 HeLa cells in 1 ml of EMEM, yielding one infectious unit per cell. After incubation for 30 min at 37°C on an orbital shaker, complete medium was added, and cells were harvested by centrifugation 39 h postinfection. The cells were ultrasonically disintegrated and centrifuged at 12,000 × *g* (average) for 15 min to sediment cell debris. The supernatants were analyzed for viral hexon antigen by RIE in agarose gels containing 0.5% anti-(N)hexon serum.

ELISA of antibodies on microtiter plates. Microtiter plates (Costar Europe, Ltd., Badhoevedorp, The Netherlands) were coated (per well) with 100 μl of hexon antigen (5 μg/ml in PBS) or virion (10 μg/ml in PBS) solutions which, prior to coating, were preincubated for 30 min at 37°C. Coating was performed for 1 h at 37°C and then for 16 to 20 h at 20°C. The plates were then blocked with 5% nonfat milk in PBS for 3 h at 20°C and washed three times with PBS containing 0.05% Tween 20. The plates were subsequently probed with anti-hexon antibodies of various dilutions in PBS containing 1% BSA and incubated for 16 to 20 h at 20°C. After three washes as described above, goat anti-rabbit IgG conjugated with alkaline phosphatase (Caltag Laboratories, San Francisco, Calif.) in PBS containing 1% BSA and 0.05% Tween 20 was added and maintained for 2 h at 20°C before the plates were washed as described above, developed, and read in a Microplate Reader 600 (Dynatech Laboratories Inc., Chantilly, Va.) at 410 nm. Enzyme-linked immunosorbent assays (ELISAs) were also performed at pH 5.0. These acid assays were done as described above, except that PBS was substituted by an acetate buffer (0.015 M), pH 5.0, containing 0.15 M NaCl.

For each antiserum, the obtained A_{410} was plotted against the logarithm of the relative serum dilution. The ELISA titers for the different antisera or immunoglobulin fractions were subsequently estimated by entering the diagram at the lowest possible absorbance value, separated from the background and in the linear part of the plot (typically, about 0.2). For each separate series of ELISA titrations, the obtained titers were standardized against the experimentally obtained anti-(N)hexon serum titer.

RESULTS

Degradation of the hexon antigen by dispase. To evaluate whether dispase showed any pH-dependent activity, native and heat-denatured ^{35}S-hexons were digested at various pHs and the enzymatic activity was measured as described in Materials and Methods. As reported earlier (7), it was shown that dispase displayed low action against native hexons at high pH, but upon lowering the pH, the susceptibility of the hexons increased continuously to pH 5.0 (Fig. 1A). Dispase displayed its expected pH optimum between pH 6 and pH 7 by using heat-denatured hexon as an enzyme substrate (10), and at pH 5.0, dispase exerted only 25% of its activity compared with the action at pH 7.5. To investigate whether the reduced activity at pH 5.0 was accompanied by an altered specificity of the active site of dispase, samples of heat-denatured ^{35}S-hexon were digested with dispase at pH 7.5 and pH 5.0 and subjected to SDS-PAGE and autoradiography. As judged by the dispase digestion patterns obtained at pH 7.5 and pH 5.0, no major difference in the dispase cleavage specificity was seen (Fig. 1B).

Sedimentation centrifugation analysis of native hexons revealed that hexons treated with low-pH buffer sedimented as control hexons maintained at neutral pH did (data not shown). Dispase-digested hexons sedimented as control hexons did, and there was no radioactivity remaining at the top of the gradients (Fig. 2A). The 15K fragment cosedimented with the remaining hexon core, indicating that the enzyme-treated hexons were fully intact (Fig. 2B).

Identification of the 15K hexon fragment. Attempts were made to establish the exact cleavage site which produces the 15K fragment when hexons are degraded by dispase at pH 5.0. Thus, hexons were cleaved and the fragments were resolved by SDS-PAGE. The 15K fragment was cut from the gel after detection with 1 M KCl, electroblotted, and subjected to N-terminal amino acid sequence analysis as described elsewhere (2). However, probably because of the high concentration of hexon (1 mg/ml) used in the cleavage mixture, the band which migrated to the 15K position of the gel was not homogeneous. About 30% of the material in the excised band revealed the amino acid sequence Met-Met-Pro-Gln-X-Ser-Tyr, indicating a cleavage after residue 4 in the intact hexon polypeptide (16).

