JOURNAL OF VIROLOGY, Oct. 1996, p. 7071–7078 Vol. 70, No. 10
0022-538X/96/$04.00+0

Domains Required for Assembly of Adenovirus Type 2 Fiber Trimers

JEONG SHIN HONG† AND JEFFREY A. ENGLER*

Department of Biochemistry and Molecular Genetics, School of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

Received 10 May 1996/Accepted 16 July 1996

Entry of human adenovirus into cells is a two-step process, mediated in the first step by a specific interaction between the trimeric fiber protein and a specific receptor on the surface of susceptible cells. Because of the interest in human adenovirus as a vector for gene therapy, we have mapped domains in the fiber protein that are important for proper assembly of this trimeric structure and for proper addition of O-linked *N*-acetylglucosamine (*O*-GlcNAc). Mutants of adenovirus type 2 fiber in this study were expressed in human cells by use of a recombinant vaccinia virus expression system that yielded protein indistinguishable from the fiber produced during adenovirus infection. The N-terminal half of the protein did not appear to influence fiber trimer formation, since deletions up to 260 amino acids (aa) from the N-terminal end as well as in-frame deletions within the shaft of the molecule still allowed trimerization; internal deletions in the shaft between aa 61 and 260 appeared to alter addition of *O*-GlcNAc, as judged by loss of reactivity to a monoclonal antibody specific for this carbohydrate addition. Deletions from the C terminus of the molecule (as small as 2 aa) appeared to prevent trimer formation. Additions of amino acids to the C-terminal end of the fiber showed variable results: a 6-aa addition allowed trimer formation, while a 27-aa addition did not. These trimer-defective mutants were also relatively less stable, as judged by pulse-chase experiments. Taken together, our results indicate that trimerization of the fiber requires at least two domains, the entire head (aa 400 to 582), and at least the C-terminal-most 15 aa of the shaft.

Adenovirus serves as a model system for the study of DNA replication, transformation, and many other intracellular processes during its lytic cycle (reviewed in reference 20). Recently, adenovirus has also enjoyed renewed interest as a possible viral vehicle for gene therapy. Among the advantages of recombinant adenovirus vectors are the ability to transduce nondividing cells; the ability to be obtained as purified, high-titer stocks; and the relatively easy manipulation of the genome to insert and express a foreign gene (37). In spite of these advantages, there are major hurdles to be overcome in the application of recombinant adenoviruses to gene therapy procedures. Among these are the immunogenicity associated with virus administration, inefficient gene delivery in vivo, and targetability of the recombinant adenovirus to specific cells. A better understanding of interaction between the cell surface receptor and the adenovirions may be essential in order to develop a new generation of vectors that will overcome these limitations.

Adenovirus is known to infect a variety of cell types in vitro. The viruses attach with high affinity to an uncharacterized cell surface receptor via the fiber protein (12). Recently, vitronectin-binding integrins have been identified as the secondary receptor for virus entry, and the interaction between the integrins and an Arg-Gly-Asp (RGD) sequence encoded in the penton base protein allows the virus to internalize (4, 42, 43); in adenovirus type 12 (Ad12) infection, this integrin- and penton base-mediated pathway may be sufficient for virus entry (3). Once in the endosomes, the virus encounters acidic pH, but exposure to this change in pH may not be important for further virus disassembly and transport (30). The virus particles are then transported to the nuclear pore complex, and the adenovirus genome is released into the nucleus.

The adenovirus fiber protein is located at each of the twelve vertices of the viral icosahedron (39). The sequences of fiber genes from several different serotypes of adenovirus have been determined (19, 22, 32), and they seem to have a common asymmetrical structure, consisting of three domains: a tail, a shaft, and a knob (13). The N-terminal tail is directly involved in the interaction of the fiber with penton base protein and encodes the signals necessary for transport of the protein to the nucleus (18); the C terminus is located in the distal knob and contains the determinants for receptor binding (9). It has been shown that the subgroup C (Ad2 and Ad5) and subgroup B (Ad7 and Ad3) adenoviruses bind to different receptors and that receptor binding specificity of adenovirus fiber can be altered by exchanging the knob domains (8, 35).

The Ad2 fiber is encoded by a single gene that expresses a polypeptide chain of 582 amino acids (aa) having a calculated molecular weight of 62,056 (16, 17). The native fibers of Ad2 have a molecular mass of 180 to 205 kDa; when denatured with sodium dodecyl sulfate (SDS), these fibers dissociate into monomeric proteins which migrate at an estimated molecular mass of 62 kDa (38). This observation suggests that the fiber is a trimeric structure in which three polypeptides are noncovalently bound into a multimeric structure. Ad2 and Ad5 fibers are also glycoproteins containing monosaccharide *N*-acetylglucosamine residues in O-glycosidic linkages (6, 21, 27); although the functional significance of this modification is not known (14), it has been suggested that *O*-GlcNAc may be important for assembly or stabilization of Ad2 and Ad5 fiber trimers (27).

Through the extensive effort of many groups, several aspects of the molecular structure of the full-length fiber have been identified. Green et al. (13) proposed a computer model for the structure of Ad5 fiber. In this model, the first 43 aa forms the N-terminal tail and the C-terminal 181 aa forms the knob. The shaft extends from residue 44 to residue 400 and contains

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Genetics, Room 460 BHSB, University of Alabama at Birmingham, Birmingham, AL 35294-0005. Electronic mail address: jengler@bmg.bhs.uab.edu.

† Present address: Department of Cell Biology and Anatomy, Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, Birmingham, AL 35294-0005.

a number of 15-residue repeating units. The fibers from different subgroups have tails and knobs of approximately the same size but contain different numbers of the repeating units in the shaft, resulting in fibers of different lengths (19, 31, 32). Each 15-residue segment contains two short β-strands and two β-bends, and these repeats together form a long, narrow amphipathic β-sheet which may be stabilized by dimer formation to form the shaft of the fiber. However, an analysis of the virion composition by van Oostrum and Burnett (41) has established that the fiber of Ad2 is a homotrimer. A left-handed triple-helical structure with threefold symmetry has also been proposed to support the proposed trimeric structure of the fiber, in which the shaft of the fiber is stabilized by interchain hydrogen bonds as well as hydrophobic interactions (36). Recently, the crystal structure of the carboxy-terminal knob domain of the Ad5 fiber was determined (44), and in that study the trimeric organization of the fiber was further characterized.

Adenovirus assembles in the nuclei of infected cells. Although fiber-deficient mutants of adenovirus seem to have an altered viral morphogenesis, more recent data suggest that the fiber plays a role in capsid stabilization rather than in capsid assembly (10). Little is known about the trimerization of fiber and its interaction with other proteins; hsp70 has been implicated in the assembly of the trimeric fiber (25). However, fiber expressed by several expression systems shows that fiber could trimerize in the absence of other adenovirus proteins (1, 18, 28), and the C-terminal domain was essential and perhaps sufficient for trimerization (15, 24, 29). A better understanding of the structure of fiber and the identification of domains necessary for trimer formation would be essential for further development of new adenovirus vectors with higher affinity for the cell surface receptor and/or with novel receptor specificities.

In this study we focused on identifying domains essential for trimerization by expressing fiber mutants in recombinant vaccinia virus-infected cells. Unique monoclonal antibodies were used as tools to assess the state of assembly of the proteins. The presence of *O*-GlcNAc on each mutant was also investigated to identify domains necessary for this modification.

MATERIALS AND METHODS

Antibodies. Two Ad2 fiber-specific monoclonal antibodies, 4D2 and 2A6, were used throughout this study. The isotype of both the 4D2 and 2A6 antibodies is immunoglobulin G2a (IgG2a) heavy chain and κ light chain, as determined by enzyme-linked immunosorbent assay (SBA clonotyping system 1; Fisher Biotech). High-titer ascites fluid from BALB/c mice injected with antibody-secreting hybridoma cells was used for all experiments.

Monoclonal antibody RL2, specific for GlcNAc-containing proteins (34), was a gift of L. Gerace (Scripps Clinic, La Jolla, Calif.). Polyclonal antibody R72, raised against Ad2 fiber, was a gift of M. Horwitz (Albert Einstein College of Medicine, New York, N.Y.). Polyclonal antibody RaF2Nat, made against the native form of purified Ad2 fiber, was a gift of P. Boulanger (Montpellier, France).

