Structure of the C-terminal head domain of the fowl adenovirus type 1 long fiber

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Avian adenovirus CELO (chicken embryo lethal orphan virus, fowl adenovirus type 1) incorporates two different homotrimeric fiber proteins extending from the same penton base: a long fiber (designated fiber 1) and a short fiber (designated fiber 2). The short fibers extend straight outwards from the viral vertices, whilst the long fibers emerge at an angle. In contrast to the short fiber, which binds an unknown avian receptor and has been shown to be essential to the invasiveness of this virus, the long fiber appears to be unnecessary for infection in birds. Both fibers contain a short N-terminal virus-binding peptide, a slender shaft domain and a globular C-terminal head domain; the head domain, by analogy with human adenoviruses, is likely to be involved mainly in receptor binding. This study reports the high-resolution crystal structure of the head domain of the long fiber, solved using single isomorphous replacement (using anomalous signal) and refined against data at 1.6 Å (0.16 nm) resolution. The C-terminal globular head domain had an anti-parallel β-sandwich fold formed by two four-stranded β-sheets with the same overall topology as human adenovirus fiber heads. The presence in the sequence of characteristic repeats N-terminal to the head domain suggests that the shaft domain contains a triple β-spiral structure. Implications of the structure for the function and stability of the avian adenovirus long fiber protein are discussed; notably, the structure suggests a different mode of binding to the coxsackievirus and adenovirus receptor from that proposed for the human adenovirus fiber heads.

INTRODUCTION

Avian adenovirus or chick embryo lethal orphan (CELO) virus (also known as fowl adenovirus type 1, species Fowl adenovirus A in the genus Adenovirus of the family Adenoviridae) is a large, non-enveloped, double-stranded (ds) DNA virus (Laver et al., 1971; McCracken & Adair, 1993). The CELO virus appears to be relatively benign and has not been associated with serious pathogenicity or economic losses (Cowen et al., 1978), nor does it give rise to any evident disease state when experimentally introduced into chickens. This apparent harmlessness has sparked interest in the possibility of using CELO virus in humans as a gene therapy vector (Kelleher & Vos, 1994; Stevenson et al., 2006) or chemotherapy vehicle (Logunov et al., 2004; Shashkova et al., 2005). Studies have also been undertaken to explore its potential as a vaccination vehicle in birds (Francois et al., 2004). The 44 kb dsDNA genome of the virus is contained within a single icosahedral capsid layer (Chiocca et al., 1996). As is the case for human adenoviruses (Fabry et al., 2005; Saban et al., 2006), the capsid vertices are comprised of penton proteins and the faces of hexon proteins, with further minor proteins incorporated as stabilizers. Each penton vertex contains a pentameric penton base and two trimeric fiber proteins, the short fiber (fiber 2) and the long fiber (fiber 1), with the short fiber emerging straight from the base and the long fiber at an angle (Hess et al., 1995). Whilst the short fiber plays a key role in the infection process in chicken cells (Tan et al., 2001), the long fiber is not essential and is thus an attractive target for modification.
The long fiber monomer contains 793 aa, with two notable glycine-rich regions (Hess et al., 1995). Twelve consecutive glycines located near the N terminus (Gly\(^{51}\)-Gly\(^{62}\)) presumably facilitate the flexibility that would be required to accommodate the long and short fibers on the same penton base and may be responsible for the fact that the long fiber emerges from the viral surface at an angle. A sequence of four consecutive glycines is present nearer the C-terminal end (aa 576–579), possibly forming a flexible linker region between the \( \beta \) triple-spiral shaft and the head domain. Crystallographic studies of the human adenovirus penton in the presence of fiber peptides show that five fiber-binding sites are available on each penton (Zubieta et al., 2005), which would imply that the N terminus of one of the fiber monomers does not bind to the penton. The most likely scenario appears to be that the short fiber occupies three binding sites and the long fiber the remaining two.

The long and short fiber head domains exhibit relatively low homology (less than 20% identical amino acids), consistent with them binding to different receptors. When the entire fiber sequences are aligned, regions of homology can be identified near the N terminus and in the putative shaft regions. Close to the N terminus, a region of around 15 highly homologous amino acids may be involved in attaching the fiber to the penton base.