Sequential degradation of virions. Radioisotope-labeled virions were treated with dispase and subsequently disrupted with pyridine, and the groups of nine hexons and the peripentonal hexons were separated by centrifugation. This was done to reveal any possible differences regarding dispase susceptibility among the two classes of hexon antigens located in the virion. No such differences were detected in the qualitative degradation patterns or in enzyme kinetic studies (data not shown).

Specificities of anti-hexon sera. Immunoblotting of Ad2 was done to ensure that the recovered anti-hexon sera did not

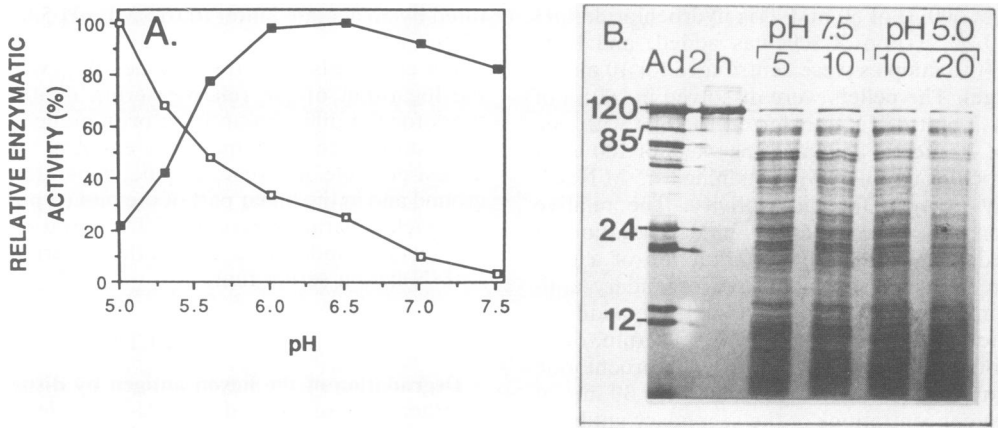

FIG. 1. pH-dependent dispase degradation of native and heat-denatured hexons. (A) Samples containing 5 μg of native (□) or heat-denatured (■) ^{35}S-hexons were treated with dispase in the following buffers: 0.01 M NaAc (acetate)-HAc, pHs 5.0, 5.3, and 5.6; 0.01 M Tris maleate, pHs 6.0 and 6.5; and 0.01 M Tris hydrochloride, pHs 7.0 and 7.5, all of which contained 0.15 M NaCl. After incubation at 37°C for 15 min, the degree of proteolysis was quantified by two different procedures as described in Materials and Methods, and maximal enzymatic activity was set at 100%. (B) SDS-PAGE patterns of heat-denatured and dispase-treated hexons. Samples from the enzyme-hexon mixtures at pH 7.5 and pH 5.0 were withdrawn at the indicated times (minutes) and subsequently analyzed by SDS-PAGE. To compensate for the lower enzymatic activity of dispase at pH 5.0, panel B shows samples after longer enzyme exposure of the mixture at low pH. The pairs of sample wells designated pH 7.5 and pH 5.0 show heat-denatured and dispase-treated hexons at pH 7.5 and pH 5.0, respectively. Lanes Ad2 and h contain a ^{35}S-Ad2 marker and buffer-treated (pH 5.0) heat-denatured hexons, respectively. On the left side, some of the Ad2 marker polypeptides are indicated by their corresponding molecular masses (in kilodaltons), according to Anderson et al. (1).

contain antibodies against other viral antigens (data not shown). The quality of antiserum specimens obtained in response to aldehyde-fixed hexons was studied by comparing native and fixed hexons by RIE, and it was estimated that less than 1% of the total antibody population was directed against aldehyde-induced epitopes.

Epitope mappings by immunoblotting of enzymatically digested hexons were performed for documentation of possible differences in the recognition of hexon epitopes among the three different anti-hexon serum specimens (Fig. 3). The most notable difference was the finding that the anti-(FA) hexon serum contained antibodies against both the dispase-produced 15K fragment and a trypsin fragment, also migrating to the 15K region upon SDS-PAGE. The anti-(N)- and anti-(FB)hexon sera contained antibodies producing a weak signal only in the 15K position of the tryptic fragment. The anti-(FB)hexon serum was completely devoid of antibodies directed to the dispase-produced 15K fragment, and even when the antibody concentration was increased to about seven times the concentration of the anti-(N)hexon serum,

