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Relative Accessibility of *N*-Acetylglucosamine in Trimers of the Adenovirus Types 2 and 5 Fiber Proteins

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Fiber is an adenovirus capsid protein responsible for virus attachment to the cell surface and contains O-linked *N*-acetylglucosamine (GlcNAc). Results of both amino acid analysis and Dionex chromatography indicated that 3 to 4 and 1.7 to 2.5 mol of GlcNAc are attached per mol of affinity-purified adenovirus type 2 (Ad2) and Ad5 fibers, respectively. Fiber shares an epitope with nuclear pore proteins containing O-linked GlcNAc, as shown by reactivity to monoclonal antibody RL2 directed against these pore proteins. GlcNAc on fiber was found to serve as an acceptor for the transfer of galactose from UDP-galactose by 4β-galactosyltransferase in Ad2 and Ad5 but not in Ad7; quantitation by labeling with UDP-[U-^{14}C]galactose in this reaction gave a 100-fold-lower estimate of the GlcNAc content of fiber, suggesting that these monosaccharides are buried within fiber trimers and are not accessible to the transferase. Affinity chromatography on lectin-bound Sepharose beads showed that Ad2 and Ad5 fibers bound weakly to wheat germ agglutinin and did not bind to ricin or concanavalin A; weak binding to wheat germ agglutinin suggests either that GlcNAc is not easily accessible or that there are not sufficient GlcNAcs for efficient binding. These data suggest that O-linked GlcNAc might be important for Ad2 and Ad5 fiber assembly or stabilization.

Proteins containing O-linked *N*-acetylglucosamine (O-GlcNAc) are found on proteins from a diverse group of biological systems such as viruses (1), schistosomes (23), rodents (8, 13, 25), insects (18), and humans (11, 16). These novel glycoproteins are localized predominantly in the nuclei and cytoplasm of cells (12). Unlike N-linked and most O-linked glycosylation events which occur within the lumen of the Golgi apparatus and the endoplasmic reticulum, recent evidence suggests that O-GlcNAc is added to these glycoproteins in the cytoplasm after they are synthesized on free polyribosomes (6, 7). Although little is known about the role that O-GlcNAc plays within a cell, suggested functions for this modification include (i) the signal for targeting of some proteins to the nucleus (16, 25), (ii) the proper assembly of multimeric complexes (5, 6, 8, 11, 13), (iii) the blocking of one or more regulatory (phosphorylation) sites in phosphoproteins (11, 16), (iv) the prevention of proteolysis (5, 6, 16, 25), and (v) in the case of SP1, the activation of transcriptional function (16).

Adenovirus is a nonenveloped virus containing a linear DNA genome. The icosahedral capsid of the virion is composed of three major proteins: hexon, penton, and fiber. These capsid proteins are synthesized in the cytoplasm of infected cells and are transported to the nucleus for incorporation into virions within minutes of synthesis (14). Fiber plays a number of important roles during adenovirus infection, such as serving as the initial binding site between the virion and cell surface receptors. Previous studies by Ishibashi and Maizel (15) demonstrated that adenovirus type 2 (Ad2) fiber could be labeled by using radioactive glucosamine and that this label could be removed by treatment with weak alkali; this observation suggested that fiber contained an O-linked amino sugar derivative (15). More recently, it has been shown that both Ad2 and Ad5 fibers have monosaccharide GlcNAc in an O-glycosidic linkage, but that fiber from Ad7 does not contain this modification (2).

In this study, both amino acid analysis and Dionex chromatography were used to show that the Ad2 and Ad5 fiber proteins contain 3 to 4 and 1.7 to 2.5 mol, respectively, of O-GlcNAc per mol of protein, whereas Ad7 lacks this modification. These GlcNAc moieties are recognized by a monoclonal antibody raised against nuclear pore proteins, suggesting that fiber may share an epitope with other cytoplasmic and nuclear GlcNAc-containing proteins. On the basis of inefficient labeling with galactosyltransferase and weak binding to wheat germ agglutinin (WGA), many of these GlcNAc monosaccharides appear be buried within the fiber structure. The lack of GlcNAc on Ad7 fiber suggests that the posttranslational glycosylation of this protein is not required for assembly or function of fibers from all serotypes. Nevertheless, the adenovirus fiber proteins from Ad2 and Ad5 may provide useful models for studies of novel cytoplasmic glycosylation pathways and for the role that glycosylation may play in assembly or stabilization of protein complexes.

