JOURNAL OF VIROLOGY, Aug. 1994, p. 5239–5246
0022-538X/94/$04.00+0

Vol. 68, No. 8

Characterization of the Knob Domain of the Adenovirus Type 5 Fiber Protein Expressed in *Escherichia coli*

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Received 21 January 1994/Accepted 5 May 1994

The adenovirus fiber protein is used for attachment of the virus to a specific receptor on the cell surface. Structurally, the protein consists of a long, thin shaft that protrudes from the vertex of the virus capsid and terminates in a globular domain termed the knob. To verify that the knob is the domain which interacts with the cellular receptor, we have cloned and expressed the knob from adenovirus type 5 together with a single repeat of the shaft in *Escherichia coli*. The protein was purified by conventional chromatography and functionally characterized for its interaction with the adenovirus receptor. The recombinant knob domain bound about 4,700 sites per HeLa cell with an affinity of 3×10^9 M^{-1} and blocked adenovirus infection of human cells. Antibodies raised against the knob also blocked virus infection. By gel filtration and X-ray diffraction analysis of protein crystals, the knob was shown to consist of a homotrimer of 21-kDa subunits. The results confirm that the trimeric knob is the ligand for attachment to the adenovirus receptor.

Human adenovirus is an icosahedral, nonenveloped, double-stranded DNA virus. Although primarily trophic for human respiratory epithelium, adenovirus type 2 (Ad2) and Ad5 of subgroup C promiscuously infect a broad range of mammalian cell types, presumably reflecting essentially ubiquitous expression of a specific cell surface receptor. This has led to a renewed interest in the use of recombinant adenovirus vectors for gene transfer into somatic cells both in vitro and in vivo. However, relatively little is currently known about the molecular basis of adenovirus infectivity. The first step in infection of mammalian cells by adenovirus is the recognition and attachment of virions to cell surface receptors in a specific and saturable manner. Ad2 and Ad5 apparently utilize the same receptor protein, although there is a different receptor for the subgroup B viruses, Ad3 and Ad7 (3). Previous studies of the virus-cell interaction have primarily used the affinity of the intact virus or its attachment protein for the detection of receptor binding. The affinity of the virus attachment protein for the receptor has also been used as an affinity purification step during attempts to purify the receptor (9, 18, 28). The receptor has been identified as a cell surface glycoprotein of variable molecular weight. Polypeptides with affinity for adenovirus fiber with M_rs of 42,000 (28), 78,000, 42,000, 34,000 (9), and 75,000 (18) have been reported. Despite these numerous studies, the identification of the adenovirus receptor protein remains an active area of research.

Recently, integrins have been implicated in the internalization of adenovirus, although these molecules are not initially involved in the specific attachment of the virus to the cell surface (7, 31). The penton base protein, which associates with fiber and forms the vertex of the icosahedral virus capsid, appears to interact with the integrin via an Arg-Gly-Asp (RGD) peptide motif that is found in a number of cell matrix molecules and is responsible for cell adhesion (1, 31). How the integrin and primary virus receptor are associated on the cell surface remains unknown.

Adenovirus binds to its cell surface receptor via the fiber protein component of its capsid, since purified fiber specifically blocks virion attachment to cells (21). Further, although the fiber protein is nonessential for virion assembly, fiber-deficient capsids are only slowly adsorbed to cells, suggesting that the binding process is dependent on fiber (6). Structurally, the adenovirus fiber protein has been proposed to consist of three domains: an NH_2-terminal tail which associates with the penton protein; a 15-residue motif repeated six times in Ad3 (26) or 22 times in Ad2 and Ad5 (8) that constitutes the shaft of the fiber; and a COOH-terminal globular domain, termed the knob, which has been assumed to be the ligand for binding to the adenovirus receptor (21). This structure is supported by both electron microscopy and X-ray crystallography (4). The subunit structure of the fiber protein has been variously proposed as a dimer (8) or trimer (23, 27) on the basis of structural models and limited structural data from crystals. However, the best available evidence is based on the polypeptide composition of purified virions and suggests that it is a homotrimer (29).

To distinguish between these two alternatives and to verify that the knob is the domain which interacts with the cellular receptor, we have cloned and expressed the knob domain together with a single repeat of the shaft (henceforth called knob) in *Escherichia coli*. The protein has been purified, and we have functionally characterized the protein for its interaction with the adenovirus receptor. The recombinant knob domain binds about 4,700 sites per HeLa cell with an affinity of 3×10^9 M^{-1} and is capable of blocking adenovirus infection of human cells. By gel filtration and X-ray diffraction analysis of protein crystals, the knob consists of a homotrimer of 21-kDa subunits.

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MATERIALS AND METHODS

Expression and purification of knob. Cloning of the knob domain was accomplished by PCR amplification using cloned Ad5 DNA (pJM17 [15]) as the template and specific oligonucleotides designed to facilitate the insertion of the PCR product directly into a bacterial expression vector. The sequences of the oligonucleotides used were CCCGAATTC TATGGGTGCCATTACAGTAGGAAA (5′ oligonucleotide) and CCCAAGCTTATTCTTGGGCAATGTATGA (3′ oligonucleotide), which are partially complementary to specific Ad5 fiber sequences (2). The positions of the ATG start and TAA stop codons within the oligonucleotides are underlined. Following the ATG start codon, the 5′ oligonucleotide overlaps those sequences encoding GAITVGN, which corresponds to the first seven residues of the 22nd repeating motif proposed by Green et al. (8) for the shaft of the fiber protein (see Fig. 1). The 3′ oligonucleotide is complementary to the last six residues of the knob domain, SYIAQE. Following amplification, the PCR product of approximately 600 bp was phenol extracted, phenol-chloroform extracted, and ethanol precipitated prior to digestion with *Eco*RI and *Hin*dIII and directional ligation into *Eco*RI-*Hin*dIII-digested pBEVpL. pBEVpL is a bacterial expression vector which contains a *trpE* promoter upstream of a pUC19 polylinker from *Eco*RI to *Hin*dIII in a pBR-derived plasmid backbone. *E. coli* TG-1 cells were used as the host strain. A clone containing the appropriate recombinant plasmid was identified by restriction enzyme digestion done according to standard techniques (24). Expression of the knob from this vector was induced by growth in modified M9 medium for 20 to 24 h with tryptophan starvation in the late stages of growth, as previously described (25).