Cells and viruses. Ad2 and Ad5 viruses were grown on plates of HeLa cells, and virus stocks were prepared as described previously (18). Ad7 virus was grown on plates of A549 cells maintained in F-12 medium (Cellgro) with 10% fetal bovine serum. HeLa, HeLa T4, and HEp-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 5% bovine calf serum (BCS) and 2% fetal bovine serum. CV1 cells were maintained in DMEM with 10% fetal bovine serum. HuTK$^-$ 143B (thymidine kinase-negative) cells were maintained in DMEM with 10% BCS and 25 μg of 5-bromodeoxyuridine per ml.

Construction of recombinant plasmids. Construction of plasmids S3-2F, S3-2F-*dl*KRAR (ΔKRAR), and S3-2F-A6c (PKRARP) has been described previously (18). Other Ad2 fiber mutant genes were also cloned into a vaccinia virus expression vector, pTKgpt-F3s (11). S3-2F-*dl*17n was constructed by cloning the *Nde*I-*Sma*I fragment of pUC2Fm (18) into the *Eco*RI site of pTKgpt-F3s after repairing the ends with Klenow fragment. S3-2F-*dl*311n, S3-2F-*dl*354n, and S3-2F-*dl*382n resulted from cloning *Nsi*I-*Sma*I, *Eco*RV-*Sma*I, and *Hin*dIII-*Sma*I fragments of pUC2Fm into the blunt-ended *Eco*RI site of the vector. S3-2F-*dl*Sph was constructed by deleting the internal *Sph*I fragment from the Ad2 fiber gene and cloning it into the *Hpa*I site of the expression vector. pUC2Fm-*dl*HP was made by cloning the *Hin*dIII fragment from pUC2Fm into the *Pst*I site of a derivative of pUC2Fm (which contains the *Pst*I-*Sma*I fragment of the fiber gene). The mutant fiber gene was then cloned into the *Hpa*I site of the expression vector to create S3-2F-*dl*HP. S3-2F-*dl*EB contains a mutant fiber gene that was created by deleting *Eco*RI to *Bgl*II (blunt-end ligated after treatment of the digested ends with T4 DNA polymerase). S3-2F-*dl*BS was created by digesting the fiber gene with *Bgl*II and *Spe*I, repairing the ends with T4 DNA polymerase, and ligating the ends together. S3-2F-*dl*444 was constructed by cloning the *Bgl*II-*Bam*HI fragment from pUC2Fm into the *Bam*HI site of the vector. S3-2F-*dl*540 was made by digesting S3-2F plasmid DNA with *Spe*I, repairing the ends with Klenow fragment, and religating the plasmid to create a premature stop codon.

Mutagenesis was performed as described previously (18) to create mutants 2F-*dl*Q581E, 2F-A27c, and 2F-REDD. 2F-*dl*Q581E had the glutamine at aa 581 replaced with glutamate and the protein truncated by 1 aa. 2F-A27c was created by deleting the existing termination codon from the Ad2 fiber gene. As a result, 27 codons downstream from the Ad2 fiber gene was further translated in addition to the fiber sequence. Mutant 2F-REDD contained a replacement of AQE at the C terminus of the Ad2 fiber (aa 580 to 582) with REDD (amino acids encoded at the C terminus of the Ad7 fiber gene). These mutants were cloned into the *Hpa*I site of the expression vector as described previously (18). Termination codons were inserted by site-directed mutagenesis following codons 410, 573, 577, 580, and 581 to create mutants 2F-*dl*410, 2F-*dl*573, 2F-*dl*577, 2F-*dl*579, 2F-*dl*580, and 2F-*dl*581, respectively. The sequences of the oligonucleotides used to create these mutants were as follows: *dl*410, 5′ TATCT GAATG AATTC TTCAG TTAGG AGATG 3′ (30-mer); *dl*573, 5′ TTCAC GATTC TTTAA GAGTT GGTAG CAAA 3′ (29-mer); *dl*577, 5′ TTCAC GATTC TTTAG GAGAA GGTGT AAGA 3′ (29-mer); *dl*579, 5′ TTCAC GATTC TTTAA ATGTA GGAGA AGGT 3′ (29-mer); *dl*580, 5′ TTCAC GATTC TTTAG GCAAT GTAGG AGAA 3′ (29-mer); *dl*581, 5′ TTCAC GATTC TTTAC TGGGC AATGT AGGA 3′ (29-mer); *dl*581E, 5′ ACGAT TCTTT ATTCG GCAAT GTAGG AGAA 3′ (29-mer); A27c, 5′ GGTTC ACGAT TCTTT CCTGG GCAAT GTAG 3′ (29-mer); and REDD, 5′ TTCAC GATTC TTTAG TCATC TTCTC TAATG TAGGA GAAGG T 3′ (41-mer).

Recombinant vaccinia viruses. Recombinant viruses were prepared as described previously (18). The vaccinia viruses were propagated in HEp-2 cells, and their titers were determined in HuTK$^-$ 143B cells. The recombinant virus stocks were aliquoted and stored at −80°C. The cells were infected at a multiplicity of infection of 10 in all experiments unless noted otherwise.

Immunoblots. Immunoblotting was performed by standard procedures using lysates prepared from recombinant vaccinia virus-infected HeLa cells (33). The cell lysates were resuspended in sample buffer (23) and then were either boiled to denature the trimers or not boiled to keep the trimeric fiber intact prior to SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The gels were blotted onto nitrocellulose or polyvinylidene difluoride membrane (Bio-Rad). When 4D2 or 2A6 antibodies were used as primary antibodies, goat anti-mouse Ig conjugated with alkaline phosphatase (Fisher Biotech) was used as a secondary antibody. Biotin-conjugated goat anti-mouse Ig and alkaline phosphatase conjugated to streptavidin (Fisher Biotech) were used to probe the specific binding of monoclonal antibody RL2 to fiber. To probe polyclonal antibody R72 on a blot, alkaline phosphatase conjugated to goat anti-rabbit Ig was used. The color reaction for alkaline phosphatase was developed with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium; Boehringer Mannheim).

Immunofluorescence. HeLa T4 cells were seeded on coverslips in six-well plates. Recombinant vaccinia virus-infected cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS). Methanol was used to permeabilize the cell membrane. Cells were incubated in 1% bovine serum albumin (BSA) in PBS to prevent nonspecific binding. All of the subsequent steps were performed in 1% BSA in PBS. 4D2, 2A6, or RaF2Nat antibody was used as the primary antibody. Goat anti-mouse Ig (for 4D2 and 2A6) or goat anti-rabbit Ig (for RaF2Nat) conjugated to fluorescein isothiocyanate (Southern Biotech, Birmingham, Ala.) was used as a secondary antibody. The cells were photographed on a Nikon Optiphot microscope equipped for fluorescent illumination at a magnification of ×400 with Kodak Tmax 400 film.

Pulse-chase and immunoprecipitation. HeLa cells were seeded in a 12-well tissue culture plate and infected with recombinant vaccinia viruses at a multiplicity of infection of 3 for pulse-chase experiments. At 17 to 18 h postinfection, the cells were starved in DMEM without methionine (GIBCO) and pulse-labeled for 10 min with 10 μCi of [^{35}S]methionine (Amersham) (100 μCi/ml) or 30 μCi of [^{35}S]Translabel (ICN) (300 μCi/ml) in each well. The radioactive medium was removed, and DMEM with 10% FBS was added. After being incubated at 37°C, the cells were collected at 0-, 2-, 4-, and 8-h time points and lysed with radioimmunoprecipitation assay (RIPA) buffer (33). The lysates were incubated with 4D2 monoclonal antibody or with polyclonal antibody R72 preloaded on protein G-conjugated beads (protein G-Sepharose 4 Fast Flow; Pharmacia). The antigen-antibody complex was collected and analyzed by SDS–12% PAGE.

Immunoprecipitation was also performed to characterize the epitopes for antibodies 4D2 and 2A6 by using the deletion mutants. HeLa cells in a 12-well tissue culture plate were infected with Ad2 or recombinant vaccinia viruses expressing mutant fibers. At 24 h postinfection the cells were labeled with 50 μCi of L-[^{35}S]methionine (250 μCi/ml) for 1 h at 37°C. The lysates were prepared

with RIPA buffer and preincubated with Immunoprecipitin (GIBCO) preloaded with rabbit and mouse sera. Each lysate was divided into three tubes and incubated with 4D2, 2A6, or R72 antibody. The antigen-antibody complexes were collected with protein G-Sepharose (Pharmacia) and analyzed by SDS–10 or 15% PAGE.