The central domain of the long fiber (aa 72–572) contains repeats compatible with a triple \( \beta \)-spiral fold (Chiocca et al., 1996; van Raaij et al., 1999b). These repeats are characterized by the presence of the consensus sequence \( XXQXQXQX-1X^\phi XQXX-2 \), where X is any amino acid, \( \phi \) is generally hydrophobic and * is typically a proline or glycine, although other small amino acids are also tolerated in this position. At position 1 in the consensus sequence above, a central \( \beta \)-turn, insertions of a few amino acids are permitted; at position 2, more extensive insertions can be accommodated as surface loops. The central domain forms the slim central fiber shaft.

The head domain is predicted to comprise aa 580–793 and is comparable in size to those fiber head domains of human adenoviruses for which structures have been reported: human adenovirus type 5 fiber head (Xia et al., 1994), type 2 (van Raaij et al., 1999a), type 12 (Bewley et al., 1999), type 3 (Durmort et al., 2001), types 37 and 19p (Burmeister et al., 2004) and the type 41 short fiber head (Seiradake & Cusack, 2005). However, sequence homology between the avian adenovirus long fiber head and these proteins is limited, ranging between 14 and 17% sequence identity.

Previously, we have reported the crystallization of the avian adenovirus long fiber head (Guardado-Calvo et al., 2006). Here, we describe the structure refined against data collected to 1.6 Å (0.16 nm) resolution, revealing the detailed tertiary fold of the molecule. The implications of the structure for the stability and receptor-binding properties of the avian adenovirus long fiber are discussed.

METHODS

Avian adenovirus long fiber head was produced and crystallized as described previously (Guardado-Calvo et al., 2006). The nucleotide sequence of the long fiber head as present in the expression vector was confirmed to be identical to the sequences reported by Hess et al. (1995) and Chiocca et al. (1996). Crystals were cryo-protected with 25% (v/v) glycerol in reservoir solution and flash-frozen in liquid nitrogen or directly in a nitrogen stream maintained at 100 K. Native data was collected on the Spanish CRG Beam Line BM16 at the European Synchrotron Radiation Facility, France. For collection of derivative data, crystals were soaked in reservoir solution containing methylmercury chloride, both at low concentration (0.25 mM) and at high concentration. For the high-concentration soak, approximately 1 mg methylmercury chloride powder was added to the reservoir (not all of the powder dissolved), the reservoir was allowed to equilibrate with the drop overnight, 1 \( \mu l \) of the reservoir solution was mixed with the drop containing the crystals, and the soak was allowed to proceed for several hours. Datasets of the derivatized crystals were measured on a Bruker-Nonius FR591 rotating anode source equipped with a KappaCCD2000 detector at the copper K-\( \alpha \) wavelength. Reflections were integrated with the HKL2000 program suite (Otwinowski & Minor, 1997). Heavy atom sites were identified by the SHELXD program (Schneider & Sheldrick, 2002) and phases were refined with MLPHARE (Collaborative Computational Project, Number 4, 1994). The non-crystallographic symmetry operator was identified using PROFESS (Collaborative Computational Project, Number 4, 1994). Further solvent flattening, threefold averaging and histogram matching were carried out with the program DM (Cowtan & Main, 1998) and automatic tracing was carried out using ARP-WARP (Perrakis et al., 1999). Molecular replacement was performed with AMORE (Navaza, 2001) or Phaser (McCoy et al., 2005) and model adjustment and manual addition of extra amino acids was done using O (Jones et al., 1991). Refinement was performed with REFMAC (Collaborative Computational Project, Number 4, 1994; Murshudov et al., 1997) and water molecules were built using ARP (Lamzin & Wilson, 1993).

The coordinates have been deposited in the protein structure database (http://www.rcsb.org) under accession code 2IUM; the structure factors, including those for the derivative data (accession code r2IU4af), have also been deposited. Fig. 1(a) was prepared with ROBSCRIPT (Ennouf, 1997), a modified version of MOLSCRIPT (Kraulis, 1991). Figs 1(b, c), 2(a, b), 3(a) and 4 were prepared using PYMOL (W. L. DeLano, The PyMOL Molecular Graphics System, 2002, DeLano Scientific, San Carlos, CA, USA; http://www.pymol.org). Fig. 3(b–d) was prepared using the program COOT (Petterson et al., 2004).