Extraction of the knob domain from *E. coli* as a soluble protein was accomplished by lysozyme treatment of the bacteria and subsequent additions of NaCl to 0.2 M and the nonionic detergent Nonidet P-40 to 0.5%, as previously described (25). Viscosity due to chromosomal DNA release was reduced by DNase I digestion in the presence of 2 mM $MnCl_2$, and the cell wall debris was removed by centrifugation at 13,000 × *g* for 20 min. The crude extract was then batch absorbed with DEAE-Sephadex equilibrated with 0.2 M NaCl–50 mM Tris-HCl (pH 7.7)–1 mM EDTA to remove most of the DNA and a few proteins. Ammonium sulfate precipitation (at 100% saturation) removed a few proteins and was also used to concentrate the material. Precipitated proteins were redissolved in 20 mM Tris-HCl (pH 8.0)–1 mM EDTA and dialyzed against that buffer overnight at 4°C. The protein was adsorbed to a Q-Sepharose FF fast protein liquid chromatography (FPLC) column (2.5 × 27 cm) equilibrated in the same buffer. Development of the column with a 0 to 80 mM NaCl gradient (550 ml) in the same buffer eluted the knob at approximately 25 mM NaCl.

Gel filtration of purified knob. Sizing of the knob protein was performed by gel filtration on a Superdex 75 HR10/30 FPLC column under nondenaturing conditions. A mixture of approximately 1 mg of knob and 1 mg each of bovine serum albumin (BSA) (M_r, 67,000), chicken ovalbumin (M_r, 45,000), and lysozyme (M_r, 14,300) in 0.5 ml of 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–100 mM NaCl was applied to the column. Fractions of 0.5 ml were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels with Coomassie blue staining. The gel was photographed with type 55 Polaroid film. Image-based computer analysis was performed on the negative with a Foto-Analyst II workstation and the Collage software package (Fotodyne) to quantify the amount of each protein present in individual fractions.

Knob blocking of adenovirus infection. Two types of assay were performed to measure the ability of the purified knob protein to bind to cell membranes and inhibit adenovirus infection. The first involved measurement of adenoviral infectivity by plaque assay. Monolayers of adenovirus-transformed human 293 cells grown on 60-mm-diameter dishes were preincubated for 15 min at room temperature with 0.5-ml samples of knob or other ligand diluted in Dulbecco's Minimal Essential Medium (DMEM; GIBCO-BRL) containing 2% fetal bovine serum (FBS) (Hyclone) to allow ligand binding to cell membranes. The irrelevant ligands consist of the fusion of glutathione *S*-transferase (GST) with the 39-kDa receptor-associated protein (GST-39K RAP) and GST alone. They were prepared as described previously (11) and were kindly provided by Joachim Herz. Cells were subsequently infected by adding approximately 400 PFU of adenovirus (titers were determined by binding for 1 h at 37°C in a plaque assay) in DMEM–2% FBS to the plates. The cell monolayers were incubated at room temperature for 30 min to allow virus adsorption. The medium was aspirated, and the monolayers were washed once with DMEM–2% FBS to remove unabsorbed virus and then overlaid with 6 ml of a 1:1 mixture of 1.3% Noble agar (Difco) and 2× MEM (GIBCO-BRL) supplemented with 4% FBS. After incubation at 37°C for 7 to 10 days, the extent of inhibition of viral infectivity was determined by counting viral plaques.

The second method of assessing viral infectivity involved infection of cells with an adenovirus vector that expresses firefly luciferase from the cytomegalovirus (CMV) promoter (AdCMVLuc [10]). The expression of luciferase activity in cultured cells infected with this virus is directly proportional to the number of infecting virus particles, as previously reported for adenovirus expressing luciferase from the mouse mammary tumor virus promoter (16). HeLa cell monolayers on a 24-well plate were washed once with phosphate-buffered saline (PBS) and preincubated with 0.25-ml samples of knob or irrelevant ligand diluted in PBS for 10 min at room temperature to allow receptor binding. After ligand binding, cells were infected by the addition of approximately 10^6 PFU of AdCMVLuc diluted in 0.25 ml of DMEM–4% FBS to the wells and incubation at room temperature for 30 min. Monolayers were washed once with DMEM–2% FBS and incubated with 1 ml of DMEM–2% FBS containing 50 μg of penicillin per ml and 10 μg of streptomycin per ml for 48 h at 37°C to allow luciferase expression. Cells were lysed, and soluble extracts were assayed for expressed luciferase activity as previously described (5).

Knob binding to cell monolayers. Iodination of knob was accomplished by incubating 500 μg of purified knob, 50 μg of Iodogen, and 500 μCi of ^{125}I for 20 min at room temperature. The labelling reaction mixture was subsequently passed over Bio-Gel P-10 to remove unincorporated iodine. The specific activity ranged from 500 to 800 cpm per ng of protein.