RESULTS

Construction and expression of Ad2 fiber mutants. Previous studies have shown that adenovirus fiber trimers can be correctly assembled, glycosylated, and transported to the nucleus in the absence of other adenovirus proteins (18, 29). On the basis of these previous findings, a series of mutations in Ad2 fiber were constructed to study the domains important for trimerization and the possibility for the addition of short polypeptides onto the fiber. We chose to use a vaccinia virus expression system because vaccinia virus can infect many of the same types of host cells as adenoviruses can and because our previous studies have already shown that the wild-type fiber protein correctly assembles into trimers and localizes into the nucleus when expressed by use of this system (18).

Mutant fiber groups. The mutant fibers described in this article belong to one of the following four groups (Fig. 1).

(i) N-terminal deletion mutants (six examples). 2F-*dl*KRAR was described previously as a nuclear translocation-defective mutant (18). It formed trimers in the cytoplasm as detected with monoclonal antibody 2A6 by immunofluorescence and immunoblotting. Four N-terminal deletion mutants (2F-*dl*17n, 2F-*dl*311n, 2F-*dl*354n, and 2F-*dl*382n) were cloned by blunt-end ligation into the *Eco*RI site of the vector after the ends were repaired with Klenow fragment. As a result, two amino acids (glycine and isoleucine) were added to the N termini of these mutants. The numbers in the designations for these mutants represent the first codons that are translated from the Ad2 fiber gene. Mutant 2F-*dl*17n contained a 16-aa deletion from the N-terminal tail of the fiber. Mutants 2F-*dl*311n and 2F-*dl*354n contained deletions of 310 and 353 aa, respectively, from the N-terminal half of the molecule. These mutants were left with an intact knob and the last five and three repeating units in the shaft, respectively. Mutant 2F-*dl*382n contained only 1.5 repeating units in the shaft and an intact knob.

(ii) Internal deletion mutants (three examples). Internal deletion mutants were constructed by using the existing restriction enzyme sites in the Ad2 fiber gene. Mutant 2F-*dl*Sph contained a 198-aa deletion from aa 62 to 260 (12.5 repeating units) in the shaft. Mutant 2F-*dl*HP has a deletion of 28 aa at the junction between the shaft and the knob with a 3-aa addition (LHA) from the cloning process. This deletion included the sequence TLWTTPDPSPN (aa 400 to 410), which has been found to be conserved in fibers (with more than 80% homology) from many adenovirus serotypes sequenced (5, 44). Mutant 2F-*dl*EB contained a deletion of 32 aa (from aa 413 to 444) from the beginning of the knob. This region contained two known sites for temperature-sensitive mutations in the fiber gene, i.e., an A-to-V change at aa 434 in H2*ts*125 (5) and an A-to-V change at aa 440 in H5*ts*142 (unpublished observation).

(iii) Mutants with deletions from the C terminus of Ad2 fiber (nine examples). The C-terminal 182 aa in Ad2 fiber form the knob domain, which is responsible for binding to the cellular receptor and also contains type-specific antigenic sites (20). Deletions as small as a 1-aa deletion (2F-*dl*581) were constructed to identify domains for trimerization. In addition, a mutant which had both been truncated by 1 aa and had E substituted for Q was tested (2F-*dl*Q581E). It has been previously reported that the C terminus is important for the trimerization of fiber expressed in baculovirus (29), and these deletions were made to map the minimum sequences required for trimerization within this region.

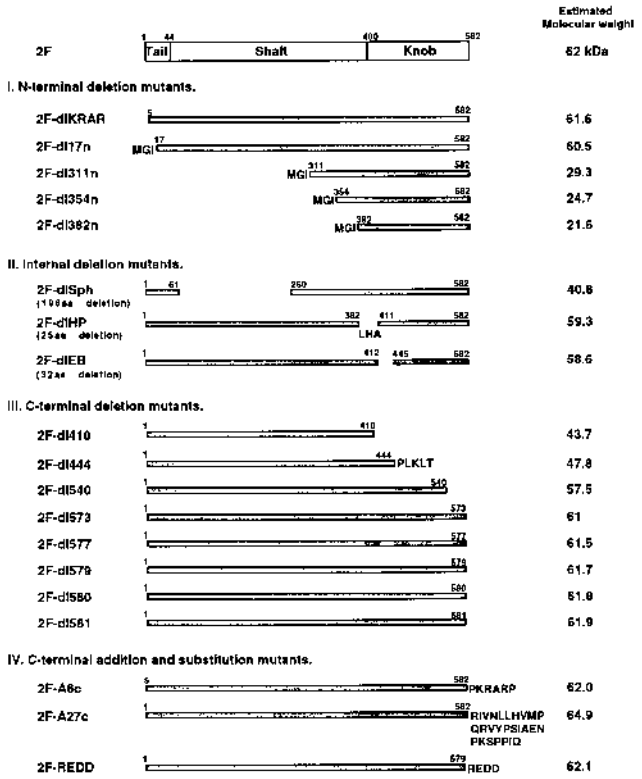

FIG. 1. Schematic representation of Ad2 fiber mutants studied. The mutants are divided into four groups depending on the nature of the mutations. 2F is the recombinant vaccinia virus full-length Ad2 fiber. (Group I) 2F-*dl*KRAR, 2F-*dl*17n, 2F-*dl*311n, 2F-*dl*354n, and 2F-*dl*382n were N-terminal deletion mutants. The number in each designation represents the position of the first codon used for each mutant. Four of these mutants (excluding 2F-*dl*KRAR) contained an additional glycine and isoleucine at their N termini because of the cloning strategy. (Group II) 2F-*dl*Sph was an internal deletion mutant which lacked 198 aa from codon 62 to 259 in the shaft of the fiber. 2F-*dl*HP and 2F-*dl*EB contained 25 (codons 383 to 410)- and 32 (codons 413 to 444)-aa internal deletions near the junction of the shaft and the knob. (Group III) 2F-*dl*410, 2F-*dl*444, 2F-*dl*540, 2F-*dl*573, 2F-*dl*577, 2F-*dl*580, and 2F-*dl*581 were C-terminal deletion mutants. The last codons used in the C-terminal deletion mutants are represented by the numbers in the designations. (Group IV) 2F-A6c and 2F-A27c were C-terminal addition mutants. 2F-A6c (previously described as 2F-ΔKRAR-PKRARP) included a 6-aa addition, PKRARP, at the end of each polypeptide. 2F-A27c was created by deleting the termination codon from the Ad2 fiber gene. As a consequence, 27 additional codons were translated and added to the C terminus of the Ad2 fiber. 2F-REDD contained a substitution of the last three codons in Ad2 fiber, AQE, to REDD, which is found at the C termini of Ad3 and Ad7 fibers. Amino acids shown for some of the mutants were added to Ad2 fiber during the cloning process. The approximate molecular mass of each mutant is shown on the right.

(iv) Addition and substitution mutants with mutations at the C-terminal end of Ad2 fiber (three examples). Mutant 2F-A6c has been described previously as 2F-ΔKRAR-PKRARP (18). This mutant was originally constructed to study whether relocating the KRARP sequence from the N terminus to the C terminus can restore nuclear translocation of 2F-*dl*KRAR. This 6-aa addition (PKRARP) did not apparently alter trimerization of the fiber protein in the cytoplasm of infected cells (18). In mutant 2F-A27c, the termination codon was deleted from the Ad2 fiber gene, so that an additional 27 codons were added onto the C terminus of Ad2 fiber. Finally, in mutant 2F-REDD, the AQE sequence at the end of the Ad2 fiber was replaced with the REDD sequence encoded at the end of the Ad3 and Ad7 fiber genes (19, 32).