RESULTS AND DISCUSSION

Structure solution

Crystals of the avian adenovirus long fiber head (Guardado-Calvo et al., 2006) were incubated in the presence of low and high concentrations of methylmercury chloride as described in Methods. Complete and redundant datasets were collected from crystals treated under different soaking conditions (for a summary of the data statistics, see Table 1). Due to problems of non-isomorphism between the mercury-soaked and native crystals, the low-concentration-soaked crystals were treated as the (pseudo)-native and the high-concentration soaked crystals as the derivative during substructure solution and phasing. Eight heavy atom positions were identified, of which the occupancies...
and temperature factors were refined. The occupancy of one of the sites refined to zero; of the seven remaining, six were related by a threefold non-crystallographic symmetry operator. Although overall phasing power was relatively low (Table 1), at low resolution it reached values above 2.0. The resulting phases and the symmetry operator were used in simultaneous solvent flattening and averaging, after which a readily interpretable map was obtained. Into this map, 613 aa were automatically traced and the resulting model was used in molecular replacement with the high-resolution, native data. The resulting model was input as a free-atom model in ARP-WARP, allowing the automatic tracing and sequence assignment of 610 residues; a further 23 aa and 857 water atoms were manually modelled into clear electron density before the refinement was completed. The final model contained residues 583–793 of each of the three monomers in the trimer; no inconsistencies between electron density and the expected amino acid sequence were observed.

Density for the three N-terminal aa 579–582 (as well as the six-histidine and T7 expression and purification tag) was missing from the maps. Presumably, this region is flexible, allowing these amino acids to adopt different conformations in the crystal. The refined structures corresponded well to the data, with good geometry and few residues in unfavoured regions of the Ramachandran plot by the PROCHECK criteria (Laskowski et al., 1993; Table 1). Only 2 aa were in ‘generously allowed’ regions, Asn694 of monomer B and Asp705 of monomer C, the first of which had clear unambiguous density supporting the modelled conformation and the second of which was part of a relatively disordered loop with poorly defined density.

**Overview of the structure**

The head domain of each monomer formed a compact β-sandwich with a topology similar to the human adenovirus fiber head domains (Fig. 1). The β-sandwich was made up...
of eight β-strands, which could be subdivided into two sheets, ABCJ and GHID, using the nomenclature from Xia et al. (1994). Most loops between the strands were short, with the exception of the DG loop, which contained aa 659–715. This loop incorporated the short β-strand E (aa 672–677), which interacted with the A strand. The A and G strands each contained a kink and crossed over from one β-sheet to the other; we designated the crossed-over...
parts of these strands A' and G', so that the two sheets could be denoted as EABCJG' and GHIDA'. The J strand also contained a notable kink, caused by two successive prolines (Pro782 and Pro783), but did not cross over between the sheets. The structure contained one cis peptide bond, between Phe676 and Pro677, Phe676 being the C-terminal residue of strand E. In human adenovirus fiber head domains of known structure, the equivalent region (which is involved in receptor binding) does not contain this feature.

When superimposed, the three monomers exhibited root mean square (r.m.s.) differences of only 0.2–0.3 Å (0.02–0.03 nm) between all C-α positions. Differences between monomers larger that 1 Å (0.1 nm) were limited to aa 641–642 and 771. These residues are part of the CD and IJ loops on the top surface of the trimer, respectively, and are involved in forming crystal contacts between monomers A and C, but not in the case of monomer B, which explains the conformational differences. Trimer contacts involved numerous residues in the N-terminal section of strands A and C, the C-terminal section of strands B and J, and the loops between strands A and A' (particularly residues Asn593 and Leu594), as well as a short stretch of the DG loop (Pro689–Thr695).