For the receptor-binding assay, HeLa cells grown on a 24-well plate were washed once with PBS. Two wells were trypsinized and counted with a hemocytometer in order to determine the total number of cells per well. The remaining wells were fixed for 10 min at room temperature with 0.5 ml of 1% formaldehyde diluted in PBS and then washed three times with PBS. Fixation with 0.25% glutaraldehyde has also been used with equivalent results, as previously reported (20). Monolayers were incubated at room temperature for 1 h with 0.25 ml of ^{125}I-knob diluted in DMEM supplemented with 0.5% FBS, washed three times with PBS, and solubilized at 37°C for 5 min in PBS containing 1% SDS. Samples were

counted in an LKB Wallac Compugamma counter to determine the quantity of bound ligand. The affinity of the knob for the receptor sites on the cell was derived from the slope of the Scatchard plot, with the number of binding sites per cell given by the x intercept. Nonspecific binding was determined by incubation with a 100-fold excess of cold ligand and was subtracted from the total binding at each concentration.

Preparation and purification of rabbit antibodies to knob. Female New Zealand White rabbits were immunized with purified knob protein on days 1, 28, 57, and 85 until high-titer antisera were obtained. Rabbits were injected with 100 μg of knob in Freund's complete adjuvant for the first injection and with 50 μg in Freund's incomplete adjuvant for each booster injection. Serum from the bleeding on day 96 was used in the experiments reported herein. Preimmune serum was collected prior to the first injection for use as a negative control. Immunoglobulin G (IgG) fractions of serum were prepared by precipitation with 18% sodium sulfate and protein A-Sepharose affinity chromatography using the MAPS buffer system (Bio-Rad, Inc.). In some instances, the antibody was further purified by affinity chromatography on a knob-AffiGel 10-agarose column prepared by coupling purified knob (10 mg/ml) to the activated resin (Bio-Rad). Elution of the bound antibody was accomplished with 5 M guanidine hydrochloride in PBS. Purified IgGs were exhaustively dialyzed against PBS prior to use.

Antibody blocking of adenovirus infection. Knob antibody was incubated with 10^6 PFU of AdCMVLuc in DMEM–2% FBS for 1 h at room temperature in a total volume of 0.25 ml. These samples were then added to HeLa cell monolayers on a 24-well plate previously washed with DMEM–2% FBS and incubated for 30 min at room temperature. The monolayers were washed once and incubated with 1 ml of DMEM–2% FBS. Cells were incubated for 48 h at 37°C for luciferase expression, after which cells were lysed and extract was assayed for luciferase activity as previously described (5).

Crystallization of knob. The solution of knob protein in 25 mM NaCl–20 mM Tris-HCl (pH 8.0)–1 mM EDTA was concentrated with an Amicon Centricon-10 concentration device at 4°C to a final protein concentration of 7 to 10 mg/ml measured by using Bio-Rad protein assay reagent with BSA as the protein standard. Crystallization trials were carried out by the hanging-drop vapor diffusion method (17). The 0.5 ml of reservoir solution consisted of 2% polyethylene glycol 400, 30% ammonium sulfate, and 100 mM imidazole at pH 7.5, over which a hanging drop was made with 3 μl of knob protein solution and 3 μl of the reservoir solution. This condition often produced numerous microcrystals at room temperature within 24 h. Furthermore, many different crystal forms were also observed under this condition. Single large crystals with cubic morphology were obtained by using a seeding technique (17). Microcrystal seeds were diluted at least 10^8 times before introduction into a freshly made hanging drop.

X-ray diffraction analysis of the knob crystal. The knob crystals were subjected to X-ray diffraction analysis using CuKα radiation from a Rigaku Rotaflex rotating anode X-ray generator at 50 kV and 100 mA. Still diffraction photographs of a crystal, aligned roughly along one of the crystal axes with a crystal-to-film distance of 100 mm, were taken. Diffraction data were collected with both a Xuong-Hamlin multiwire area detector system and a Rigaku R-AXIS II image plate system. With the Xuong-Hamlin system, the distances between the crystal and the two detectors were 615 and 555 mm, respectively. Detectors were positioned at 2Θ angles of 15 and 25°, respectively. The rotation angle for each data frame was 0.1°, with 60 s of exposure time. With the R-AXIS system, the crystal-to-image plate distance was set to 80 mm with an oscillation angle of 3° for each image. The Xuong-Hamlin detector data were processed with the XDS program package (12, 13), and the R-AXIS data were processed with the R-AXIS oscillation film-processing package (22).

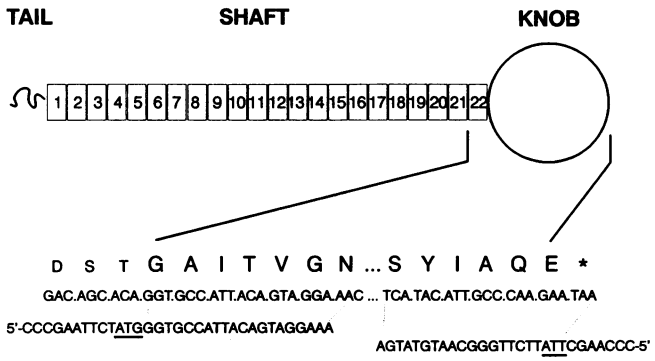


FIG. 1. Diagrammatic representation of the Ad5 fiber protein indicating the various structural domains. The protein is divided into the tail, shaft, and knob domains. Each of the 22 repeating motifs of the shaft is indicated by a numbered box. The region of the protein cloned by PCR and expressed in *E. coli* has been expanded, and the amino acid sequence is shown in one-letter code from the beginning of the 22nd repeat (GAITVGN) through the C terminus (SYIAQE), with the corresponding nucleotide sequence given immediately below. The sequences of the two oligonucleotide primers used for PCR are shown on the bottom line, with the ATG initiation and TAA termination codons underlined.

RESULTS

Cloning, expression, and purification of the knob domain. PCR amplification of Ad5 DNA yielded a fragment of approximately 600 bp which corresponds to 181 residues of the knob domain and a single 15-residue repeat of the shaft of the fiber protein (Fig. 1). This region was chosen for heterologous expression for two reasons. First, we wanted to express the knob domain and use it in functional assays to verify its proposed role as the ligand for adenovirus attachment to the cell surface receptor. Second, we wanted to express at least one of the 15-residue β-repeat motifs that constitute the shaft domain for structural reasons. Since the repeats might be necessary for proper folding of the subunits of the fiber into a trimer, we reasoned that inclusion of at least one repeat might be sufficient for this purpose. Additionally, we hoped that crystallization and X-ray structure determination of the knob would be possible. If the inclusion of one repeat was sufficient for proper folding of the shaft, then this structure might be amenable to X-ray diffraction analysis.