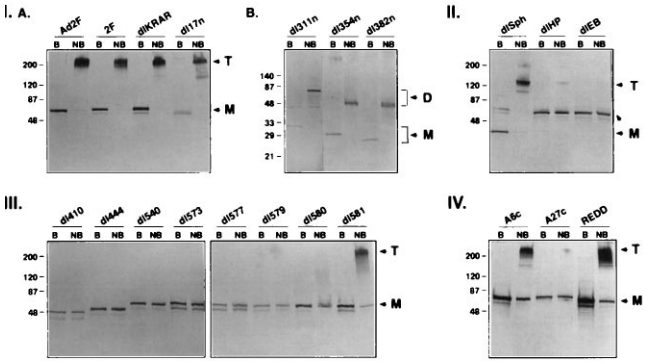

FIG. 2. Immunoblot of HeLa cell lysates infected with recombinant vaccinia viruses expressing fiber mutants. Samples were either boiled or not boiled prior to loading to detect multimer formation of each mutant. The samples were separated on SDS–4 to 20% precast gradient PAGE gels (Bio-Rad) and blotted, and the resulting blots were developed with R72, a polyclonal antibody against Ad2 fiber. The lanes marked B represent samples boiled prior to loading on the gel, and the lanes marked NB represent unboiled samples. The numbers on the left of each panel show the positions and sizes of molecular weight standards. T, trimers; D, dimers; M, monomers. Panel I.A shows wild-type Ad2 fiber (2F) and N-terminal deletion mutants that formed trimers. Panel I.B shows N-terminal deletion mutants that formed putative dimers. Panel II shows three internal deletion mutants. Panel III shows eight C-terminal deletion mutants. Panel IV shows C-terminal mutants with addition or substitution.

Most mutants were cloned into the *Hpa*I site of pTKgpt-F3s (11), downstream from the first termination codon in the vector. The expressed polypeptide was then translated by reinitiating the protein synthesis 15 nucleotides downstream from the termination codon at the bona fide translation initiation (ATG) codon in the fiber gene. Other N-terminal deletion mutants marked in Fig. 1 with MGI began translation at the ATG provided in the vector.

Effect of deletions on trimerization of fiber. The assembly of fiber trimers can be assessed by several criteria, including the following: (i) sedimentation in sucrose gradients, (ii) resistance to trypsin proteolysis, and (iii) electrophoretic mobility in polyacrylamide gels. On the basis of assays for criterion iii, the fiber protein complex has been reported to be very stable and to run at a molecular weight consistent with that of a trimer when the protein sample is not boiled prior to SDS-PAGE (29); when the fiber is boiled prior to SDS-PAGE, however, the trimeric structure is disrupted and the protein runs at a size consistent with a protein monomer. Since the relative mobility of the trimer as determined by SDS-PAGE corresponded well with other criteria identified in previous studies (29, 35), this assay was used throughout the present study as one means for identification of the oligomeric status of wild-type and mutant fibers: stable trimers retained high molecular weights, while monomers (and perhaps weaker trimers) had lower apparent molecular weights.

After SDS-PAGE of lysates from cells infected with recombinant vaccinia virus that expressed the various mutant fibers shown in Fig. 1, the gels were blotted and probed with R72 polyclonal antibody which was raised against Ad2 fiber (Fig. 2). Fibers from both Ad2- and recombinant vaccinia virus 2F-infected HeLa cells were detected as monomers (62 kDa) when the lysates were boiled and as trimers (180 to 200 kDa) when the lysates were not boiled, indicating that recombinant vaccinia virus 2F fiber behaved in a manner similar to that of fiber isolated from adenovirus infection. N-terminal deletion mutants, 2F-*dl*KRAR and 2F-*dl*17n, also formed stable trimers (Fig. 2, panel IA). 2F-*dl*17n always appeared as a doublet in boiled samples (*dl*17n, lane B); this could result from either internal initiation at the methionine at aa 17 or truncation of the *dl*17n fiber by proteases. These data agreed with those of a previous report that showed that deletions of up to 60 aa from the N terminus of the fiber did not affect the trimerization of the fiber (29).

Further deletions from the N terminus of the fiber were made to study the possible role of the shaft in trimer formation. Deletions of the N-terminal tail and up to 260 aa (leaving 9.5 repeating units in the shaft) did not prevent trimerization (data not shown). This observation is consistent with recent reports from several groups who showed that the knob of Ad2 or Ad5 fiber could trimerize (24, 45) when it was expressed in insect cells or in *Escherichia coli*. Surprisingly, three of our N-terminal deletion mutants, 2F-*dl*311n, -*dl*354n, and -*dl*382n (which contained 5, 3, and 1.5 repeating units, respectively, and an intact knob domain), seem to form stable dimers (as judged by their approximate sizes of 49 and 42 kDa when unboiled samples are analyzed by SDS-PAGE) rather than trimers (with expected sizes of 72 and 63 kDa) (Fig. 2, panel IB). This result suggests that not only the C-terminal knob but also a short part of the shaft is required for the stable trimerization of fiber.

The effects of internal deletion mutations were also examined. One internal deletion mutant, 2F-*dl*Sph (lacking amino acid residues 62 to 259 [12.5 repeats in the shaft {13}]) was also able to form a stable trimer (Fig. 2, panel II). Two other internal deletion mutants, 2F-*dl*HP and *dl*EB, contained deletions near the junction of the knob and shaft and were unable to form trimers, consistent with the previous observations that the C-terminal knob and a part of the shaft are required for trimerization. In addition, C-terminal truncation mutants, i.e., 2F-*dl*410, -*dl*444, -*dl*540, -*dl*573, -*dl*577, -*dl*579, and -*dl*580, were detected as monomers in both boiled and unboiled samples by an immunoblotting technique (Fig. 2, panel III). Novelli and Boulanger (29) previously demonstrated that mutants similar to 2F-*dl*410 and -*dl*540 ($AcNPV^{fib}CT410$ and $AcNPV^{fib}CT540$) were not able to form stable trimers in insect cells. From these data, it can be concluded that deletions of the C terminus of the fiber could block trimer formation.

Several short regions of partial homology between Ad2, Ad3, Ad5, and Ad7 can be identified (19, 32). One of these homologous segments (YTFSYIAQE) is located at the very end of the C terminus, but short deletions in this segment were not expected to show any effect on trimerization, since the C-terminal four amino acids (IAQE) were not involved in hydrogen bond interaction and V-sheet formation, which are thought to be important for trimerization (44). Mutant 2F-*dl*573 contained a deletion of this domain, and the resulting protein product was unable to form a stable trimer (Fig. 2, panel III). Additional mutations in this domain (deleting 5 [YIAQE; *dl*577], 3 [AQE; *dl*579], or 2 [QE; *dl*580] aa from the C-terminal end) also blocked trimer formation. Deletion of either the next-to-last (Q; *dl*581) or the last amino acid (E; *dl*Q581) allowed trimerization (Fig. 2, panel III; see Fig. 4B). Since the 2F-*dl*580 mutant showed that the deletion of the last 2 aa was sufficient to block trimerization of Ad2 fiber, the AQE sequence was replaced with the REDD sequence from the corresponding regions of the Ad3 and Ad7 fibers to determine if the role of this region in trimerization depended on the specific sequence or on the serotype. The replacement mutant 2F-REDD formed a stable trimer (Fig. 2, panel IV), suggesting that the charges of these amino acids rather than the specific sequence may play a role in trimerization.

To further test the possibility that addition of short peptides to the C-terminal end of the fiber might disrupt trimer formation, two mutants were tested. Previously, we constructed a mutant that contained six additional amino acids at the C-

terminal end (2F-A6c; PKRARP); this mutant retained the ability to form trimers (18) (Fig. 2, panel IV). However, the addition of a longer 27-aa peptide to the C terminus of the fiber (2F-A27c) blocked trimer formation (Fig. 2, panel IV). Taken together, these results indicate that the C terminus of the fiber must be involved in intersubunit or intrasubunit interaction for trimer formation.

Epitopes of monoclonal antibodies 4D2 and 2A6. Two monoclonal antibodies, 4D2 and 2A6, were used throughout this study to assess the integrity of the trimeric fiber structure. Initially, these two antibodies were isolated from a pool of monoclonal antibodies prepared from mice injected with UV-irradiated Ad2 virus as an antigen, characterized, and shown to recognize Ad2 (Fig. 3, lanes 1) or Ad5 (data not shown) fibers; we have also determined that Ad7 fiber monomers and trimers were recognized on immunoblots by 4D2 but not by 2A6 (data not shown). Both antibodies recognized Ad2 and Ad5 fibers in the nuclei of the infected cells by indirect immunofluorescence (18), and antibody 4D2 also recognized Ad7 fiber in the nucleus by immunofluorescence (unpublished data). Occasionally 2A6 antibody recognized bands at the monomer size in boiled samples on an immunoblot (data not shown); this seemingly aberrant behavior was probably due to refolding of the fiber monomers on the blot, restoring a structure similar to that of a trimer that could be recognized on the blot during incubation with monoclonal antibody 2A6. Neither 4D2 nor 2A6 antibodies inhibit Ad2 attachment to and infection of HeLa cells (unpublished data).