MATERIALS AND METHODS

Purification of virion proteins from infected cells. HeLa cells (American Type Culture Collection, Rockville, Md.) were maintained in Dulbecco modified Eagle medium (purchased from Cellgro or from GIBCO Laboratories) with 5% fetal bovine serum (Hyclone, Logan, Utah), gentamicin (50 μg/ml), and nystatin (240 U/ml). HeLa cells at a density of 2 × 10^6 to 6 × 10^6 cells per ml in suspension culture were

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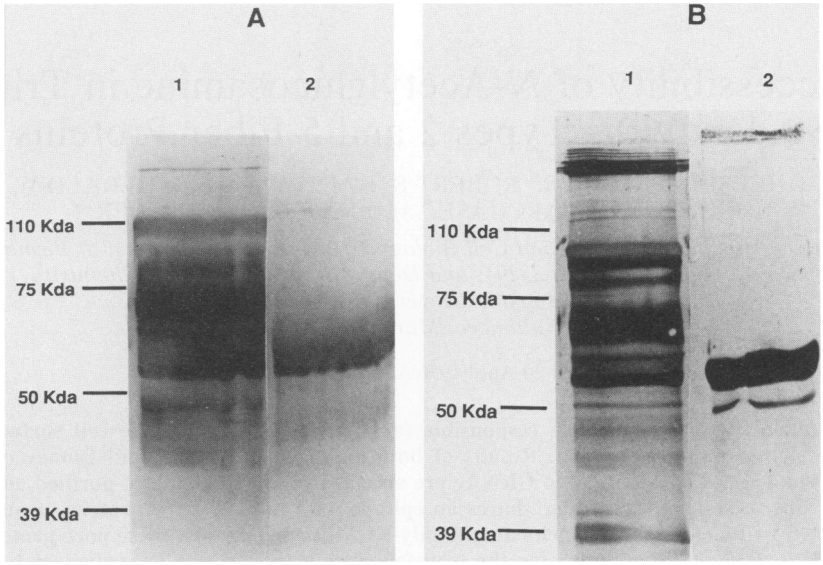

FIG. 1. Purification of adenovirus fiber on a monoclonal antibody affinity column. Monoclonal antibody 4D2-5 was coupled to CNBr-Sepharose 4B as described in Materials and Methods. Adenovirus-infected cell lysates were continuously cycled over the affinity column overnight at 4°C before elution. (A) Ad2-infected cell lysates; (B) Ad5-infected cell lysates. Lanes: 1, unfractionated cell lysate; 2, protein eluted from the column.

infected with Ad2 or Ad5 (American Type Culture Collection) and were harvested at 60 h postinfection to prepare virus seed (21). Soluble viral proteins and banded virions were purified by CsCl density gradient centrifugation.

Labeling of adenovirus-infected cell proteins with [^{3}H] leucine (100 μCi/ml; 142 Ci/mmol; Amersham) was accomplished by addition of label at the time of virus infection and incubation for 48 h before preparation of a cell lysate (21). Immunoprecipitation and analysis of virus proteins were carried out on samples as described by Harlow and Lane (10), using one of three different antibodies: (i) a polyclonal antibody directed against whole adenovirus (from the American Type Culture Collection), (ii) a monoclonal antibody (4D2-5) that recognizes the Ad2 and Ad5 fiber proteins (J. S. Hong, unpublished observation), or (iii) a monoclonal antibody specific for a GlcNAc-containing epitope found on nuclear pore proteins (RL2; 13, 26). Fractions of the immunoprecipitated cell lysate were analyzed by electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (20). The gels were stained (0.2% Coomassie blue–20% methanol–10% acetic acid), destained (20% methanol–10% acetic acid), impregnated with En^{3}Hance (New England Nuclear, Boston, Mass.), and visualized by autofluorography. Alternatively, Western immunoblots of virus protein samples were prepared and analyzed by standard techniques (10); Western blots were developed by using biotinylated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch or Bethesda Research Laboratories) and streptavidin-conjugated alkaline phosphatase (Southern Biotech Associates, Birmingham, Ala.).

Purification and quantitation of GlcNAc on fiber. Ad2 and Ad5 fibers were purified from infected-cell lysates by immunoaffinity chromatography on a column made by linking antifiber monoclonal antibody 4D2-5 to CNBr-activated Sepharose 4B beads. The bound fiber proteins were eluted from the column with 100 mM sodium acetate (pH 4.0)–200 mM NaCl; fractions were immediately neutralized by dropwise addition of a 2 M Tris hydrochloride solution (pH 8.1). Fractions containing fiber were desalted on a PD-10 column (Pharmacia). Samples were subjected to acid hydrolysis (6 N HCl for 110°C for 20 h) and quantitated for amino acid and amino sugar content by using the Pico-Tag system (Millipore/Waters). Norleucine was included as an internal standard to correct for physical losses during transfer. The data obtained were corrected for loss during acid hydrolysis by a factor that measures the recovery of known amounts of amino sugars.

Alternatively, 100 μg of affinity-purified fiber was precipitated with 10% trichloroacetic acid and subjected to acid hydrolysis (either 4 or 6 M HCl) for 5 h at 100°C under nitrogen; additional 100-μg samples of desalted fiber were also acid hydrolyzed in 6 M HCl under similar conditions. Percent recovery of GlcNAc in each experiment was determined by inclusion of a small amount of [^{3}H]glucosamine with the fiber sample. Amino sugars were isolated from the hydrolysate on Dowex AG 50W-X8 (H^+ form) as described by Spiro (27), quantitated on a Dionex chromatography system as described by Hardy et al. (9), and corrected for the percent recovery of GlcNAc.