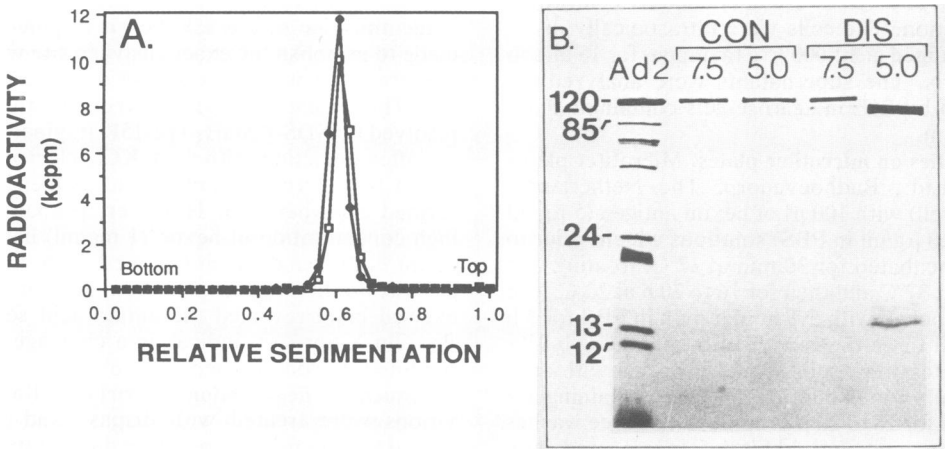

FIG. 2. Sucrose gradient sedimentation analysis of hexons. (A) [^{35}S]methionine-labeled hexons (22 μg) were treated with dispase at pH 5.0 (□) and buffer at pH 5.0 (◆) and centrifuged at 86,700 × *g* (average) for 17 h in 10 to 25% (wt/vol) sucrose gradients buffered with 20 mM HAc (acetate)-NaAc (pH 5.0) containing 0.15 NaCl and 2 mM EDTA. Similar sedimentation analyses, in sucrose gradients buffered with 20 mM Tris hydrochloride (pH 7.5) containing 0.15 M NaCl and 2 mM EDTA, were performed for hexons treated at pH 7.5. These analyses revealed sedimentation patterns identical to those of the acid series (data not shown). (B) Each peak from the four analyses was collected, and the same amount of radioactivity was withdrawn from each pool and subjected to SDS-PAGE. The pairs of sample wells designated pH 7.5 and pH 5.0 show hexons recovered from neutral and acid sucrose gradients, respectively. Lanes Ad2, CON, and DIS show a ^{35}S-Ad2 marker, buffer-treated hexons, and dispase-treated hexons, respectively. Some of the Ad2 marker polypeptides are indicated by their corresponding molecular masses (in kilodaltons) (1).