Immunoblots were used to map the epitope recognized by antibody 4D2 (Fig. 3A). This antibody would not recognize fibers with as few as 17 aa deleted from the amino terminus of the protein (Fig. 3A); on the other hand, fibers with only 6 aa deleted from the amino terminus were recognized by 4D2 (lanes 3) (18). To confirm previous results indicating that monoclonal antibody 2A6 was specific for fiber trimers, we compared the reactivities of polyclonal antibody R72 and 2A6 on duplicate immunoblots of unboiled samples (Fig. 3B); all of the recombinants that formed trimers were recognized by 2A6 (2F, 2F-*dl*KRAR, 2F-*dl*17n, 2F-*dl*Sph, 2F-*dl*581, 2F-A6c, and 2F-REDD), while those that did not form trimers were not recognized.

These results were confirmed by further experiments in which mutant fibers were immunoprecipitated with 4D2 or 2A6 antibody (data not shown). Antibody 4D2 was able to immunoprecipitate all of the mutants tested except 2F-*dl*17n and 2F-*dl*382n. Antibody 2A6 was able to immunoprecipitate the mutants that were previously shown to form trimers (2F, 2F-*dl*KRAR, 2F-*dl*17n, 2F-*dl*Sph, and 2F-A6c).

Localization of mutant fibers. Adenovirus particles assemble in the nuclei of infected cells. Although it was previously demonstrated that the nuclear translocation signal resides at the very N terminus of Ad2 fiber (18), these mutants provided an opportunity to study the possibility of activation of a secondary nuclear translocation signal (as had been suggested by studies with baculovirus [29]) and to confirm previous results that suggested that trimerization and nuclear localization processes act independently (18). Indirect immunofluorescence was performed to localize the mutant fibers; antibodies 4D2, 2A6, and RaF2Nat all recognized full-length fiber in the nuclei of infected cells (data not shown). Mutant 2F-*dl*Sph was recognized by all three antibodies used in this study (Fig. 4A) and was localized to the nucleus. Mutant 2F-*dl*17n was localized in the cytoplasm of the cells and was excluded from the nuclei, since the nuclear localization signal was deleted (Fig. 4A) (18); this mutant was recognized only by the 2A6 antibody, further confirming the epitope mapping results for 4D2. Several N-terminal deletion mutants (2F-*dl*311n and 2F-*dl*354n [data not shown] and 2F-*dl*382n [Fig. 4]) were localized in both the cytoplasm and the nucleus, suggesting either that a weak cryptic nuclear localization signal had been activated or that these proteins could diffuse into the nucleus because of their small size (less than 30 kDa); these mutants had also lost most or all of the epitopes for 4D2 and 2A6 and were detected only by a polyclonal antibody made against full-length fiber. Mutant 2F-A27c showed strong nuclear fluorescence with antibodies 4D2 and RaF2Nat but little or no fluorescence with 2A6, confirm-

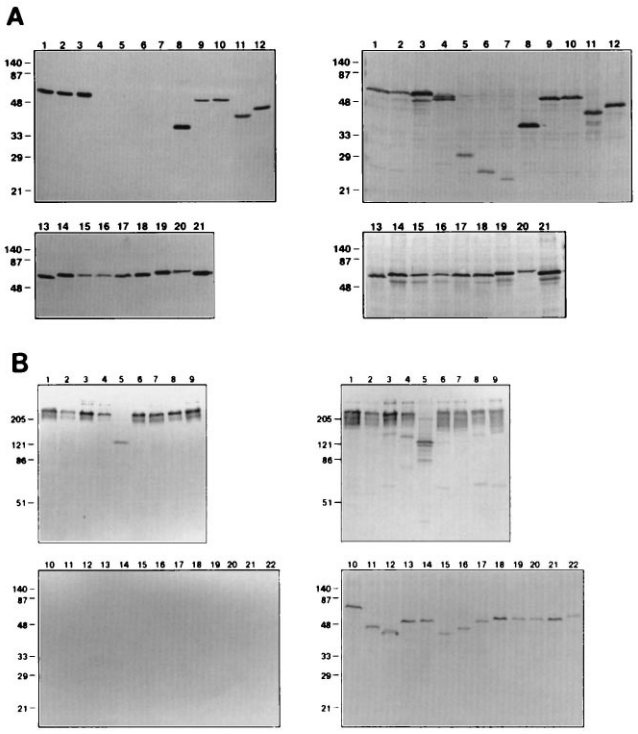

FIG. 3. (A) Immunoblot of the fiber mutants developed with monoclonal antibody 4D2. The lysates were prepared from recombinant vaccinia virus-infected cells by resuspending the cells in Tris-EDTA and sonicating them. The amount of the recombinant protein was not adjusted in each lysate so that it should only be observed qualitatively. Anti-mouse IG conjugated with alkaline phosphatase was used as a secondary antibody for 4D2, and anti-rabbit IG conjugated with alkaline phosphatase was used for R72 anti-fiber polyclonal antibody. The left panels show blots of boiled lysates that were developed with 4D2 monoclonal antibody. The right panels are identical blots of boiled samples, developed with antibody R72. The lysates were boiled for 5 min prior to being loaded in 2× sample buffer recommended in a standard protocol. (23). 4D2 recognizes most of the fiber mutants with intact N termini in both monomeric and trimeric (data not shown) forms. Lanes: 1, Ad2 fiber from adenovirus-infected cells; 2, 2F; 3, 2F-*dl*KRAR; 4, 2F-*dl*17n; 5, 2F-*dl*311n; 6, 2F-*dl*354n; 7, 2F-*dl*382n; 8, 2F-*dl*Sph; 9, 2F-*dl*HP; 10, 2F-*dl*EB; 11, 2F-*dl*410; 12, 2F-*dl*444; 13, 2F-*dl*540; 14, 2F-*dl*573; 15, 2F-*dl*577; 16, 2F-*dl*579; 17, 2F-*dl*580; 18, 2F-*dl*581; 19, 2F-A6c; 20, 2F-A27c; 21, 2F-REDD. (B) Immunoblot of the fiber mutants developed with monoclonal antibody 2A6. The lysates were prepared as described for panel A. However, they were not boiled prior to being loaded onto SDS-PAGE gels in this assay. The 2× sample buffer used in this experiment contained only 0.2% SDS instead of the 2% SDS used in standard denaturing gels. The left panels show blots developed with 2A6, and the right panels show identical blots developed with R72 polyclonal antibody. 2A6 recognized fiber trimers, but not monomers, from Ad2 and Ad5 (shown in Fig. 1). Trimeric fibers were as follows: Ad2 fiber from adenovirus infected cells (lanes 1), 2F (lanes 2), 2F-*dl*KRAR (lanes 3), 2F-*dl*17n (lanes 4), 2F-*dl*Sph (lanes 5), 2F-*dl*581 (lanes 6), 2F-*dl*Q581 (lanes 7), 2F-A6c (lanes 8), and 2F-REDD (lanes 9). Dimeric fibers were as follows: 2F-*dl*311n (lanes 10), 2F-*dl*354n (lanes 11), and 2F-*dl*382n (lanes 12). Monomeric fibers were as follows: 2F-*dl*HP (lanes 13), 2F-*dl*EB (lanes 14), 2F-*dl*410 (lanes 15), 2F-*dl*444 (lanes 16), 2F-*dl*540 (lanes 17), 2F-*dl*573 (lanes 18), 2F-*dl*577 (lanes 19), 2F-*dl*579 (lanes 20), 2F-*dl*580 (lanes 21), and 2F-A27c (lanes 22).