**Stability and folding**

The surface area of each monomer was around $9.6 \times 10^3$ Å$^2$, of which 23% was buried upon trimer formation ($2.2 \times 10^3$ Å$^2$). When the theoretical solvation energies of the trimer were compared with those of the monomer, a solvation energy gain of 150–200 kJ mol$^{-1}$ was predicted (around 40 kcal mol$^{-1}$) on trimer formation (Henrick &

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Fig. 3. Surface properties of avian adenovirus long fiber head and human adenovirus fiber heads. All panels are side views, maintaining the same orientation. (a) Surface representation of the human adenovirus type 12 fiber head in red with CAR-binding residues in blue (Bewley et al., 1999). (b–d) Electrostatic potential surface of the human adenovirus type 5 fiber head (b), human adenovirus type 12 fiber head (c) and avian adenovirus long fiber head (d).
but the avian adenovirus long fiber head does not.

where the human adenovirus fiber head contacts CAR domain 1
overlap with CAR domain 1 and a hash sign marks the region
the region where the avian adenovirus long fiber head would
the human adenovirus type 12 fiber head is shown. Asterisks mark
one of the three CAR domain 1s that could bind simultaneously to
in red. (b) Top view using the same colour scheme as in (a). Only
domain 1 structure in blue and the avian adenovirus long fiber head
human adenovirus type 12 fiber head structure in yellow, the CAR
complex structure (Bewley et al. 2012). Superposition of the avian adenovirus long fiber head
Fig. 4. Superposition of the avian adenovirus long fiber head structure onto the human adenovirus type 12–CAR domain 1 complex structure (Bewley et al., 1999). (a) Side view with the human adenovirus type 12 fiber head structure in yellow, the CAR domain 1 structure in blue and the avian adenovirus long fiber head in red. (b) Top view using the same colour scheme as in (a). Only one of the three CAR domain 1s that could bind simultaneously to the human adenovirus type 12 fiber head is shown. Asterisks mark the region where the avian adenovirus long fiber head would overlap with CAR domain 1 and a hash sign marks the region where the human adenovirus fiber head contacts CAR domain 1 but the avian adenovirus long fiber head does not.

Thornton, 1998). This relatively large buried surface area (Jones & Thornton, 1996) and large solvation energy gain at least partly explain the extraordinary stability of the trimeric long fiber head, as revealed by the fact that it does not dissociate into monomers during SDS-PAGE, unless previously boiled in SDS-containing buffer (Guardado-Calvo et al., 2006). The surface buried within the trimer interface is of mixed nature, containing both hydrophobic and polar contacts. In human adenovirus (van Raaij et al., 1999a), σ1 (Chappell et al., 2002), σC (Guardado-Calvo et al., 2005) and the PRD1 p5 spike protein (Merckel et al., 2005), a similar range of mixed contacts is observed in the interface between the head domains. Moreover, the total surface area buried in the trimer correlates closely between the avian and human adenoviruses (around 2 × 10^3 Å^2 or 20% of the available surface area).

Comparison of the structure with known protein structures

Superposition of the structurally equivalent residues from the avian adenovirus long fiber head and human adenovirus head domain monomers using the DALLI server resulted in an overall r.m.s. difference of just over 3 Å (0.3 nm) (153 superposed C-α atoms when compared with human adenovirus type 2; Fig. 2a). Not only did all adenovirus head domains have the same topology, but the loops connecting the β-strands were also highly similar, with mainly short interconnecting loops apart from one long DG loop. The kink in the A strand was also conserved in both types of fiber head, although the cross-over of the A strand to the other β-sheet did not occur in human adenovirus fiber heads. When trimers were superposed (3 × 153 structurally equivalent C-α atoms; Fig. 2b), the agreement was only slightly worse [r.m.s. differences of 4.2 Å (0.42 nm) when compared with human adenovirus type 2] and the relative orientation of the monomers in the trimer was very similar.

In comparison with the human adenovirus type 2 fiber head, the avian fiber head contained an additional 16 aa, which were located in the AB loop, the G strand, the GH loop and the IJ loop, all of which were longer in the avian long fiber head (Fig. 2c). Furthermore, the CD and HI loops on the top of the trimer were oriented differently, and the DG loop displayed a different conformation. These variations give rise to what are likely to be functionally significant differences in the external surfaces of the trimer unit, whilst maintaining a highly similar internal structural framework. One notable difference was in the conformation of the long DG loop, which in human adenovirus type 2 has more residues at the monomer–monomer interface (on the left in Fig. 2a and indicated with an ‘I’ in Fig. 2b), whilst in the avian adenovirus fiber head it covers more of the outer GHIDA’ sheet surface (on the right in Fig. 2a and indicated with an ‘S’ in Fig. 2b). Inspection of the electrostatic surfaces (Fig. 3) revealed that human types 5 and 12 were more similar to each other than to the avian adenovirus long fiber head.