The DNA fragment was directionally cloned into a bacterial expression vector containing the *trpE* promoter. Upon amino acid starvation of the bacterial culture in the later stages of growth in minimal medium, the knob domain was efficiently expressed in *E. coli* and was readily extracted as a soluble protein. As Fig. 2 shows, knob is visualized as a 21-kDa polypeptide on denaturing SDS-PAGE, which agrees with the predicted molecular mass of 21,279 Da based on the protein sequence. This band represents the monomer species of knob and is the most prominent band in the soluble protein extract from the bacteria. No such polypeptide was observed in extracts from untransformed *E. coli* TG-1 cells grown under the same conditions. High-level expression of knob (10 mg/liter) was routinely observed.

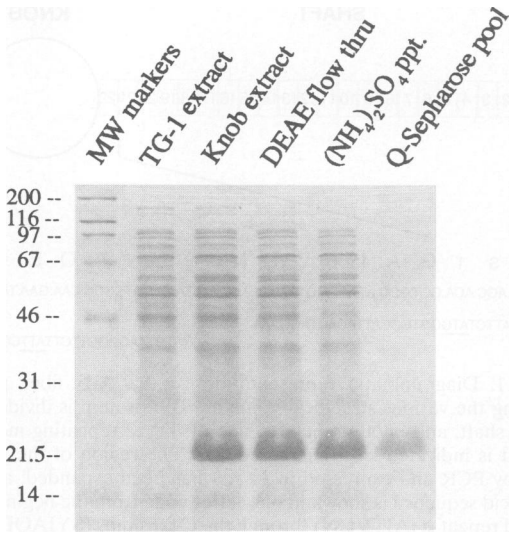


FIG. 2. SDS-polyacrylamide gel analysis of steps in the purification of the bacterially expressed knob domain. TG-1 extract corresponds to host cells not transformed with the expression vector. The final purified product from Q-Sepharose ion exchange is shown on the right. MW, molecular weight; ppt., precipitate.

A relatively simple purification procedure was devised for the expressed knob. A batch absorption step was first performed with the extract to remove DNA fragments. Under the ionic conditions used (0.2 M NaCl), knob was not bound to the matrix. Solid ammonium sulfate was added to 100% saturation to precipitate the knob. After the precipitate had been redissolved in Q-Sepharose starting buffer, the knob was dialyzed against the same buffer and clarified by centrifugation before the ion-exchange chromatography step. A shallow NaCl gradient eluted the knob in a single peak that was pooled and concentrated with a Centricon-10 device. The purified protein was electrophoretically pure as judged by SDS-PAGE and Coomassie blue staining (Fig. 2).

Gel filtration of purified knob on a Superdex 75 column was performed in order to estimate the molecular weight of the knob domain in solution by comparison to the purified protein standards BSA, ovalbumin, and lysozyme. SDS-PAGE was performed with individual fractions across the elution profile to determine the precise elution volume of the individual protein species. The Coomassie blue-stained gel is shown in the inset of Fig. 3 (top). Densitometric analysis of a photograph of the gel was performed and is shown in Fig. 3 (bottom). This analysis demonstrates that all four proteins are resolved as distinct peaks with a normal Gaussian distribution, even though the individual proteins are not completely separated from one another on the column. By comparison to the protein standards, the peak of the knob protein (fractions 21 and 22) elutes between the BSA (fraction 20) and ovalbumin (fractions 22 and 23) peaks, i.e., between the 67- and 45-kDa proteins. This is consistent with a molecular mass of approximately 60 kDa. Since the monomer is 21 kDa by SDS-PAGE, this suggests that the knob protein most likely exists in solution as a trimer of 21-kDa subunits rather than as a dimer.

Functional analysis of knob binding to cell membranes. Functional studies of the knob were performed to verify that it is the ligand responsible for adenovirus binding to the cellular receptor. These assays involve preincubation of cell monolayers with various concentrations of purified ligand and subsequent infection of the monolayer with adenovirus. A decrease in adenovirus binding and internalization would be expected if the adenovirus receptor were already occupied by ligand. Such a decrease in adenovirus infectivity is readily quantifiable by two types of infectivity assay. With cells in which the virus is able to grow lytically, a plaque assay can be performed in which the number of infecting adenovirus particles (PFU) is directly counted. The results of such an assay performed with a recombinant adenovirus vector to infect human 293 cells are shown in Fig. 4A. Preincubation of the monolayer of 293 cells with recombinant knob domain blocked adenovirus infection by over 90%. This block was dependent on the concentration of knob in the medium, with half-maximal inhibition of infectivity occurring at a concentration of about 0.04 μg/ml. In contrast, the irrelevant ligands, recombinant GST and GST-39K RAP, showed no effect on adenovirus infectivity, and this indicates that the binding of knob to the adenovirus receptor on the cell surface is specific. These results also suggest that the adenovirus receptor and the LRP/α2-macroglobulin receptor (known to bind GST-39K RAP with high affinity [11]) are distinct proteins.

Alternatively, with cells infected by a recombinant adenovirus vector harboring a gene encoding the firefly luciferase driven from the CMV immediate-early promoter, the number of infecting adenoviruses can be measured indirectly by quantitation of expressed luciferase enzyme activity. With human HeLa cells preincubated with knob to allow receptor binding, inhibition of AdCMVLuc infectivity was also observed (Fig. 4B). Again, the inhibition was dose dependent with half-maximal inhibition of infectivity occurring at 0.1 μg/ml, and the irrelevant ligand, GST protein, showed no effect on adenovirus infectivity.