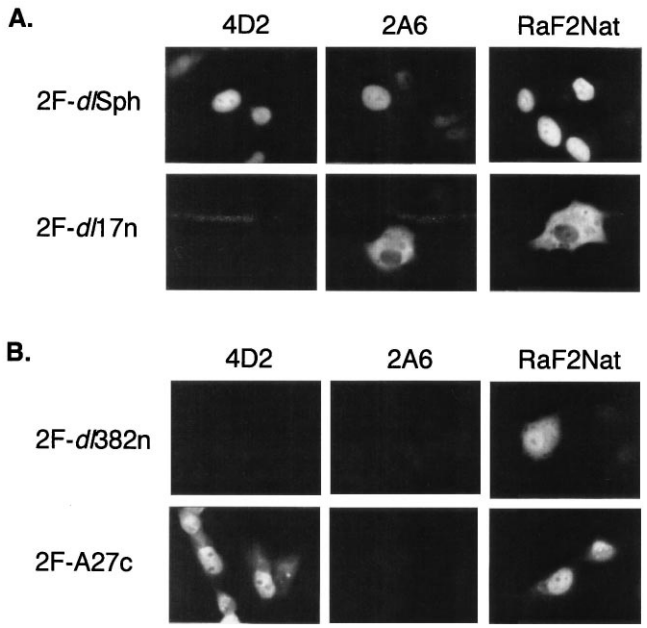

FIG. 4. Indirect immunofluorescence of recombinant fiber mutants. Each recombinant was probed with the 4D2, 2A6, and RaF2Nat antibodies. (A) Two examples of fiber mutants that form trimers are shown. An internal deletion mutant, 2F-*dl*Sph, represents a typical immunofluorescence pattern of trimeric fiber in the nuclei of recombinant virus-infected cells. 2F-*dl*17n was also located in the nucleus but was not identified by 4D2. Three fiber mutants (2F-*dl*581, 2F-REDD, and 2F-A6c) showed the same immunofluorescence in the nuclei of infected cells as did 2F-*dl*Sph shown in panel A (data not shown). (B) Two examples of fiber mutants defective in trimer formation are shown. 2F-*dl*382n was identified only by RaF2Nat polyclonal antibody and located in both cytoplasm and the nucleus. Two other N-terminal deletion mutants (2F-*dl*311n and 2F-*dl*354n) looked the same as 2F-*dl*382n did with all three antibodies (data not shown). The bottom row in panel B shows cells infected with vaccinia virus expressing fiber mutant 2F-A27c. Two internal deletion mutants (2F-*dl*EB and 2F-*dl*HP) and all of the C-terminal deletion mutants except 2F-*dl*581 (i.e., 2F-*dl*410, 2F-*dl*444, 2F-*dl*440, 2F-*dl*573, 2F-*dl*577, 2F-*dl*579, and 2F-*dl*580) showed the same profile of reactivity and nuclear fluorescence as did 2F-A27c (data not shown); they were all recognized by the 4D2 and the RaF2Nat antibodies but not by the 2A6 antibody.

ing that this mutant was unable to form trimers; two other internal deletion mutants (2F-*dl*HP and 2F-*dl*EB) also showed this same pattern of immunofluorescence reactivity (data not shown).

Glycosylation of mutant fibers. Ad2 and Ad5 fibers are glycoproteins that contain multiple monosaccharide *O*-GlcNAc carbohydrates, but the function of this unusual modification has not been determined (21, 27). Monoclonal antibody RL2, which was developed against nuclear pore protein (34), recognizes *O*-GlcNAc residues on Ad2 and Ad5 fibers and has been a useful tool to characterize the glycosylation status of fiber mutants (27). When an immunoblot with mutant fiber was probed with the RL2 monoclonal antibody, all of the mutants except 2F-*dl*311n, -*dl*354n, -*dl*382n, and -*dl*Sph (Fig. 5, lanes 5 through 8, respectively) could be detected. The glycosylation defect in these mutants could be due to either deletion or alteration of the GlcNAc addition sites. When these results are taken together with those of other studies (6, 29), it is reasonable to predict that *O*-GlcNAc addition sites reside in the shaft between residues 60 and 260 in Ad2 fiber.

Stability of mutant fibers. Several of the fiber mutants that were unable to form trimers also did not appear to make the same quantity of recombinant protein as the mutants that formed trimers did, even when variables (i.e., virus titer, cell

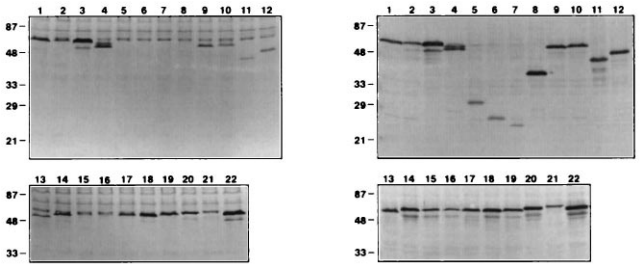

FIG. 5. Glycosylation of Ad2 fiber mutants characterized with *O*-GlcNAc-specific antibody RL2. The left panels are blots of boiled lysates from vaccinia virus-infected cells that were developed with RL2 monoclonal antibody. The right panels are identical blots of boiled samples developed with R72. The lysates were boiled for 5 min prior to being loaded in 2× sample buffer recommended in a standard protocol. The blots on the left were probed with biotin-labeled goat anti-mouse IG as a secondary antibody and alkaline phosphatase-conjugated streptavidin. The blots on the right were probed with alkaline phosphatase-conjugated anti-rabbit IG as a secondary antibody. The amounts of protein in these blots were not quantitated and should be considered only qualitatively. Lanes: 1, Ad2 fiber from adenovirus-infected cells; 2, 2F; 3, 2F-*dl*KRAR; 4, 2F-*dl*17n; 5, 2F-*dl*311n; 6, 2F-*dl*354n; 7, 2F-*dl*382n; 8, 2F-*dl*Sph; 9, 2F-*dl*HP; 10, 2F-*dl*EB; 11, 2F-*dl*410; 12, 2F-*dl*444; 13, 2F-*dl*540; 14, 2F-*dl*573; 15, 2F-*dl*577; 16, 2F-*dl*579; 17, 2F-*dl*580; 18, 2F-*dl*581; 19, 2F-*dl*Q581; 20, 2F-A6c; 21, 2F-A27c; 22, 2F-REDD.

numbers, and duration of infection) were kept constant. To test the possibility that these proteins might be more labile in infected cells, a pulse-chase experiment was performed. As shown in Fig. 6, fiber mutants that form trimers (2F, *dl*KRAR, *dl*17n, *dl*Sph, and *dl*580) or dimers (*dl*382n) seemed to be stable for up to 8 h of chase. However, trimer-defective mutants (*dl*410, *dl*444, *dl*540, *dl*573, *dl*577, *dl*579, *dl*580, and A27c) were much less stable. These results suggest that the monomeric form of the molecule might be more susceptible to proteolytic degradation within the infected cell. Because of this increased lability, one possible interpretation of the inability of these mutants to form trimers could be that the concentration of fiber protein is too low to allow the individual monomers to interact to form trimers.

DISCUSSION

The focus of this article has been to describe the effects of deletions and additions on the trimerization of fiber complexes

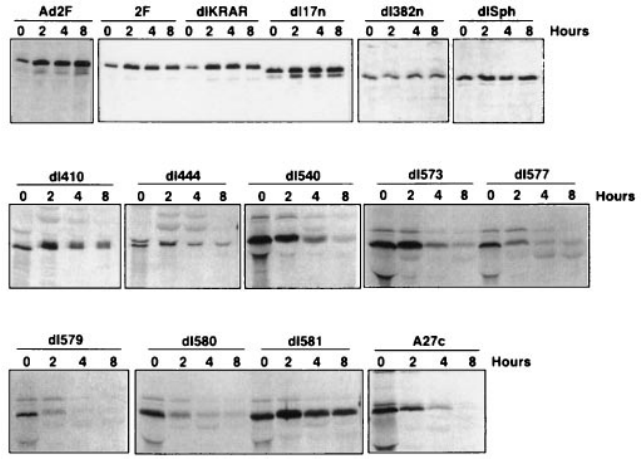

FIG. 6. Pulse-chase labeling of each mutant. The cells were pulse-labeled for 10 min in [^{35}S]methionine or Translabel (ICN) and chased in enriched medium for the times indicated. The lysates were then immunoprecipitated with R72 antibody and analyzed by SDS-PAGE.

and to identify domains that might be manipulated without disrupting the trimeric structure. For these studies, a recombinant vaccinia virus system has been used to express Ad2 fiber mutants in human HeLa cells, a cell line known to be susceptible to infection by human adenovirus. The wild-type fiber protein expressed in this vaccinia virus vector is indistinguishable from that expressed during adenovirus infection on the basis of electrophoretic mobility, reactivity to antifiber antibodies as determined by immunoblotting and immunoprecipitation, resistance to digestion by proteases, ability to form trimers, posttranslational modification by *O*-GlcNAc, and transport of the protein to the nucleus.