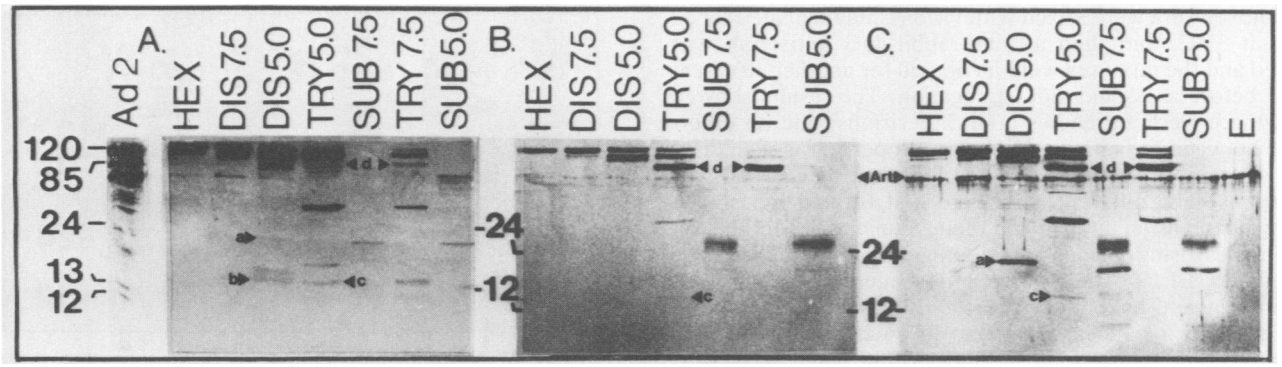

FIG. 3. Western immunoblotting of enzyme-digested hexons with the different anti-hexon sera. Hexons were digested with dispase (DIS), trypsin (TRY), and subtilisin (SUB) at pH 7.5 and pH 5.0 for 15 min at 37°C. From each sample mixture, 1.7 μg of hexon was withdrawn and subjected to SDS-PAGE. After electrophoretic transfer of the digested hexons to Zeta-Probe filters, the blots were incubated with the same amount of polyclonal antibodies [equivalent to 200 μl of the anti-(N)hexon serum] from the anti-(FA)hexon (panel A), anti-(FB)hexon (panel B), or anti-(N)hexon (panel C) serum. The primary antibodies were detected by secondary ^{125}I-labeled goat anti-rabbit IgG antibodies. The panels show three separate gels which were run and exposed to film in parallel. Lanes Ad2, HEX, and E show a ^{35}S-Ad2 marker, hexons incubated in pH 5.0 buffer, and a well containing only SDS-disruption buffer, respectively. Arrowheads and a, b, c, and d indicate the 23K, the dispase-produced 15K, the trypsin-produced 15K, and the 85K fragments, respectively, which are discussed in the text. The arrowheads and Art indicate the artifactual band observed for the anti-(FB)- and anti-(N)hexon sera. Numbers to the sides of panels A, B, and C show the molecular masses (in kilodaltons) of some of the Ad2 marker polypeptides (1). The polypeptides of the basic proteins V and VII are faintly visualized, since these species have been shown to be poorly retained by the cationized nylon membrane (32).

no staining of the 15K fragment could be detected. The anti-(N)hexon serum displayed a high titer of antibodies against a dispase-generated fragment with an estimated M_r of 23,000 (23K peptide). This fragment was also revealed by the anti-(FA)hexon serum, but to a much lower extent. Interestingly, this 23K fragment was seen only faintly in autoradiograms of dispase-cleaved [^{35}S]methionine-labeled hexons or in Coomassie brilliant blue-stained gels of digested hexons. Both antisera against high-pH-treated hexons (native and fixed) showed a high content of antibodies recognizing a trypsin fragment migrating in the 85K region. Such specificities were less pronounced in the anti-(FA)hexon serum.

The anti-(N)- and the anti-(FB)hexon sera gave rise to an artifactual band with an electrophoretic mobility corresponding to 65K. The presence of this band was markedly reduced when DL-dithiothreitol was used instead of 2-mercaptoethanol during the sample preparation. These observations suggest that the artifact was skin keratins which have been introduced during the process of sample preparation (22).

Immunoaggregation of hexons and virions by different anti-hexon sera. The anti-(N)-, anti-(FA)-, and anti-(FB)hexon sera were titrated by RIE and diluted to give the same relative titers. Hexons and virions were mixed with the three series of antisera at pH 5.0 and pH 7.5 to give final serum dilutions ranging from 1/2 to 1/1,000. After incubation at 37°C for 30 min, the samples were diluted 30 times and analyzed for possible aggregation by centrifugation. The aggregation patterns for the hexon antigen were essentially the same for all sera irrespective of the pH, thus corroborating the RIE titrations (Table 1).

Virions displayed a tendency to precipitate spontaneously with an accompanying loss of infectivity at pH 5.0 despite physiological ionic strength conditions, especially when virions which had been frozen prior to use were used. This was avoided by diluting the virus stock preparation to a concentration of 5×10^{10} virions per ml. It should be noted that the critical virion concentration for spontaneous aggregation varied between virus pools.

The anti-(N)hexon serum was the only serum causing aggregation of virions, and the precipitation was strongly pH dependent. Thus, the two fixed anti-hexon sera were both totally devoid of antibodies causing aggregation of virions at any dilution or pH (Table 1). To investigate whether the anti-hexon antibodies in these sera were capable of binding virions, the following experiment was carried out. The

TABLE 1. Properties of anti-hexon sera

Serum	Relative titer of antibodies against[a]:				Reciprocal serum dilution producing:					
	Hexons		Virions		50% aggregation of hexons[b]		50% aggregation of virions[b]		50% neutralization of infectivity[c]	
	pH 7.5	pH 5.0	pH 7.5	pH 5.0	pH 7.5	pH 5.0	pH 7.5	pH 5.0	pH 7.5	pH 5.0
Anti-(N)hexon	1	1	0.5 ± 0.1	0.7 ± 0.1	60	50	65	160	320 ± 20	350 ± 20
Anti-(FB)hexon	0.9 ± 0.2	1.0 ± 0.2	0.4 ± 0.1	0.57 ± 0.05	70	60	NA[d]	NA	80 ± 20	60 ± 10
Anti-(FA)hexon	1.0 ± 0.1	1.2 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	80	70	NA	NA	125 ± 5	115 ± 5

[a] Measured by ELISA and expressed as antibody titers normalized to the anti-hexon titers of the anti-(N)hexon serum obtained at the corresponding pH. Typical titer values against hexons for the anti-(N)hexon serum under the experimental conditions described in Materials and Methods were 25,000 and 18,000 at pH 7.5 and pH 5.0, respectively. Each value represents the mean of three separate assays ± standard deviation.