Apart from human adenovirus fiber heads, the search also indicated structural similarity with reovirus (Chappell et al., 2002; Guardado-Calvo et al., 2005) and lactobacillus phage fibers (Spinelli et al., 2006a, b). These proteins contain β-sheet structures in their head domains; however, they are topologically different, forming circular β-barrels rather than β-sandwiches. When comparing the avian reovirus fiber head with the mammalian reovirus fiber head, differences in how the monomers self-associate in the trimer were observed, with the individual monomers of the avian reovirus fiber being more ‘splayed out’ than in their mammalian counterpart. It appeared that there was no such variability in quaternary structure between avian and human adenovirus fiber heads.
Receptor binding

Experiments carried out to compare the effects of infecting coxsackie and adenovirus receptor (CAR)-deficient Chinese hamster ovary (CHO) cells with avian adenovirus, with the CHO cells expressing human CAR, suggest that avian adenovirus binds to human, and consequently avian, CAR. Subsequent fiber-deletion studies have implicated the long fiber in CAR binding, whilst the receptor for the short fiber remains to be identified (Tan et al., 2001). Therefore, we tried to measure the binding of the purified avian adenovirus long fiber head domain with domain 1 of CAR; this domain was shown previously to be necessary and sufficient for high-affinity human adenovirus fiber head binding (Freimuth et al., 1999; Lortat-Jacob et al., 2001).

Our results from gel filtration (P. Guardado-Calvo & M. J. van Raaij, unpublished results) and surface plasmon resonance experiments (Hugues Lortat-Jacob, personal communication) did not show any interaction. This suggests either that, as is the case with coxsackievirus binding, domain 2 of CAR may be required for efficient binding of the avian adenovirus long fiber head, or that the long fiber shaft contains elements necessary for CAR binding. The other alternative is that CAR may not be a physiological receptor for avian adenovirus. It should be noted that these experiments were carried out with domain 1 of human CAR, not chicken CAR (NCBI RefSeq no. XW-416681; Pruitt et al., 2005), presumably because the chicken CAR sequence was not known at the time and due to the interest in using avian adenovirus as a human gene therapy or

Table 1. Crystallographic data and refinement statistics

The crystallographic space group is C2. Values in parentheses are for the highest resolution bin, where applicable.

<table>
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<th>Data collection</th>
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<th>High-concentration derivative</th>
<th>Native</th>
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<td>KappaCCD2000</td>
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<tr>
<td>Cell parameters (α, β, γ) (°)</td>
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<td>90.0, 101.3, 90.0</td>
<td>90.0, 101.5, 90.0</td>
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<td>1.5418</td>
<td>0.9799</td>
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<td>43.0–2.2 (2.28–2.19)</td>
<td>25.0–1.6 (1.66–1.60)</td>
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<td>36315 (3624)</td>
<td>88294 (6836)</td>
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<td>4.5 (2.3)</td>
<td>3.1 (2.5)</td>
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<td>10.4 (2.8)</td>
<td>20.9 (2.3)</td>
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Phasing (using high-concentration derivative as ‘derivative’ and low-concentration derivative as ‘native’)

Resolution range used (Å)                  | 43.0–2.2 (2.39–2.30)          |
| Number of reflections                     | 31459 (2900)                  |
| Number of heavy atom sites                | 8                            |
| Phasing power (centric/acentric)‡         | 1.08/0.90                    |
| Rcullis (centric/acentric)§               | 0.82/0.71                    |

Refinement

Resolution range (Å)                       | 16–1.6 (1.69–1.60)            |
| No. reflections used in refinement        | 85836 (9941)                  |
| No. reflections used for R-free           | 2034 (230)                    |
| R-factor||                                | 0.180 (0.31)                  |
| R-free                                    | 0.213 (0.31)                  |
| No. of protein/water atoms (Å²)           | 4797/857                     |
| Mean B protein/water atoms (Å²)           | 23.44/39.74                  |
| Ramachandran statistics (%)¶             | 91.2/8.4/0.4/0.0              |
| R.m.s. deviations [bonds (Å)/angles (°)]#  | 0.015/1.618                  |