A previous deletion analysis of the fiber gene (29) used recombinant baculovirus to express Ad2 fiber in insect cells; in that work, two different polypeptide domains for fiber trimerization were described, and these domains appeared to act antagonistically. Also in that study, the C-terminal sequence confined to the distal knob of the fiber (between residues 540 and 582) was involved in the fiber's trimerization and the shaft portion of the fiber (between Met-61 and Asn-410) contained information for formation of stable dimers; a second domain, between aa 1 and 16, seemed to retard fiber trimerization.

In the present study, we have extended these observations to localize one domain required for trimerization to the C-terminal end of the fiber molecule. This region of the fiber is strikingly conserved among all human adenoviruses (7, 19, 22, 32), and mutants with deletions of as few as 2 aa from the C-terminal end of the fiber aborted trimer formation. Xia et al. (44) demonstrated with their crystal structure of the knob of Ad5 fiber that the first six residues in this conserved segment, YTFSYI, form β-strand J in a V sheet which plays a significant role in trimerization by providing intersubunit interactions with the adjacent β-strand G. The last two amino acids in the predicted peptide sequence are not defined in the published crystal structure of fiber knobs expressed in *E. coli* (45), and so it is hard to rationalize how deletion of these amino acids might disrupt trimerization of the complex. Furthermore, the addition of certain short peptide sequences (up to 16 aa in length) to the end of the fiber gene open reading frame resulted in formation of trimeric structures (18, 26), although perhaps with lower levels of stability; on the other hand, addition of another longer sequence (A27c) did disrupt fiber trimerization. Since the end of the fiber protein seems to protrude into the interior of the trimer, it is also hard to rationalize why certain additions to the C terminus might be tolerated.

In the present study, the trimerization of Ad2 fiber required not only the very C-terminal end but also other parts of the knob when the protein was expressed in mammalian cells by a recombinant vaccinia virus. Mutant 2F-*dl*HP contained a deletion of the TLWTTPDPSPN sequence (aa 400 to 410) which is conserved in fibers from many serotypes; this motif was predicted to participate in the formation of a hydrophobic core in the fiber monomer. The deletion in 2F-*dl*HP could disrupt the formation of this hydrophobic core, so that monomeric fiber could not adopt a trimeric structure. Mutant 2F-*dl*EB contains a deletion of the B and C β-strands in the V sheet on the basis of the structure determined by Xia et al. (44). The V sheets appear to play a significant role in trimerization by providing many contacts between monomers. Therefore, this deletion could have disrupted hydrogen-bonding interactions within the fiber monomer and eventually disrupted trimer formation. Failure of these two mutants to assemble correctly suggests that much of the knob domain may be crucial for fiber trimer formation. The importance of a C-terminal domain in the assembly of trimeric structures has also been described for the reovirus cell attachment protein, sigma 1, wherein mutations in conserved residues in the knob caused a complete or partial abrogation of cell binding function (1, 2, 19, 32, 40).

Portions of the shaft nearest to the knob may also be required to form stable trimers. A mutant with an intact knob with 18 aa from the shaft according to Green's model (23) (2F-*dl*382n) formed dimers rather than trimers when expressed in recombinant vaccinia virus, suggesting that a part of the shaft may be required for stable trimer formation. On the other hand, the fiber protein expressed in *E. coli* and used for determination of the crystal structure of the knob contained one 15-aa repeat from the shaft and appeared to form trimers (44). The discrepancy in these two results might be due to the presence of three extraneous amino acids beyond the end of the proposed 15-aa repeat unit in the shaft (13); perhaps this additional short segment alters the structure of the molecule in such a way as to block trimer formation.

Deletion mutants which were made by introducing deletions at the N-terminal end of the fiber and expressed in HeLa cells did not seem to destabilize fiber trimers to the same extent as they did in insect cells (29). Mutant 2F-*dl*260n, lacking the first 260 aa of the molecule, remained as a trimer when the sample was not boiled prior to SDS-PAGE, suggesting that the N-terminal half of Ad2 fiber did not significantly influence the trimerization of the fiber. This conclusion is also supported by the fact that fibers from Ad3 and Ad7 contain only six repeating units in the shaft and yet are able to form stable trimers (1, 2, 19, 32). On the other hand, the addition of a fusion peptide (14 aa long) to the N terminus of Ad3 fiber prevented correct trimer assembly (1). In addition, deletion mutants that lack the knob of Ad2 fiber, i.e., 2F-*dl*410 (a vaccinia virus recombinant) and CT410 (a baculovirus recombinant), mostly existed as monomers. In contrast, double-truncation mutants DT17-410 and DT61-410 formed significant amounts of dimers (29). These results suggest that the N-terminal end of the fiber might play a role in preventing premature dimer formation, possibly by binding to a molecular chaperone (25). When they are taken together, it appears that the N-terminal half of the fiber is not required for fiber trimerization but that alterations in this region might influence the propensity of the fiber to form aberrant dimeric complexes.

Fibers from Ad2 and Ad5 contain monosaccharide *O*-GlcNAc residues (6, 21, 27). Caillet-Boudin et al. (6) suggested that the *O*-GlcNAc is located within the N-terminal two-thirds of the fiber and more probably in the N-terminal one-third (between residues 18 and 194). Novelli and Boulanger (29) reported that the shaft domain of the fiber, between Met-61 and Asn-410, contains at least one glycosylation site. The studies reported here have further limited the region in which *O*-GlcNAc addition might take place (to that between residues 61 and 260). The deletion in mutant 2F-*dl*Sph blocked *O*-GlcNAc addition to the fiber, although this mutant did not contain any of the *O*-GlcNAc addition sites proposed by Mullis et al. (27). The defect in glycosylation of this mutant could result from either direct deletions of *O*-GlcNAc attachment sequences or indirect deletions of the sites by conformational change. Although the extent of glycosylation of each mutant was not tested in this study, trimer-defective mutants seemed to show less reactivity towards RL2 than did trimer-competent fiber mutants, suggesting that the trimerization of fiber might create additional sites for *O*-GlcNAc attachment.

The function of GlcNAc residues on fiber still remains unclear. It has been proposed elsewhere that the *O*-GlcNAc residues on fiber may help to stabilize trimers (27). When Ad2 fiber is synthesized in vitro, only 25 to 30% of the fiber polypeptides are trimerized after 4 h of in vitro translation reaction (28). This could be explained by the absence of *O*-

GlcNAc residues if they play a role in the stabilization of fiber trimers. Another possible function for the *O*-GlcNAc residues may be a role in receptor binding. Fibers from subgroup B viruses (Ad3 and Ad7) are not glycosylated. The membrane receptor components recognized by the infectious adenovirus particles, the transcytoplasmic routes, and the alterations of the cytoskeletal network associated with adenovirus infection are different for members of subgroups B and C (8). *O*-GlcNAc residues on fibers from group C viruses might play a role in the potential differences in the interactions between the virus and the host cell.

Taken together, these results indicate that there are regions in the fiber knob and part of the shaft that are essential for formation of the stable trimeric complexes required for virion assembly. Surprisingly, loss of amino acid sequences at the very C terminus of the molecule had a dramatic effect on trimer formation, even though these amino acids were not apparent in the crystal structure of the knob (44). On the other hand, a number of alterations and additions at the C terminus still made relatively stable trimers, and by judicious choice of these added sequences, one might allow attachment of new epitopes to the fiber (26). Whether these new epitopes can serve as a means for redirecting adenovirus to infect novel cell types remains to be determined.

ACKNOWLEDGMENTS

We thank Pierre Boulanger for the gift of polyclonal antibody RaF2Nat and Marshall Horwitz for the gift of polyclonal antibody R72. We thank Barbara Mulach for critical comments about the manuscript.

This work was supported by grant GM38234 from the National Institutes of Health and grant R464 from the Cystic Fibrosis Foundation. Synthesis of oligonucleotide primers for site-directed mutagenesis and for DNA sequencing was supported by NCI grant CA13148 to the UAB Comprehensive Cancer Center.