[b] Mean of two separate assays.

[c] Mean of five separate assays ± standard deviation.

[d] NA, No aggregation above background (10%) was observed.

anti-hexon sera were mixed with virions and incubated for 30 min at 37°C, and then an anti-rabbit IgG antiserum was added and the mixtures were incubated for another 30 min at 37°C before assessment of aggregation. The results showed that there were antibodies bound to virions, and no difference between the sera regarding this property was seen (data not shown). These results were also confirmed by ELISA titrations. The different sera were first titrated by ELISA using hexon as an antigen and then examined for anti-hexon antibodies using virions as an antigen in the ELISA. The estimated ELISA titers were subsequently standardized against the anti-hexon titer of the anti-(N)hexon serum at the pH specific for the assessment. In such assays, all sera displayed the same relative titers within each pH series, when the anti-hexon titer was compared with the anti-virion titer (Table 1). It should be noted that the anti-hexon ELISA titers were about twice as high as the corresponding anti-virion ELISA titers. The low titers of the latter could be explained by hidden hexon epitopes in the virions. However, the differences between the anti-hexon and the anti-virion titers could also be due to differences among the two antigens in efficiency of coating the ELISA plates. The absolute titers obtained from ELISA titrations performed at low pH revealed lower values at low pH than at pH 7.5. This result could be due to a lower efficiency of binding at low pH of any of the following factors: the anti-hexon antibodies, the secondary antibodies, or the antigens at the coating step.

Neutralization of virus infectivity by various anti-hexon sera. RIE-titrated and heat-inactivated anti-hexon sera were mixed with virus at low or high pH and incubated for 30 min at 37°C before virions were brought back to ca. pH 7 and allowed to attach to cells. At 39 h postinfection, the extent of virus neutralization was assayed by the immunotitration method described in Materials and Methods. The anti-(N)- and anti-(FA)hexon sera displayed a neutralization of virus infectivity of ≥95% at final serum dilutions of 1/10 and 1/6, respectively. Upon further dilution, the extent of neutralization gradually decreased, reaching 50% levels at serum dilutions of 1/320 and 1/125 for the anti-(N)- and anti-(FA)hexon sera, respectively (Table 1). The anti-(FB)hexon serum caused a maximum of around 75% neutralization and reached 50% neutralization at a final serum dilution of 1/80. The neutralization level for all sera was independent of the pH at which the antibodies were allowed to react with virions, and all neutralizations performed in the acid neutralization assay mirrored the physiological ones.

Isolation of antibodies against the 15K fragment. Hexons were fixed in an alkaline buffer and then coupled to CNBr-activated Sepharose 4B. Samples of the two fixed antisera in PBS, containing the same amount of anti-hexon antibodies as judged by RIE, were applied to the affinity column. Material that passed unadsorbed and eluted after adsorption was collected, pooled, and dialyzed against double-distilled water and then lyophilized and suspended in PBS, as described in Materials and Methods. The amounts of anti-hexon antibodies in the two different fractions were determined by ELISA against hexons at pH 7.5. When the anti-(FA)hexon serum was applied to the column, about 0.6 ± 0.2% (standard deviation) (n = 4) of the total anti-hexon activity passed through the column unretained by the alkaline-fixed hexon matrix. This is compared with the anti-(FB)hexon serum, for which only 0.06 ± 0.03% (standard deviation) (n = 2) of the total anti-hexon activity of the serum was recovered in the flowthrough fraction. Even when the amount of the anti-(FB)hexon serum was increased about 10 times, the relative anti-hexon activity that passed through the column unadsorbed was the same, indicating that the flowthrough of anti-hexon antibodies was not due to an overloading effect.