Galactosylation reactions. Autogalactosylation reactions of the galactosyltransferase preparation were carried out as described previously (28), using 60 μM cold uridine diphospho-D-galactose (UDP-Gal; Boehringer Mannheim) and 9 μU 4β-galactosyltransferase (4.7 U/mg; Sigma Chemical) for 30 min at 37°C. Virions and viral proteins were galactosylated in a reaction mixture (25-μl final volume) consisting of 60 μM $MnCl_2$ and 200 mM sodium cacodylate (pH 6.8). The labeling reaction was initiated by the addition of 0.4 μCi of UDP-[U-^{14}C]Gal (257 mCi/mmol; Amersham), followed by 9 μU of the autogalactosylated bovine galactosyltransferase (containing 60 μM cold UDP-Gal carried over from the prior pregalactosylation of the enzyme); the reaction was carried out at 37°C for 30 min. The labeled sample was immunoprecipitated by using polyclonal or monoclonal antibodies directed against virions as described above. A fraction of this precipitate was suspended in cracking buffer (20) and ana-

TABLE 1. Quantitation of GlcNAc by amino acid analysis[a]

Amino acid (no.) or GlcNAc	mol expected	Determination			
		Expt 1		Expt 2	
		nmol observed	Calculated aa/molecule	nmol observed	Calculated aa/molecule
Ad2 fiber					
Ala	37	1.45	40	5.17	57
Asp (29) + Asn (47)	76	2.46	68	4.58	50
Glu (18) + Gln (18)	36	1.36	37	2.70	30
Gly	46	1.83	50	4.34	48
Phe	17	0.66	18	1.78	20
His	5	0.20	6	1.17	13
Ile	32	1.13	31	3.42	38
Lys	34	1.37	38	3.08	34
Leu	56	2.24	62	5.10	56
Met	12	0.17	5	1.04	11
Pro	34	1.27	35	3.09	34
Arg	10	0.44	12	1.00	11
Ser	62	2.07	57	5.24	58
Thr	68	2.41	66	6.23	69
Val	33	1.15	32	3.02	33
Tyr	17	0.60	17	1.26	14
Total	575	20.80	575	52.20	575
GlcNAc	?	0.11	3.0	0.28	3.1
Ad5 fiber					
Ala	44	1.11	50	5.72	52
Asp (25) + Asn	72	1.01	46	5.10	46
Glu (19) + Gln	38	0.69	31	3.42	31
Phe	19	0.44	20	2.18	20
Gly	48	1.20	54	6.26	57
His	7	0.35	16	1.55	14
Ile	24	0.63	28	3.08	28
Lys	30	0.74	34	3.68	33
Leu	70	1.63	74	8.11	73
Met	10	0.20	9	1.01	9
Pro	39	0.74	33	3.97	36
Arg	10	0.25	11	1.22	11
Ser	52	1.13	51	5.54	50
Thr	66	1.51	68	7.43	67
Val	31	0.84	38	4.09	37
Tyr	15	0.24	11	1.29	12
Total	575	12.71	575	63.62	575
GlcNAc	?	0.06	2.5	0.29	2.6

[a] Affinity-purified fiber was subjected to acid hydrolysis, and the contents of the resulting amino acids (aa) and amino sugar were determined by using the Pico-Tag system as described in Materials and Methods. In the case of Ad2, experiment 1, the fiber sample was blotted onto an Immobilon membrane (Dupont) before hydrolysis. The system used was capable of quantitating both galactosamine and glucosamine; no galactosamine was detected in any of the experiments.

lyzed by SDS-polyacrylamide electrophoresis (PAGE) as described above.

When used as a method for quantitation of GlcNAc, 15 μg of affinity-purified fiber was galactosylated to completion by addition of excess UDP-[U-^{14}C]Gal in vitro as described above. To ensure the completeness of the reaction, two controls were performed. (i) An additional 15 μg of affinity-purified fiber was added to an identical reaction after 1 h; after an additional 1-h incubation, twice as many counts were incorporated in immunoprecipitable fiber, indicating that there was sufficient enzyme and UDP-Gal to galactosylate all accessible acceptors in the initial 15-μg sample of fiber after the first 1 h incubation. (ii) The amount of ^{14}C label transferred to fiber from UDP-[U-^{14}C]Gal was the same after a 1- or a 2-h incubation at 37°C.