REFERENCES

1. **Albiges-Rizo, C., A. Barge, R. W. H. Ruigrok, P. A. Timmins, and J. Chroboczek.** 1991. Human adenovirus serotype 3 fiber protein: comparison of native and recombinant proteins. J. Biol. Chem. **266:**3961–3967.
2. **Albiges-Rizo, C., and J. Chroboczek.** 1990. Adenovirus serotype 3 fibre protein is expressed as a trimer in E. coli. J. Mol. Biol. **212:**247–252.
3. **Bai, M., L. Campisi, and P. Freimuth.** 1994. Vitronectin receptor antibodies inhibit infection of HeLa and A549 cells by adenovirus type 12 but not by adenovirus type 2. J. Virol. **68:**5925–5932.
4. **Bai, M., B. Harfe, and P. Freimuth.** 1993. Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. J. Virol. **67:**5198–5205.
5. **Caillet-Boudin, M.-L., P. Lemay, and P. Boulanger.** 1991. Functional and structural effects of an Ala to Val mutation in the adenovirus serotype 2 fibre. J. Mol. Biol. **217:**477–486.
6. **Caillet-Boudin, M.-L., G. Strecker, and J.-C. Michalski.** 1989. O-linked GlcNAc in serotype-2 adenovirus fibre. Eur. J. Biochem. **184:**205–211.
7. **Chroboczek, J., and B. Jacrot.** 1987. The sequence of adenovirus fiber: similarities and differences between serotypes 2 and 5. Virology **161:**549–554.
8. **Defer, C., B. Marie-Thérèse, M.-L. Caillet-Boudin, and P. Boulanger.** 1990. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. J. Virol. **64:**3661–3673.
9. **Devaux, C., M.-L. Caillet-Boudin, B. Jacrot, and P. Boulanger.** 1987. Crystallization, enzymatic cleavage, and the polarity of the adenovirus type 2 fiber. Virology **161:**121–128.
10. **Falgout, B., and G. Ketner.** 1988. Characterization of adenovirus particles made by deletion mutants lacking the fiber gene. J. Virol. **62:**622–625.
11. **Falkner, F. G., and B. Moss.** 1988. Escherichia coli *gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62:**1849–1854.
12. **Greber, U. F., M. Willetts, P. Webster, and A. Helenius.** 1993. Stepwise dismantling of adenovirus 2 during entry into cells. Cell **75:**477–486.
13. **Green, N. M., N. G. Wrigley, W. C. Russell, S. R. Martin, and A. D. McLachlan.** 1983. Evidence for a repeating cross-β sheet structure in the adenovirus fibre. EMBO J. **2:**1357–1365.
14. **Haltiwanger, R. S., G. D. Holt, and G. W. Hart.** 1990. Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. J. Biol. Chem. **265:**2563–2568.
15. **Henry, L. J., D. Xia, M. E. Wilke, J. Deisenhofer, and R. D. Gerard.** 1994. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. J. Virol. **68:**5239–5246.
16. **Hérissé, J., and F. Galibert.** 1981. Nucleotide sequence of the EcoRI E fragment of adenovirus 2 genome. Nucleic Acids Res. **9:**1229–1240.
17. **Hérissé, J., M. Rigolet, S. D. de Dinechin, and F. Galibert.** 1981. Nucleotide sequence of adenovirus 2 DNA fragment encoding for the carboxylic region of the fiber protein and the entire E4 region. Nucleic Acids Res. **9:**4023–4042.
18. **Hong, J. S., and J. A. Engler.** 1991. The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal. Virology **185:**758–767.
19. **Hong, J. S., K. G. Mullis, and J. A. Engler.** 1988. Characterization of the early region 3 and fiber genes of Ad7. Virology **167:**545–553.
20. **Horwitz, M. S.** 1990. Adenoviridae, p. 1679–1742. *In* B. N. Fields and D. M. Knipe (ed.), Virology, vol. 1 Raven Press, New York.
21. **Ishibashi, M., and J. V. Maizel.** 1974. The polypeptides of adenovirus. VI. Early and late glycoproteins. Virology **58:**345–361.
22. **Kidd, A. H., M. J. Erasmus, and C. T. Tiemessen.** 1990. Fiber sequence heterogeneity in subgroup F adenoviruses. Virology **179:**139–150.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
24. **Louis, N., P. Fender, A. Barge, P. Kitts, and J. Chroboczek.** 1994. Cell-binding domain of adenovirus serotype 2 fiber. J. Virol. **68:**4104–4106.
25. **Macejak, D., and R. B. Luftig.** 1991. Association of HSP70 with adenovirus type 5 fiber protein in infected HEp-2 cells. Virology **180:**120–125.
26. **Michael, S. I., J. S. Hong, D. T. Curiel, and J. A. Engler.** 1995. Addition of a short peptide ligand to the adenovirus fiber protein. Gene Ther. **2:**660–668.
27. **Mullis, K. G., R. S. Haltiwanger, G. W. Hart, R. B. Marchase, and J. A. Engler.** 1990. Relative accessibility of *N*-acetylglucosamine in trimers of the adenovirus types 2 and 5 fiber proteins. J. Virol. **64:**5317–5323.
28. **Novelli, A., and P. Boulanger.** 1991. Assembly of adenovirus type 2 fiber synthesized in cell-free translation system. J. Biol. Chem. **266:**9299–9303.
29. **Novelli, A., and P. Boulanger.** 1991. Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. Virology **185:**365–376.
30. **Rodriguez, E., and E. Everitt.** 1996. Adenovirus uncoating and nuclear establishment are not affected by weak base amines. J. Virol. **70:**3470–3477.
31. **Ruigrok, R. W. H., A. Barge, C. Albiges-Rizo, and S. Dayan.** 1990. Structure of adenovirus fibre II. Morphology of single fibres. J. Mol. Biol. **215:**589–596.
32. **Signäs, C., G. Akusjärvi, and U. Petterson.** 1985. Adenovirus 3 fiber polypeptide gene: implications for the structure of the fiber protein. J. Virol. **53:**672–678.
33. **Smith, J. A.** 1992. Analysis of proteins, p. 10.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Short protocols in molecular biology. John Wiley & Sons, New York.
34. **Snow, C. M., A. Senior, and L. Gerace.** 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. **104:**1143–1156.
35. **Stevenson, S. C., M. Rollence, B. White, L. Weaver, and A. McClelland.** 1995. Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. J. Virol. **69:**2850–2857.
36. **Stouten, P. F. W., C. Sander, R. W. H. Ruigrok, and S. Cusack.** 1992. New triple-helical model for the shaft of the adenovirus fiber. J. Mol. Biol. **226:**1073–1084.
37. **Stratford-Perricaudet, L. D., and M. Perricaudet.** 1994. Gene therapy: the advent of adenovirus, p. 344–362. *In* J. A. Wolff (ed.), Gene therapeutics: methods and applications of direct gene transfer. Birkhauser, Boston.
38. **Sunquist, B., U. Pettersson, L. Thelander, and L. Philipson.** 1973. Structural proteins of adenoviruses. IX. Molecular weight and subunit composition of adenovirus type 2 fiber. Virology **51:**252–256.
39. **Svensson, U., R. Oersson, and E. Everitt.** 1982. Virus-receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. J. Virol. **38:**70–81.
40. **Turner, D. L., R. Duncan, and P. W. K. Lee.** 1992. Site-directed mutagenesis of the C-terminal portion of reovirus protein s1: evidence for a conformation-dependent receptor binding domain. Virology **186:**219–227.
41. **van Oostrum, J., and R. M. Burnett.** 1985. Molecular composition of the adenovirus type 2 virion. J. Virol. **56:**439–448.
42. **Wickham, T. J., E. J. Filardo, D. A. Cheresh, and G. R. Nemerow.** 1994. Integrin αvβ5 selectively promotes adenovirus mediated cell membrane permeabilization. J. Cell Biol. **127:**257–264.
43. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrin αvβ3 and αvβ5 promote adenovirus internalization but not virus attachment. Cell **73:**309–319.
44. **Xia, D., L. J. Henry, R. D. Gerard, and J. Deisenhofer.** 1994. Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution. Structure **2:**1259–1270.
45. **Xia, Y.-P., C.-T. Yeh, J.-H. Ou, and M. M. C. Lai.** 1992. Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex. J. Virol. **66:**914–921.