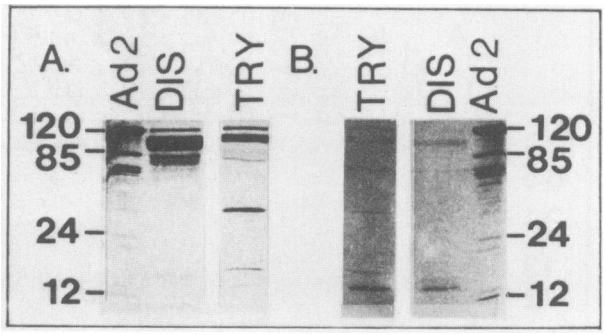

FIG. 4. Western immunoblotting of enzyme-digested hexons with antibody fractions obtained after affinity chromatography of the anti-(FA)hexon serum. Hexons were treated with dispase (DIS) and trypsin (TRY) for 15 min at pH 5.0, and then samples containing 3 μg of hexons were withdrawn and loaded onto a 13% polyacrylamide gel. After separation, the digested hexons were transferred to Zeta-Probe membranes, and each blot was incubated with 400 μl of the retained anti-hexon fraction (A) or 400 μl of the anti-hexon antibodies that passed through the alkaline-fixed hexon-Sepharose 4B column unadsorbed (B). The primary antibodies were detected by a secondary ^{125}I-labeled goat anti-rabbit IgG antibody. Lanes Ad2 show ^{35}S-Ad2 markers with some of the marker polypeptides indicated by their corresponding molecular masses (in kilodaltons) (1).

Upon Western blotting analysis of the two fractions, obtained after affinity chromatography of the anti-(FA)hexon serum, it was shown that the anti-hexon antibodies passing through the column mainly recognized the 15K fragment (Fig. 4). In contrast, the retained anti-hexon activity was devoid of antibodies against the 15K fragment but revealed a signal against the somewhat slower-migrating band in the same region (Fig. 3). Blotting analysis of the flowthrough fraction of the anti-(FB)hexon serum displayed only activity directed towards the artifactual 65K band (data not shown).

Anti-hexon antibodies in the flowthrough fraction of the anti-(FA)hexon serum, that passed through the column, displayed a high neutralizing capacity. It was possible to dilute this fraction about 3.8 ± 0.5 (standard deviation) (n = 2) times before reaching a point which gave 50% neutralization of virus infectivity. Considering the small amount of anti-hexon antibodies in this fraction (0.6%), the isolated anti-15K antibody fraction was about 5, 7, and 2 times more potent per antibody in inhibiting virus infectivity than the total anti-(FA)-, anti-(FB)-, and anti-(N)hexon sera, respectively (Table 1; pH 7.5). The material from the anti-(FB) hexon serum passing through the column unadsorbed displayed no neutralizing activity. Despite its origin, the anti-15K fraction from the anti-(FA)hexon serum displayed no preferential neutralization of virus infectivity at low pH.

ELISAs were performed at low and high pHs with the two immunoglobulin fractions of the anti-(FA)hexon serum, and for each separate titration the anti-(N)hexon serum was included as a standard. The results revealed that the anti-15K antibodies displayed a higher relative titer against hexons at low pH than at pH 7.5 (Table 2). In contrast, the retained anti-hexon fraction displayed a reduction of the titer when the antibody titer against hexons at low pH was compared with the titer at pH 7.5. When virions were employed as the antigen, the relative titer of the anti-15K antibody fraction also increased at pH 5.0. However, in this

TABLE 2. Relative titers of antibody fractions recovered after affinity chromatography

Serum or immunoglobulin fraction	Relative titer of antibodies against[a]:			
	Hexons		Virions	
	pH 7.5	pH 5.0	pH 7.5	pH 5.0
Anti-(N)hexon	1	1	0.5 ± 0.1	0.7 ± 0.1
Retained anti-(FA)hexon fraction	0.9 ± 0.1	0.7 ± 0.2	0.31 ± 0.05	0.60 ± 0.07
Anti-15K fraction	0.0082 ± 0.001	0.017 ± 0.002	0.003 ± 0.001	0.012 ± 0.004

[a] The titers were measured by ELISA and expressed as antibody titers normalized to the anti-hexon titers of the anti-(N)hexon serum obtained at the corresponding pH. Each value represents the mean of three separate assays ± standard deviation.

case, the retained anti-hexon antibody fraction also displayed an increase in relative anti-virion titer when the titers at pH 5.0 and pH 7.5 were compared. After comparison of the retained antibody titers with the corresponding titers of the unseparated anti-(FA)hexon serum, it is evident that some of the antibodies (20 to 40%) were lost during the process of separation (Tables 1 and 2).