TABLE 2. Quantitation of amino sugar on fiber by Dionex chromatography[a]

Sample	Hydrolysis method	mol of GlcNAc/mol of fiber
Ad2 fiber		
Expt 1	TCA precip; 4 M HCl	3.6
Expt 2	TCA precip; 6 M HCl	5.4
Expt 3	Desalted sample; 6 M HCl	3.1
Avg		4.0
Ad5 fiber		
Expt 1	TCA precip; 4 M HCl	1.9
Expt 2	TCA precip; 6 M HCl	1.6
Expt 3	Desalted sample; 6 M HCl	1.7
Avg		1.7

[a] Affinity-purified fiber was trichloroacetic acid precipitated (TCA precip) and hydrolyzed with HCl as described in Materials and Methods; some samples were desalted rather than acid precipitated before acid hydrolysis. Samples were purified on Dowex 50 (proton form) and then analyzed and quantitated by Dionex chromatography.

Samples were analyzed by SDS-PAGE on a 10% gel, stained, and destained. The fiber-specific band was excised, solubilized by using SOLVABLE (Dupont), and counted in a scintillation counter to determine incorporated [^{14}C]galactose.

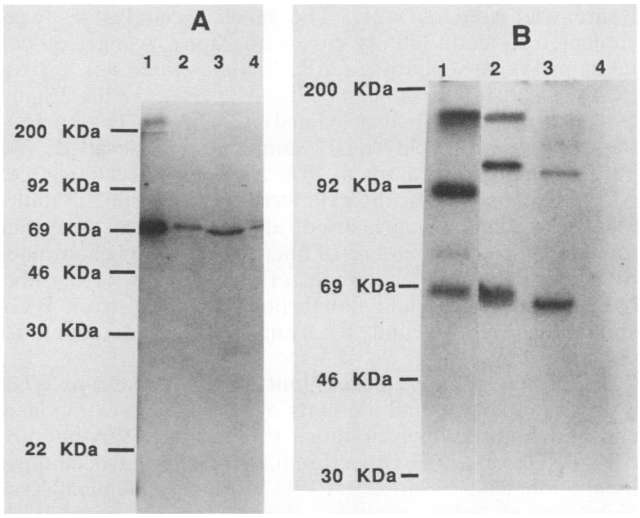

FIG. 2. Galactosylation in vitro of Ad2, Ad5, and Ad7 fibers. HeLa cells were infected with Ad2, Ad5, or Ad7 at 37°C; after 60 h of infection, purified virus and soluble viral proteins were purified as described in Materials and Methods. (A) Galactosylation of Ad2 and Ad5 purified virions or soluble protein pools with UDP-[^{14}C]Gal and bovine milk 4β-galactosyltransferase, followed by immunoprecipitation with monoclonal antibody 4D2-5. Lanes: 1, soluble Ad2 viral protein pool; 2, purified Ad2 virions; 3, soluble Ad5 viral protein pool; 4, purified Ad5 virions. (B) Galactosylation of cell lysates from Ad2, Ad5, and Ad7. Lane 1 contains [^{3}H]leucine-labeled Ad2-infected cell lysate and shows the positions expected for fiber, penton base, and hexon; lanes 2 through 4 are Ad2-, Ad5-, and Ad7-infected cell lysates, respectively, that have been ^{14}C labeled by galactosylation as in panel A, followed by immunoprecipitation with a serotype-specific polyclonal antibody. The identities of higher-molecular-weight bands in these gels are not known.

TABLE 3. Quantitation of amino sugars on fiber, using 4β-galactosyltransferase and UDP-[^{14}C]Gal to label terminal GlcNAcs[a]

Sample	pmol of fiber added	pmol of galactose added	Calculated mol of GlcNAc/molecule
Ad2 fiber			
Expt 1	246	5.0	0.02
Expt 2	246	5.3	0.02
Ad5 fiber			
Expt 1	242	1.8	0.007
Expt 2	242	3.3	0.014

[a] A 15-μg amount of affinity-purified fiber was galactosylated to completion with labeled UDP-Gal as described in Materials and Methods. After SDS-PAGE on a 10% gel, bands corresponding in size to fiber were cut from the gel, counted, and converted to picomoles galactose added per picomole of fiber.

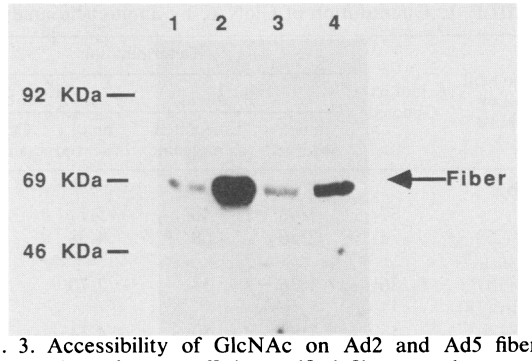

FIG. 3. Accessibility of GlcNAc on Ad2 and Ad5 fibers to 4β-galactosyltransferase. Affinity-purified fiber samples were disrupted with 4 M guanidine hydrochloride, followed by dialysis in deionized water and labeling with UDP-[U-^{14}C]galactose and 4β-galactosyltransferase, as described in Materials and Methods. Size markers are shown on the left; the arrow shows the position expected for fiber monomers. Lanes: 1, untreated Ad2 fiber; 2, denatured Ad2 fiber; 3, untreated Ad5 fiber; 4, denatured Ad5 fiber.