*No sigma cut-off or other restrictions were used for inclusion of observed reflections.
‡Rmerge = ΣhΣi |Ih| – <Ih>)/ΣhΣi |Ii|, where Ih is the intensity of the ith measurement of the same reflection and <Ih> is the mean observed intensity for that reflection.
‡Phasing power = (|Fh|/|Fh| + |Fh| + |Fh|)/(|Fh| + |Fh| + |Fh|).
§Rcullis = (Σ|Fobs(hkl)| – Σ|Fcalc(hkl)|)/Σ|Fobs(hkl)|.
||R = Σ||Fobs(hkl)|| – Σ|Fcalc(hkl)|)/Σ|Fobs(hkl)|.
¶According to the program PROCHECK (Laskowski et al., 1993). The percentages indicated are of residues in the most favoured, additionally allowed, generously allowed and disallowed regions of the Ramachandran plot, respectively.
#Estimates provided by the program REFMAC (Murshudov et al., 1997).
anti-cancer vehicle. However, domain 1 of the predicted CAR homologue is 61% identical to human CAR, and residues of human CAR known to interact with adenovirus fiber head (Bewley et al., 1999; Law & Davidson, 2005) are identical (Glu56, Leu73, Ser75, Tyr80, Tyr83, Lys121 and Lys123) or similar (Asp\(^{69}\)(Glu and Val\(^{70}\)/Thr) in chicken CAR. The location of the CAR-binding site on adenovirus fiber heads has been determined by mutation analysis for human adenovirus type 5 (Kirby et al., 1999; Roelvink et al., 1999) and from structural data for human adenovirus type 12 (Bewley et al., 1999) and type 37 (Seiradake et al., 2006); it is located on the side of the molecule and involves residues of the AB, CD and DG loops (Law & Davidson, 2005). Structural alignments of the avian adenovirus long fiber head with fiber heads from the CAR-binding human adenoviruses revealed that few of the amino acids involved in CAR binding were conserved in the long fiber head. Moreover, the 2 aa that were absolutely conserved (a lysine in the B strand and a proline in the DG loop) were lacking in the long fiber head. In fact, the equivalent surface was significantly different in shape and charge (Fig. 3). It should be noted that the canine adenovirus type 2 fiber head does bind CAR, both in surface plasmon resonance experiments and crystals (Seiradake et al., 2006), although it does not contain the conserved lysine and proline residues. The structure of the CAR-binding region of the DG loop in the canine adenovirus type 2 fiber head is more similar to that of the human adenovirus fiber head than the avian adenovirus long fiber head.

Superposition of the avian adenovirus long fiber head structure onto that of the human adenovirus type 12–CAR domain 1 complex (Fig. 4) indicated that the different orientation of the DG loop was incompatible with CAR domain 1 binding in the same way that it binds to adenovirus type 12 (or any of the other adenovirus fiber head–CAR complexes analysed structurally so far), suggesting that either CAR binds in a different fashion to the avian adenovirus long fiber, as discussed above, or that CAR is not the real receptor. Site-directed mutagenesis experiments in conjunction with cross-linking or co-crystallization studies with (avian) CAR or an alternative receptor are needed to reveal the actual long fiber head residues responsible for receptor binding.

**Conclusions and perspective**

We have solved the structure of the receptor-binding domain of the avian adenovirus long fiber. The structure provides insights at the molecular level into the interactions required to form an intact fiber head and reveals what are likely to be functionally important differences at the putative receptor-binding site from the human adenovirus fiber head structures. The structural data will be of use in efforts focused on elucidating the early steps of the avian adenovirus replication cycle and may lead to therapeutic applications for avian (and human) diseases, by facilitating the design of artificial, chimeric fibers based on the structure, which could be used as vaccination or gene therapy vectors, or as anti-adenoviral peptides, which could block receptor binding through competitive inhibition.

Future structural work will target the shaft domain and is likely to require the deletion or modification of the four-glycine hinge domain, as carried out by Merckel et al. (2005) for the bacteriophage PRD1 p5 spike protein; alternatively, fusion to the foldon trimerization domain may be employed (Papanikolopoulou et al., 2004). Co-crystallization of the long fiber head with its receptor may also be undertaken once the receptor elements necessary for fiber head binding have been identified.

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induce protection against bursal disease in chickens. Vaccine 22, 2351–2360.