Knob binding to HeLa cell monolayers was quantified by Scatchard analysis (Fig. 4C). The results demonstrate approximately 4,700 high-affinity binding sites per HeLa cell. Calculation of the apparent association constant (K_{ass}) on a per-receptor basis was as follows. There were 4.7×10^3 sites per cell in a 0.25-ml binding volume containing 1.6×10^6 cells; that is, 3.0×10^{13} sites per liter, of which 15% were occupied (from the y intercept of the Scatchard plot), or 4.5×10^{12} sites per liter. Dividing by Avogadro's number gives a K_{ass} of 3×10^9 M^{-1}.

Antibodies to knob. Purified knob was used to immunize rabbits in order to generate antiknob polyclonal antibodies for use in biochemical assays. Purified IgG was prepared from rabbit serum by protein A-Sepharose affinity chromatography. The antibody specifically recognized the bacterially expressed knob domain on Western blots (immunoblots), and no bands were immunoblotted in extracts prepared from untransformed TG-1 cells (data not shown).

The rabbit antibody was further purified by binding to a knob affinity column prepared by coupling purified knob to AffiGel 10 resin. This antibody was capable of efficiently neutralizing adenovirus infectivity when premixed with virus prior to infection of HeLa cell monolayers (Fig. 5). This effect was dose dependent, with half-maximal inhibition occurring at a concentration of 1 μg of IgG per ml. Control IgG did not block adenovirus infection of HeLa cells at any concentration used. These results demonstrate that antibody binding to the knob domain of fiber can selectively block virus attachment to mammalian cells.

Crystallization of the knob and preliminary X-ray diffraction analysis. Crystallization of the knob and preliminary X-ray diffraction analysis were performed as a prelude to determination of the tertiary structure. The knob protein at a concentration of 7 mg/ml was easily precipitated with 30%

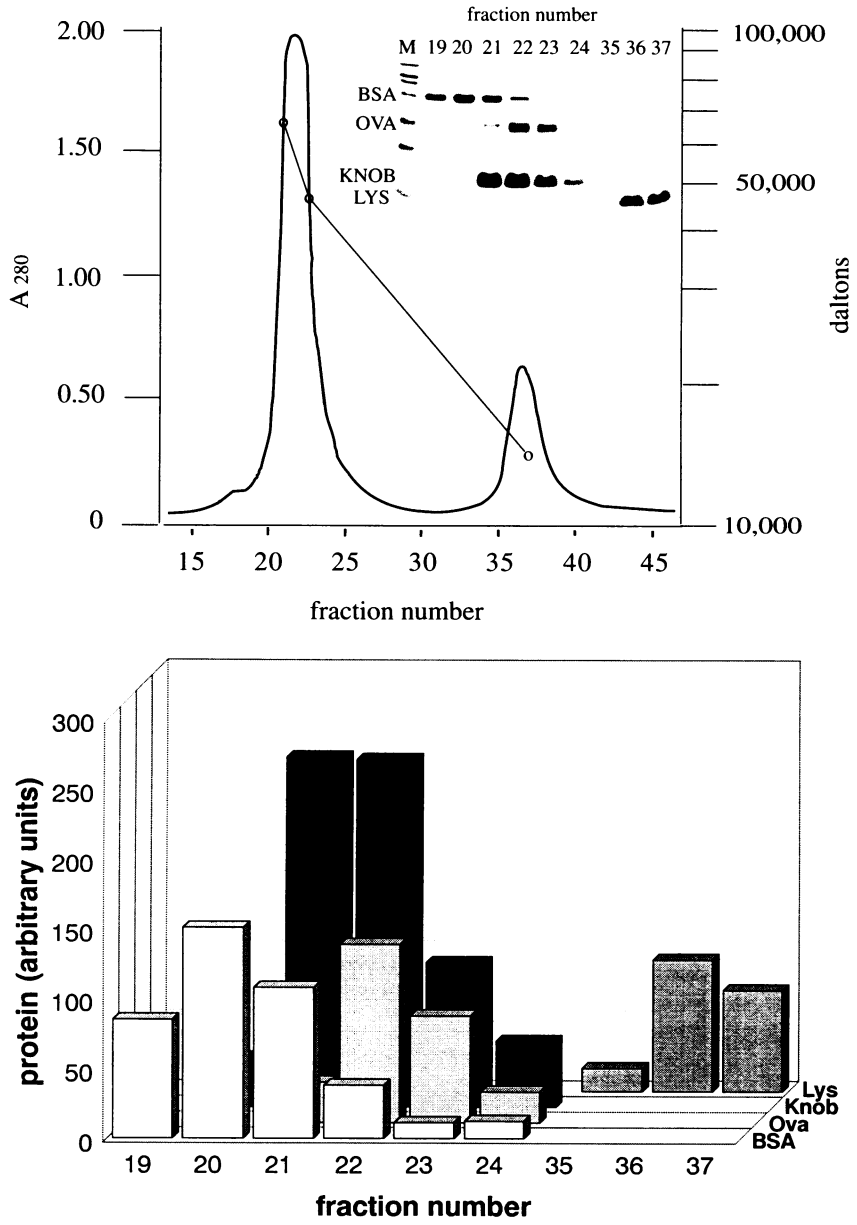
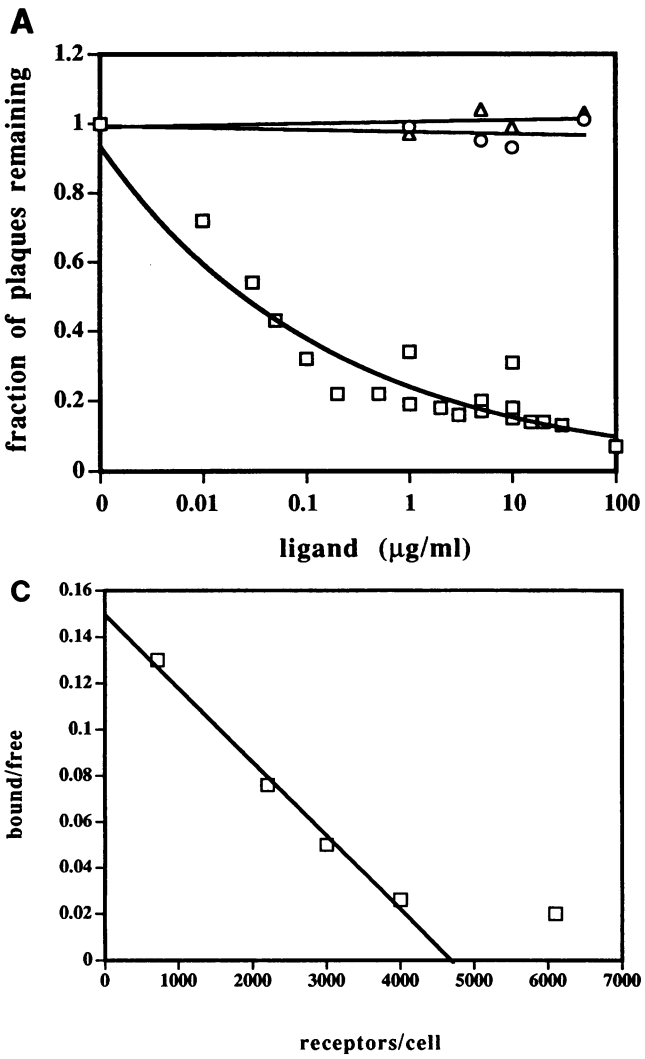