When used to measure relative accessibility of native and denatured protein, a sample of antibody-affinity purified fiber was denatured with 4 M guanidine hydrochloride for 15 min on ice and then dialyzed overnight in water at 4°C before the galactosyltransferase reaction. Equal amounts of native trimer and of denatured fiber were used in these experiments. Gel slices corresponding to fiber in each experiment were excised from the gel, and counts incorporated were determined as described above.

Lectin affinity chromatography. Adenovirus-infected HeLa cells were labeled with 100 μCi of [^{3}H]leucine per ml throughout a 48-h viral infection, after which time a cell lysate was prepared (21). The labeled cell lysates were subjected to lectin affinity chromatography, using 1-ml columns of WGA-Sepharose 6MB, *Ricinus communis* agarose (RCA) beads, and concanavalin A-agarose beads (Sigma Chemical) as described previously (22). After elution from the column with cold sugar, samples were desalted, lyophilized, and loaded on a 10% SDS-polyacrylamide gel. After electrophoresis, the gels were treated with En^{3}Hance (New England Nuclear), dried, and exposed to X-ray film for 7 days. Relative binding of fiber to WGA was determined by densitometric measurement of the intensity of the fiber band in the bound and flowthrough fractions from WGA shown in Fig. 4A and B, using a Bio-Rad model 620 densitometer.

To measure relative accessibility of the protein to WGA on immunoblots, equal amounts of native trimer (unboiled) and of denatured fiber monomer (boiled) were separated by SDS-PAGE on a 10% gel before blotting onto nitrocellulose, incubation with biotinylated WGA (Sigma Chemical), and development with alkaline phosphatase conjugated to streptavidin.

RESULTS

Quantitation of GlcNAc attached to fiber. Fiber protein was antibody affinity purified from lysates of Ad2- and Ad5-infected HeLa cells as described in Materials and Methods. The protein bound to the column was judged to be 99% pure by SDS-PAGE (Fig. 1). The purified fiber was then subjected to acid hydrolysis, and the products were analyzed by chromatography on a Waters Pico-Tag chromatography system; peak areas were quantitated relative to known standards and corrected for losses of each amino acid due to the acid hydrolysis step. The amount of most amino acids recovered agreed within 20% of the expected values calculated from the DNA sequence (4, 24). In these analyses, only GlcNAc was recovered as an amino sugar: when compared with the molar recovery of total amino acids (575 mol of recoverable amino acids per mol of fiber), 3 and 2.5 mol of GlcNAc were found attached to Ad2 and Ad5 fibers, respectively (Table 1).

As an alternative method of quantitation, known amounts of purified fiber were subjected to acid hydrolysis and the sugar composition was analyzed by using the Dionex chromatography system (9). This system allows the separation and identification of monosaccharides released from glycoproteins by acid hydrolysis. Averages of 4 and 1.7 mol of GlcNAc were found per mol of Ad2 and Ad5 fiber protein hydrolyzed, respectively (Table 2).

Fibers from Ad2 and Ad5 but not Ad7 contain terminal GlcNAc residues that serve as acceptors for the addition of galactose by galactosyltransferase and UDP-Gal. To confirm the identity of the saccharide detected by the in vivo labeling of fiber with [^{3}H]glucosamine (2, 15), virions and free virus protein pools from Ad2 and Ad5 were subjected to in vitro galactosylation, using bovine milk 4β-galactosyltransferase and UDP-[U-^{14}C]Gal. Only terminal GlcNAc residues can serve as an acceptor for UDP-Gal in the presence of this transferase, creating a Gal-GlcNAc moiety in a β-1,4 linkage (28). After galactosylation with UDP-[U-^{14}C]Gal, virion and protein pool fractions were immunoprecipitated with a monoclonal antibody directed against fiber and were analyzed by SDS-PAGE. The autoradiogram of the labeled fiber protein is shown in Fig. 2A. Both Ad2 and Ad5 virion and protein pools contained a radioactive band of 65 kDa, the expected molecular size of fiber. The presence of the radiolabeled bands indicated that Ad2 and Ad5 fiber contained terminal GlcNAc residues both in purified virions and in soluble protein pools. Treatment of infected cells with tunicamycin, a potent inhibitor of N-linked glycosylation pathways, did not affect the ability of fiber to act as an acceptor in the galactosyltransferase reaction (data not shown).