DISCUSSION

In a previous study, we have shown that the Ad2 hexon antigen undergoes a low-pH-induced conformational change as judged by different proteolytic cleavage patterns at low pH compared with high pH (7). Both virion-associated hexons and free hexons, from the pool of excess antigen, display different cleavage patterns at pH 5.0 than at pH 7.5 when cleaved by the bacterial endoproteolytic enzyme dispase. In this paper we demonstrate, by comparing heat-denatured hexon and native hexon as an enzyme substrate for dispase, that the different cleavage patterns of the hexon at different pH values were not due to an increased enzymatic activity of dispase at low pH and that dispase altered its specificity upon lowering of the pH. Sedimentation analysis of dispase-cleaved hexons showed that these were fully intact, indicating that the obtained 15K fragment, which is the major low-molecular-weight polypeptide produced by acid dispase cleavage of the hexon, was in fact released by cleavage of the native hexon antigen and was not a product due to extensive degradation of the hexon antigen. A comparison of the N-terminal end of the 15K fragment with the established primary structure of the hexon protein (16) revealed that the fragment was cleaved from the N-terminal part of the hexon polypeptide. The fragment starts at amino acid residue 5, Met, and ends somewhere between residues 135 and 150. The three-dimensional structure of the hexon suggests that the N-terminal end of the polypeptide lies within the interior of the capsid and acts as an anchor. However, the exact position of the polypeptide stretch containing the first 41 amino acid residues in the three-dimensional model of the hexon antigen is uncertain (24). Our results suggest that upon lowering of the pH, the N-terminal part of the hexon polypeptide becomes available for dispase digestion. This could be explained by two different mechanisms. Either the N-terminal part of the hexon undergoes a conformational change and becomes exposed or another segment of the polypeptide chain moves and exposes the N-terminal part of the hexon protein. We believe that the result from the sedimentation centrifugation indicates that the hexon undergoes a minor conformational change, and this strengthens the hypothesis that only the N-terminal part was involved in the pH-induced change. This alteration of the hexon configuration could be mediated by the highly acidic stretch of amino acids ranging from residue 146 to residue 161 (16, 24).

The conformational change of the hexon antigen was not restricted to any subpopulation of hexons in the virus particle, as judged by sequential degradation of dispase-treated intact virions. Both the peripentonal hexons and the groups of nine hexons were equally sensitive regarding their quantitative and qualitative susceptibility towards dispase treatment at low pH. This suggests that all hexons in the virus particle have the ability to undergo a low-pH-induced conformational change.

Although the neutralization of adenoviruses has been studied extensively (17, 29, 31, 34), the precise mechanism of the antibody-mediated inactivation remains controversial. Adenoviruses display the following three structural antigens to which antibodies can bind and subsequently inactivate the infectivity: the fiber, the penton base, and the hexon. It has been proposed that the anti-fiber antibodies perform their activity mainly by aggregation of the virions and that the anti-penton base antibodies neutralize by blocking the release of virions from the acid endosome into the cytoplasm (31, 34). For anti-hexon antibodies, it has been suggested that the antiserum reduces infectivity both by aggregation of the virions and by inhibition of the low-pH-induced conformational change of the hexon, since it has been shown that anti-hexon antibodies prevent dispase digestion of the hexon specifically at low pH. The anti-hexon-mediated inactivation of adenovirus infectivity reveals no lag period in the kinetics, thus indicating a single-hit model for neutralization (31). According to this model, a single antibody binding to any one of a number of critical sites on the virus surface is sufficient for neutralization of virus infectivity (20).

To investigate the possible functional aspects of the N-terminal part of the hexon protein in Ad2 infection, we have tried to raise a polyclonal anti-hexon serum with antibodies against the dispase-produced 15K fragment. To achieve such an antiserum, we used acid glutaraldehyde-fixed hexons as immunogens, thereby anticipating a "freezing" of the hexon in the low-pH conformation by chemical cross-linking. Possible exposure of hidden antigenic sites would induce a subsequent immunological response. Glutaraldehyde used at high concentrations (e.g., 0.5 to 0.25%) and for prolonged times (e.g., 2 h at room temperature) has been reported to produce configurational changes and impairment of immunogenicity (12, 14). Such adverse effects of glutaraldehyde were not seen in this study. Interestingly, the anti-hexon sera raised against the different fixed hexon preparations were totally devoid of antibodies causing precipitation of intact virions, although they contained the same amount of hexon-aggregating antibodies as the anti-(N)hexon serum. This suggests that the glutaraldehyde treatment somehow changed the immunogenicity of the hexon antigen as judged by comparison with the anti-(N)hexon serum. The facts that both the liquid-phase aggregation assays with anti-IgG and the ELISAs revealed that antibodies indeed recognize and

bind to the virions to the same extent as the anti-(N)hexon serum suggest that virion-binding antibodies are unable to properly cross-link a neighboring virus particle.