FIG. 3. FPLC gel filtration analysis of purified knob and molecular weight standards. A mixture of BSA, ovalbumin (OVA), lysozyme (LYS), and knob proteins was chromatographed on a Superdex 75 10/30 column. (Top) Elution profile of the column. The column effluent was monitored at 280 nm, and 0.5-ml fractions were collected; the inset shows SDS-PAGE (15% polyacrylamide) performed with individual fractions to determine the elution volume of individual proteins. The Coomassie blue-stained gel was scanned with an Epson ES-800C scanner and Adobe Photoshop 2.5 software. The image was imported into Aldus Freehand 3.1 to generate the composite figure. (Bottom) Densitometric analysis of the gel shown in the top panel was performed, and the amount of protein present in individual bands (in arbitrary units) was plotted against the fraction number.

$(NH_4)_2SO_4$ at pH 7.5. Several different crystal forms were observed under this condition. By the microcrystal seeding technique, large crystals of >0.5 mm were routinely obtained. Shown in Fig. 6A is a photograph of a single knob crystal about 1 mm in each dimension produced by this technique. This crystal form, which is cubic, diffracted very well to 2-Å (0.2-nm) resolution.

Figure 6B shows a still photograph of the diffraction pattern obtained from the crystal shown in Fig. 6A. The photograph was taken with one of the crystal axes roughly parallel to the X-ray beam. Diffraction spots were clearly observed at the edge of the film, which corresponds to 2-Å (0.2-nm) resolution. Precession photography showed that the crystal possesses both twofold and threefold symmetry axes, indicating a possible cubic space group. A native data set was collected with both the Xuong-Hamlin multiwire area detector and the Rigaku R-AXIS image plate system. The systematic absence of every second reflection along the reciprocal lattice axis indicated the presence of a twofold screw axis. The refined cell dimensions were $a = b = c = 86.4$ Å (8.64 nm) and $\alpha = \beta = \gamma = 90°$. Assuming 12 molecules per unit cell, the Matthews' coefficient, which is the ratio of crystal unit cell volume to the total