To determine whether GlcNAc is also found on fiber from other groups of adenovirus, the glycosylation of Ad7 fiber was also checked. Lysates from Ad2-, Ad5-, and Ad7-infected cells were subjected to in vitro galactosylation, again using bovine milk 4β-galactosyltransferase and UDP-[U-^{14}C]Gal. These galactosylated lysates were immunopre-

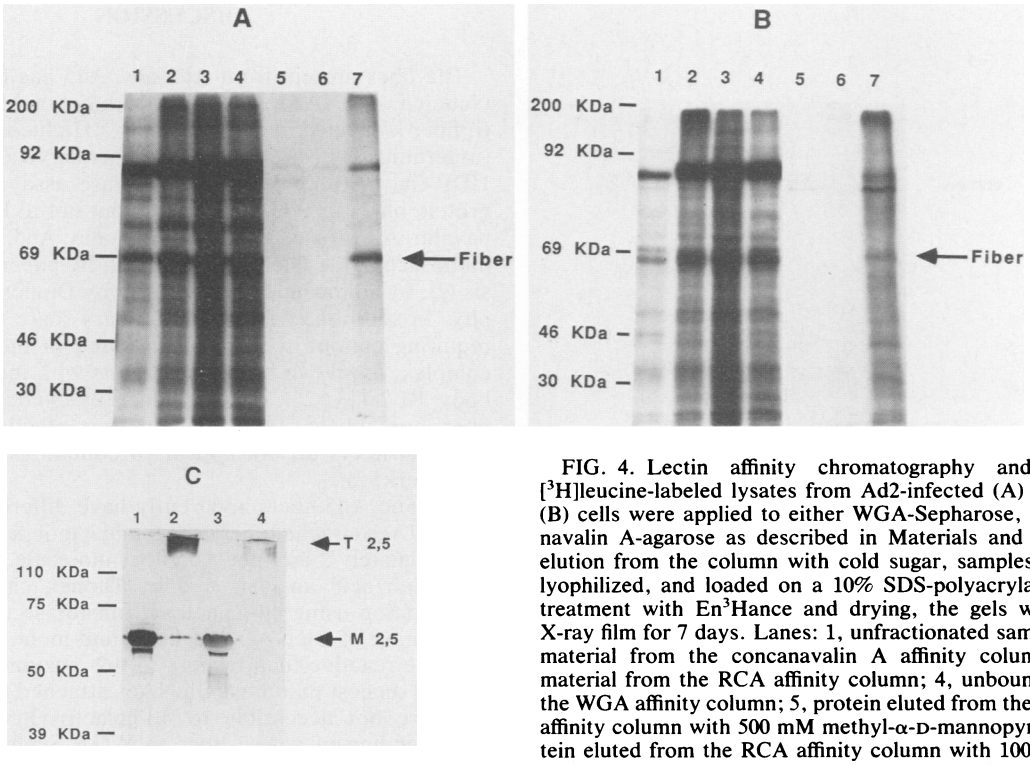

FIG. 4. Lectin affinity chromatography and immunoblots. [^{3}H]leucine-labeled lysates from Ad2-infected (A) or Ad5-infected (B) cells were applied to either WGA-Sepharose, RCA, or concanavalin A-agarose as described in Materials and Methods. After elution from the column with cold sugar, samples were desalted, lyophilized, and loaded on a 10% SDS-polyacrylamide gel. After treatment with En^{3}Hance and drying, the gels were exposed to X-ray film for 7 days. Lanes: 1, unfractionated sample; 2, unbound material from the concanavalin A affinity column; 3, unbound material from the RCA affinity column; 4, unbound material from the WGA affinity column; 5, protein eluted from the concanavalin A affinity column with 500 mM methyl-α-D-mannopyranoside; 6, protein eluted from the RCA affinity column with 100 mM lactose; 7, protein eluted from the WGA affinity column with 200 mM GlcNAc. Molecular size markers are shown on the left. (C) Western blot of affinity-purified Ad2 and Ad5 fibers developed with biotinylated WGA and streptavidin-alkaline phosphatase. Molecular size markers are shown on the left. Arrows show the positions expected for Ad2 and Ad5 fiber monomers (M 2,5) and trimers (T 2,5). Lanes: 1, boiled Ad2 fiber; 2, unboiled Ad2 fiber; 3, boiled Ad5 fiber; 4, unboiled Ad5 fiber.

cipitated with a polyclonal antibody directed against whole virus of the same serotype and were analyzed by SDS-PAGE; radioactive galactose was added onto fibers from both Ad2 and Ad5 but not from Ad7, as shown by the autoradiogram of the galactosylated fiber samples (Fig. 2B, lanes 2 to 4). The level of detectable GlcNAc in this technique depends both on the specific activity of the UDP-[U-^{14}C]Gal used and on the particular protein used; under optimal conditions, as little as 10 fmol of O-GlcNAc can be detected on a protein (R. S. Haltiwanger and G. W. Hart, unpublished observation).