Comparison of the different staining patterns of the anti-hexon sera in a Western immunoblotting assay of enzymatically cleaved hexons revealed that the anti-(FA)hexon serum contained antibodies against the dispase-produced 15K fragment and that control sera were missing such specificities. It is tempting to conclude that this immunoresponse could be induced by the acid fixation of the immunogen. However, one should bear in mind that the immunization process is a complex series of events which involves some unpredictable steps. The Western immunoblotting method has a certain limitation, since it may not detect antibodies against noncontiguous epitopes or antibodies that have stringent requirements for a native conformation. However, it has been reported that some antigens are not totally denatured even after SDS-PAGE, and it has also been proposed that some antigens may renature during the step of electrophoretic transfer (13).

Taken together, the facts that dispase cleaved off a 15K polypeptide at pH 5.0 and that an immune serum against acid-fixed hexon antigen contained antibodies recognizing this particular 15K fragment make us believe that the 15K fragment is involved in the hypothetical pH-induced conformational change of the adenovirus hexon antigen. This view is strengthened by the finding that it was possible to specifically isolate anti-15K antibodies from the anti-(FA)hexon serum by negative-affinity chromatography on a column of alkaline-fixed hexons. This finding suggests that the anti-15K antibodies should not be able to recognize and interact with the hexon antigen at pH 7.5. On the other hand, a strict pH-dependent neutralization by these anti-15K antibodies was not demonstrated; the antibodies had the same neutralizing capacity at pH 7.5 as at pH 5.0. These contradicting results could be explained by any of the following mechanisms. (i) The hypothetical equilibrium between the two conformational states of the hexon antigen is such that at pH 7.5, some of the hexon capsomers in the virus particle are still in the low-pH conformational state. This would explain why dispase indeed cleaves a minor fraction of the total amount of hexons at pH 7.5 and why the anti-15K antibodies neutralize at pH 7.5. (ii) The antibody fraction contains different subpopulations, some of which recognize the low-pH conformation of the hexon but are affected by the low pH and fail to properly react with the hexon. (iii) The isolated antibody fraction contains antibodies which are not detected in the Western blotting assay but which recognize and bind to hexons at pH 7.5. (iv) Even if the virion-antibody interaction was performed at pH 5.0, the antibody-virion complex must be returned to pH 7.5 in order to achieve proper attachment to living cells. This change of pH to neutrality might easily cause weakly bound antibodies to dissociate from the hexon antigen. Although it displayed a high specific neutralizing capacity, the isolated anti-15K antibody fraction did not play a significant role in the neutralization performed by the unfractionated anti-(FA)hexon serum, because of the small amount of antibodies directed towards the 15K antigenic determinant.

ELISAs performed at low pH and at pH 7.5, using the two immunoglobulin fractions obtained from the negative-affinity column, revealed higher relative titers at pH 5.0 than at pH 7.5 for the anti-15K antibody fraction. This result shows that some of the antibodies in the anti-15K fraction indeed react with the hexon antigen preferentially at pH 5.0, but a large fraction (about 50%) of the antibody population also is able to react with hexons at pH 7.5.

In summary, the finding of neutralizing anti-hexon antibodies that do not aggregate virions and the isolation of a neutralizing subpopulation of antibodies against a defined segment of the hexon polypeptide suggest that the hexon capsomer of the virion may play a critical role in the initial steps of adenovirus infection. We believe that upon lowering of the pH in the endocytotic pathway, the hexon protein undergoes a conformational change and exposes the N-terminal end of the molecule. This pH-induced conformational change of the hexon either could be necessary for the subsequent proper exposure of the penton base, which then interacts with the endosomal membrane, or may be involved in the step of uncoating the virion. Further studies of the neutralization process performed by anti-penton base and anti-hexon antibodies using monoclonal antibodies which are in preparation would be rewarding.

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