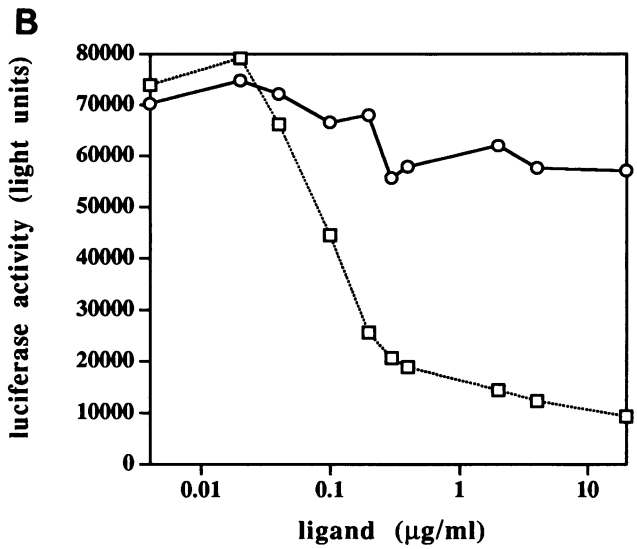


FIG. 4. Analysis of purified ligand binding to human cells. (A) Binding of ligands to 293 cells and inhibition of adenovirus infection. Purified ligands were incubated with 293 cell monolayers at the indicated concentrations for 15 min at room temperature to allow receptor binding. A defined number of infectious adenovirus particles was added, and the incubation was continued for 30 min to allow virus adsorption. Monolayers were overlaid with agar and incubated for 10 days to allow plaque formation. Plaques were counted, and the results for duplicate dishes were averaged to generate each datum point. The results of four separate experiments are shown. Circles denote GST protein, and triangles represent GST-39K fusion protein, which can block the binding of a variety of ligands to the LRP/α2-macroglobulin receptor. Squares represent the knob domain. (B) Binding of ligands to HeLa cells and inhibition of adenovirus infection. Purified ligands were incubated with HeLa cell monolayers at the indicated concentrations for 15 min at room temperature to allow receptor binding. Approximately 10^6 PFU of AdCMVLuc was added, and the incubation was continued for 30 min to allow virus adsorption. After 2 days, cells were extracted and luciferase activity was determined. Datum points are the averages for duplicate wells. Circles denote GST protein, and squares represent the recombinant knob domain. (C) Scatchard analysis of knob binding to HeLa cells. Cells (1.6×10^6) in a 24-well tray were washed once with PBS, formalin fixed, and washed three times with PBS. Cells were incubated with various concentrations of ^{125}I-knob diluted in DMEM–0.5% FBS (0.25 ml), washed three times with PBS, and solubilized in PBS containing 1% SDS at 37°C. Samples were counted to determine the quantity of bound ligand. Datum points are the averages for duplicate wells. Nonspecific binding was determined by incubation with a 100-fold excess of cold ligand and was subtracted from the total binding at each concentration.

molecular weight in the unit cell (14), was 2.56 Å^3/Da (0.00256 nm^3/Da). These results suggest a trimeric arrangement of the knob monomers in the unit cell, with the threefold molecular axis coincident with the crystallographic threefold symmetry axes and four trimers of knob in each crystal unit cell. A complete data set has been collected to 2.5-Å (0.25-nm) resolution with an R_{merge} of 4.1%. R_{merge} was defined as $\Sigma(|F_{hi}|-\langle F_h\rangle)/\Sigma\langle F_h\rangle$, where F_{hi} is a reflection with Miller index h on data frame i and $\langle F_h\rangle$ is a mean for all measured F_hs and Friedal pairs. The summation was carried out over all reflections.

DISCUSSION

Fiber is the virus attachment protein mediating adenovirus binding to mammalian cells. The globular knob domain located at the carboxyl terminus of the fiber protein has been presumed to be the receptor-binding domain despite any direct evidence. This presumption has been based largely on the physical shape of the protein, which appears as a ball and stick protruding from the icosahedral virus. The knob is therefore the domain expected to first contact the adenovirus receptor on the cell membrane. To obtain direct evidence supporting this assumption, we have cloned, expressed, and purified the knob domain in order to characterize its ability to functionally interact with mammalian cell membranes.

For the first time, direct evidence that the knob is the ligand for the adenovirus receptor has been obtained. First, recombinant knob expressed in *E. coli* is functionally capable of blocking adenovirus infection of mammalian cells. Using two different assays with both HeLa and 293 cells, we have been able to demonstrate efficient blocking of adenovirus infectivity. The concentration-dependent inhibition of adenovirus infectivity by the knob domain is similar to those observed previously by Philipson et al. (21) for blocking of labelled Ad2 and Ad5 virion attachment to HeLa cells by purified Ad2 fiber protein. These results confirm that the knob is the ligand for adenovirus binding to its cellular receptor.

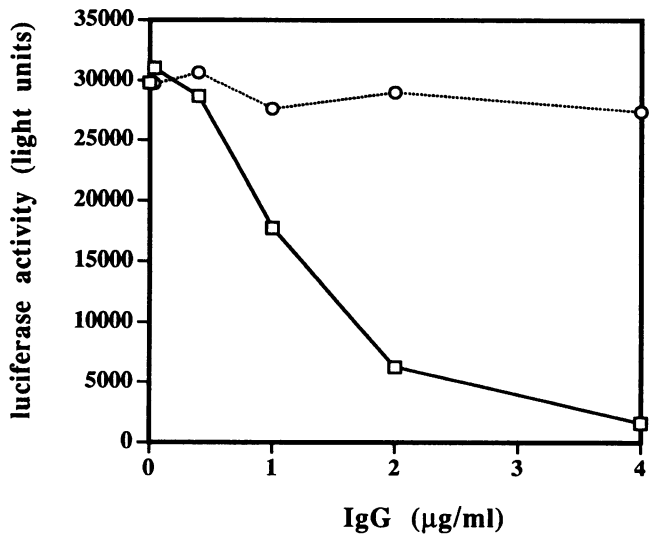


FIG. 5. Antibody to knob blocking of adenovirus infectivity. Normal rabbit antibody (circles) or rabbit antibody to knob (squares) was incubated at the indicated concentration with approximately 10^6 PFU of AdCMVLuc. Virus was then used to infect HeLa monolayers grown in 24-well trays. After 2 days, cells were extracted and the expressed luciferase activity was determined. Results are the averages for duplicate wells. Background was determined by using uninfected wells.

Previously, the number of receptor sites per HeLa cell was estimated at 10^5 for purified fiber protein but only 10^4 for intact adenovirus (21). This difference was attributed to fiber attachment to cell membrane components either different from those recognized by virus or inaccessible to the intact particle. Competition assays performed with virions and purified fiber suggested the existence of two classes of recognition sites with high and low affinities with respect to binding (3). Additionally, initial underestimates of the molecular weight of the fiber protein led to an overestimate of the number of fiber receptors. A somewhat lower estimate of 3,000 to 6,000 fiber receptor sites per cell, with an affinity of 7×10^8 M^{-1}, was obtained by others (19, 20). More recently, high-affinity fiber binding to HeLa cells was determined to have a K_d of 1.7 nM and only 16,000 sites per cell (31). Therefore, both the number of high-affinity knob binding sites (4,700) and their affinity (3×10^9 M^{-1}) for the receptor determined by our experiments are within the experimental range of values obtained previously for adenovirus and fiber binding to HeLa cells. Our data, however, do not rule out the existence of a large number of lower-affinity receptors for knob.

Antibody binding to the knob domain of fiber was shown to interfere with adenovirus attachment to cell membranes. The neutralizing activity that we observed using the rabbit antibody to the knob domain is significantly higher than that obtained previously with antibody to whole fiber (30). This may be due in part to the additional purification of the antibody on the knob-agarose column that we performed. However, antibodies raised against the whole fiber that are directed against the shaft or amino-terminal tail may not sterically interfere with the ability of adenovirus to recognize its receptor, whereas antibodies raised against the knob domain would be more likely to do so.