Accessibility of GlcNAc in fiber multimers. In addition to the chemical analyses described above, the amount of GlcNAc attached to fiber was quantitated by in vitro labeling of known amounts of fiber with excess UDP-[^{14}C]galactose of known specific activity and bovine milk 4β-galactosyltransferase. Only about 0.02 and 0.01 mol of GlcNAc were observed per mol of Ad2 and Ad5 fiber proteins, respectively (Table 3). The fiber proteins used in these experiments were primarily trimers and were not denatured by the conditions of the galactosylation reaction; controls to measure the completeness of the reaction showed that both enzyme and UDP-Gal concentrations were in vast excess in these reactions. However, when denatured with guanidine hydrochloride before the reaction, both the Ad2 and Ad5 fibers were much better substrates for 4β-galactosyltransferase (Fig. 3); there were 18 and 3 times more counts incorporated in denatured than in native Ad2 and Ad5 fibers, respectively. This result suggests that most GlcNAc monosaccharides attached to the fiber trimer are not accessible to galactosyltransferase and may be buried within this protein complex. Even on denatured fibers, most O-GlcNAc acceptors appear to be undetectable. The 4β-galactosyltransferase enzyme normally adds galactose to longer N-linked oligosaccharides on proteins in the Golgi apparatus; since fiber contains GlcNAc monosaccharides, it may serve as a poorer substrate for this transferase because of nearby peptides that may limit accessibility of the enzyme to the sugar acceptor.

Lectin-binding analyses of terminal GlcNAc monosaccharides on fiber. WGA is a lectin that recognizes terminal GlcNAc residues. The efficiency of binding to WGA depends on the number of GlcNAc sugars and on their relative proximity (17); binding may also be affected by the relative accessibility of the GlcNAcs within a protein complex. Cell lysates prepared from HeLa cells infected with Ad2 or Ad5 labeled with [^{3}H]leucine were analyzed by lectin affinity chromatography on WGA-Sepharose. This lectin could bind [^{3}H]leucine-labeled fibers from Ad2 (Fig. 4A, lane 7) and Ad5 (Fig. 4B, lane 7), confirming the presence of terminal GlcNAc residues. The affinity of Ad5 fiber for WGA-Sepharose seemed to be lower than that for Ad2 fiber: approximately 20 and 10% of the ^{3}H-labeled Ad2 and Ad5 fibers, respectively, could be bound to WGA-Sepharose, as determined by densitometry of the fiber-specific bands in lanes 4 and 7 of Fig. 4A and B. Lectin affinity chromatography using RCA (specific for β-galactosides) and concanavalin A-agarose (which has high affinity for either the biantennary complex or the high-mannose oligosaccharides of N-linked glycoproteins [22]) was unable to bind Ad2 or Ad5 fiber in these experiments (Fig. 4A and B). These data support the conclusion that fibers from both Ad2 and Ad5 contain terminal GlcNAc residues but do not appear to contain galactose or mannose.

Earlier attempts to show the glycosylation of fiber by

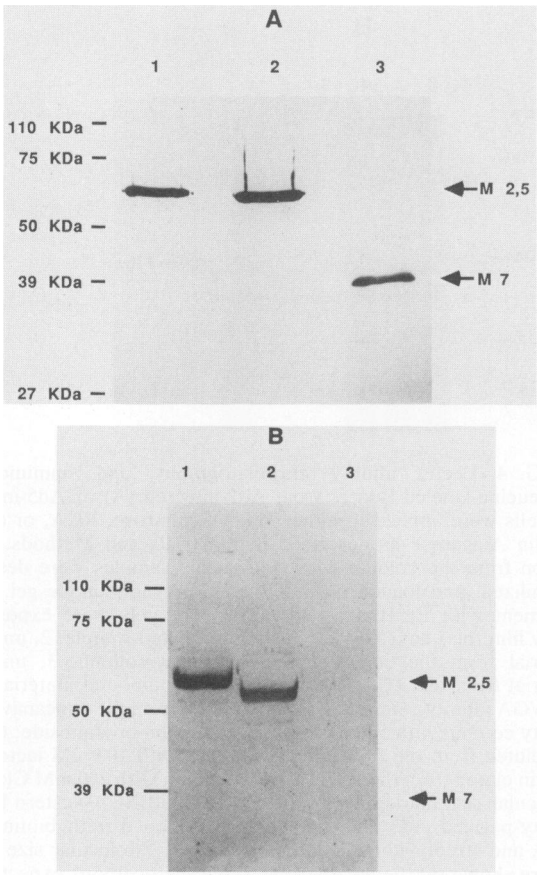

FIG. 5. Western blot of fiber with 4D2-5 and RL2 antibodies. Adenovirus-infected cell lysates were resolved by SDS-PAGE on a 10% gel, blotted onto nitrocellulose, and developed as described in Materials and Methods. Arrows show the positions expected for Ad2 and Ad5 fiber monomers (M 2,5) and Ad7 fiber monomers (M 7). (A) Blot developed with monoclonal antibody 4D2-5 directed against fiber protein; (B) blot developed with monoclonal antibody RL2, which recognizes an epitope containing O-GlcNAc. The apparent difference in mobility of Ad2 and Ad5 fiber in this gel is an artifact of the electrophoresis conditions. Lanes: 1, Ad2-infected cell lysate; 2, Ad5-infected cell lysate; 3, Ad7-infected cell lysate.