The knob has been structurally characterized by gel filtration chromatography. Comparison of the elution behavior of the knob with molecular weight standards suggests that the knob exists in solution as a trimer of 21-kDa subunits. Although there are two cysteine residues in each monomer, the chro-

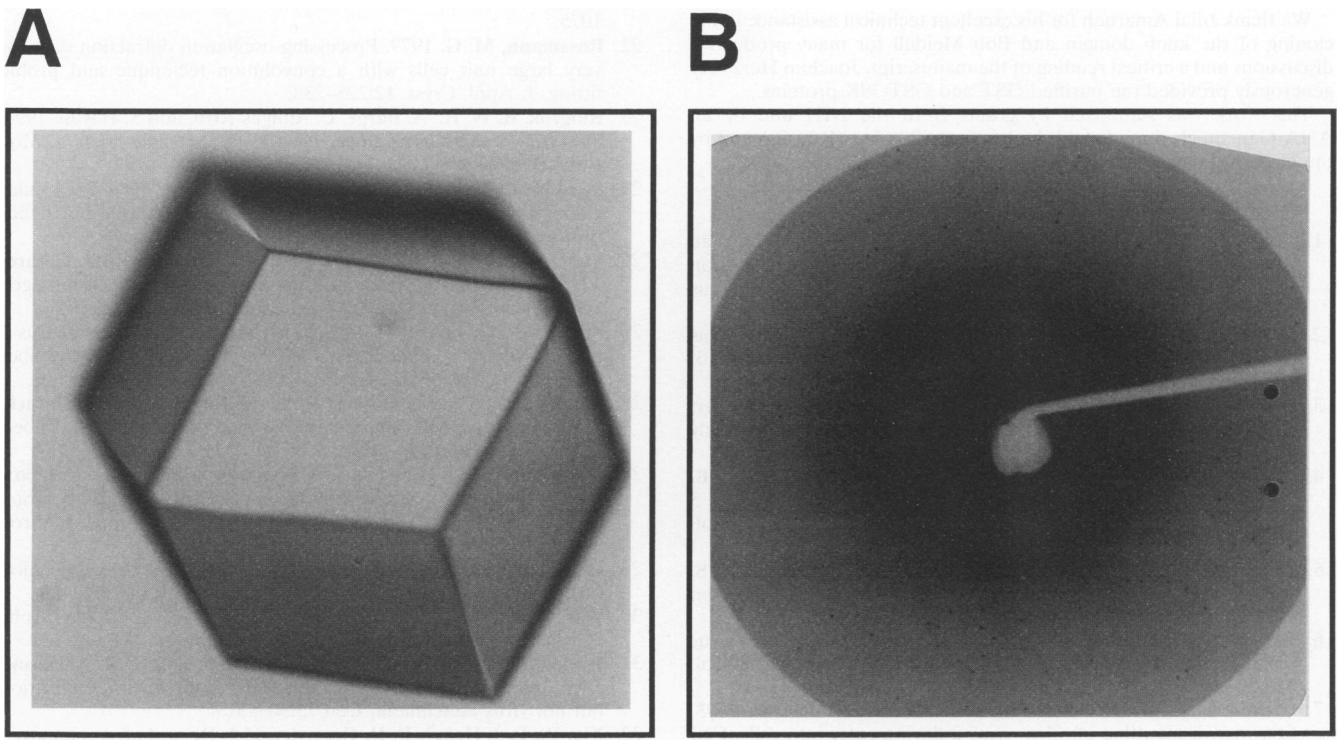


FIG. 6. (A) Crystal of recombinant knob domain. (B) Still photograph of the crystal shown in panel A.

matographic behavior was unaffected by reduction of the protein with mercaptoethanol (data not shown). This suggests that disulfide bonds are either not present or are unimportant to the trimeric nature of the globular knob. The existence of the knob in solution as a trimer suggests that the individual monomers self-associate in the absence of 21 of the 22 repeating protein motifs of the shaft normally found in the Ad5 fiber. The data are consistent with the possibility that the trimeric assembly of the fiber protein is energetically driven by the association of the individual globular subunits of the knob, as previously predicted (8).

A trimeric arrangement of monomers in the knob is also supported by the preliminary X-ray diffraction analysis of crystals of purified knob. The knob crystal has the symmetry of space group $P2_13$, which implies 12 symmetry-related molecular units. In addition, the $P2_13$ space group requires four threefold crystallographic axes, suggesting four trimers in one crystal unit cell. The Matthews' coefficient for the knob crystal is 2.56 $Å^3$ (0.00256 nm^3)/Da. With 12 monomers in the unit cell, the solvent volume analysis excludes a possible dimeric arrangement of monomers. This was confirmed by structure analysis, which clearly showed three discrete monomers. The complete crystallographic analysis and structure determination will be published elsewhere (32).

The availability of crystals of the recombinant knob which diffract to high resolution allows the structure of the knob domain of the fiber protein to be determined. The structure will provide insight into the nature of the interactions of the subunits of the trimeric knob and may also provide hints as to the interaction of the knob with the adenovirus receptor. The structure will also serve as a rational guide for site-directed mutagenesis to alter the interactions of knob with the adenovirus receptor and characterize in detail the molecular interactions of the two proteins.

ACKNOWLEDGMENTS

We thank Bilal Amarneh for his excellent technical assistance in the cloning of the knob domain and Bob Meidell for many productive discussions and a critical reading of the manuscript. Joachim Herz very generously provided the purified GST and GST-39K proteins.

This work was supported by grants from the NIH and by an AHA-Genentech, Inc., Established Investigatorship (R.D.G.) and by the Howard Hughes Medical Institute (J.D.).

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