using a lectin affinity blotting technique gave mixed results: GlcNAc could be detected on Ad2 fiber but not on Ad5 fiber (2). When Western blots with affinity-purified fiber were probed with biotinylated WGA and a streptavidin-alkaline phosphatase conjugate, both trimers (in unboiled samples) and monomers (in boiled samples) of Ad2 and Ad5 fibers could be detected (Fig. 4C). Consistent with the results obtained by using 4β-galactosyltransferase, boiling the fiber samples to denature trimers before separation by SDS-PAGE and blotting resulted in better reactivity to the lectin probe, suggesting that GlcNAc monosaccharides in trimers are less accessible to WGA.

Fiber shares an epitope with proteins in the nuclear pore complex. A monoclonal antibody that recognizes an O-GlcNAc-containing epitope on proteins of the nuclear pore complex (26) was used to develop Western blots containing Ad2, Ad5, and Ad7 fiber proteins. Both Ad2 and Ad5 fibers were recognized by this monoclonal antibody, but Ad7 fiber was not (Fig. 5B). Identical Western blots developed with antibody 4D2-5 show that all lanes contained approximately the same amount of fiber (Fig. 5A).

DISCUSSION

The fiber protein from Ad2 and Ad5 has been shown to contain O-GlcNAc residues by a number of different criteria: (i) fiber is labeled in vivo by using [^{3}H]glucosamine (2, 15), (ii) terminal GlcNAc residues on fiber serve as acceptors for UDP-Gal in the galactosyltransferase assay, (iii) the fiber protein binds to WGA-Sepharose but not to RCA or concanavalin A-agarose, and (iv) Ad2 and Ad5 fibers contain monosaccharide GlcNAc, as shown by paper electrophoresis (2), by amino acid analysis, and by Dionex chromatography. In addition, Ad2 and Ad5 fibers share an O-GlcNAc-requiring epitope with proteins found in the nuclear pore complex, as shown by Western blots with monoclonal antibody RL2 (13, 26). The presence of O-GlcNAc on these fibers probably has little effect on virus infectivity, since Ad7 and Ad4 fibers do not appear to contain this modification (this work; 2).

Ad2 and Ad5 fibers apparently have different numbers of O-GlcNAcs attached: between 3 and 4 mol per Ad2 fiber and approximately 2 mol per Ad5 fiber molecule, as determined by amino acid analysis and by Dionex chromatography. Quantitation using 4β-galactosyltransferase underestimated the number of GlcNAcs per fiber, and monomers appear to be more reactive than trimers to this enzyme; these observations suggest that most GlcNAcs attached to undenatured fiber are not accessible to 4β-galactosyltransferase. The weak binding of native fiber to WGA-Sepharose (both on columns and on immunoblots) also supports this possibility; when completely denatured by boiling, fiber was more reactive on Western blots. One possible explanation is that GlcNAc plays a role in the assembly or stabilization of Ad2 and Ad5 fiber trimers and that once assembled, each attached monosaccharide is buried within the structure of the trimer. Since fibers from some other adenovirus serotypes (such as Ad7) lack GlcNAc (2; this work), fiber trimers from other serotypes may have alternate means for stabilization of their tertiary structures and do not require this amino sugar for their functions in viral infection. Experiments to further define the potential role of GlcNAc in formation of Ad2 and Ad5 fiber trimers are under way.

The adenovirus fiber protein belongs to a class of proteins containing O-GlcNAc monosaccharides. Interestingly, these GlcNAc-containing proteins are localized predominantly in the nuclei and the cytoplasm of cells and are especially prevalent among the proteins of the nuclear envelope (12). The function of this O-GlcNAc-containing domain recognized by RL2 and shared by fiber and by nuclear pore proteins is not clear.

Viruses such as Sindbis virus and vesicular stomatitis virus have often been used as models to elucidate glycosylation events within the lumen of the endoplasmic reticulum and the Golgi apparatus (19). In a similar way, adenovirus fiber may serve as a model system for studying this novel cytoplasmic glycosylation pathway. For example, others have suggested that O-GlcNAc may play a role in the transport of some glycoproteins to the nucleus (16; M. Schindler, J. Cell Biol. **99**:99a, 1984); this possibility can now be tested by using fiber, since a nonglycosylated temperature-sensitive mutant of Ad5 fiber (H5*ts*142) has been described (3). In addition, it is not clear how many sites on fiber may have GlcNAc attached; it is possible that there are many sites that are partially occupied, a few sites that are completely modified, or a combination of these two possibilities. Adenovirus fiber may also be a useful substrate for

further studies of the transferases responsible for these glycosylation reactions.

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