Thiamine diphosphate binds to intermediates in the assembly of adenovirus fiber knob trimers in *Escherichia coli*

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Abstract

Assembly of the adenovirus (Ad) homotrimeric fiber protein is nucleated by its C-terminal knob domain, which itself can trimerize when expressed as a recombinant protein fragment. The non-interlocked, globular structure of subunits in the knob trimer implies that trimers assemble from prefolded monomers through a dimer intermediate, but these intermediates have not been observed and the mechanism of assembly therefore remains uncharacterized. Here we report that expression of the Ad serotype 2 (Ad2) knob was toxic for *thi*[−] strains of *Escherichia coli*, which are defective in de novo synthesis of thiamine (vitamin B1). Ad2 knob trimers isolated from a *thi*⁺ strain copurified through multiple chromatography steps with a small molecule of mass equivalent to that of thiamine diphosphate (ThDP). Mutant analysis did not implicate any specific site for ThDP binding. Our results suggest that ThDP may associate with assembly intermediates and become trapped in assembled trimers, possibly within one of several large cavities that are partially solvent-accessible or buried completely within the trimer interior.

Keywords: oligomeric proteins; assembly; chemical chaperone; subunit interface; adenovirus fiber knob; thiamine diphosphate

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Oligomeric proteins can assemble from either identical or different polypeptide subunits and fall into two classes depending on whether their subunits are interlocked or globular (Goodsell and Olson 2000). While the interlocking of subunits must result from simultaneous folding and assembly, globular subunits potentially can fold and assemble in separate, sequential steps. Isolated globular subunits would have exposed surfaces that become buried in the subunit interfaces of the assembled oligomer, and these surfaces could require stabilization to block non-

specific interactions. Molecular chaperones generally do not interact with folded protein species (Bukau et al. 2000); therefore, other factors and alternative mechanisms may be required to stabilize globular subunits prior to their assembly into oligomeric protein species. The mechanisms for assembly of this class of oligomeric proteins are not well understood.

The underlying molecular symmetry of oligomeric proteins is frequently exploited by viruses to build symmetrical capsid structures, and investigation of these systems could provide useful insights into general mechanisms for assembly of oligomeric proteins. The facets of the adenovirus icosahedral capsid, for example, are built primarily from hexons (San Martin and Burnett 2003), homotrimeric proteins with interlocking subunits (Athappilly et al. 1994). Earlier genetic analysis indicated that a virally encoded molecular chaperone (100K

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protein) is required for assembly of hexon trimers (Oosterom-Dragon and Ginsberg 1981; Morin and Boulanger 1986), and hexon polypeptides were shown to associate with this dedicated chaperone in large, heterogeneous complexes (Cepko and Sharp 1982; Hong et al. 2005). The 12 vertex capsomers are homopentamers of the penton base protein. Recent structural analysis indicates the pentamer consists of globular subunits that have extensive intersubunit interactions (Zubieta et al. 2005), but the assembly mechanism has not been characterized. In addition, a homotrimeric spike protein, the fiber, is attached to each penton base pentamer (Zubieta et al. 2005) and protrudes from the capsid surface to mediate binding of virus particles to cellular receptors.

Adenovirus fibers have a complex oligomeric structure where the subunits are intertwined in the β -spiral N-terminal shaft domain (van Raaij et al. 1999b) and globular in the C-terminal knob domain (Xia et al. 1994; Cusack 2005). The molecule is rod-shaped overall and terminates in the distal knob domain, which has a receptor-binding function (Henry et al. 1994) and contains three identical, symmetry-related binding sites for the coxsackie-adenovirus receptor that are centered over the trimer subunit interfaces (Bewley et al. 1999). Assembly of the intact fiber trimer depends on proper trimerization of the knob domain (Hong and Engler 1996), suggesting that the knob trimer surface could function as a template to initiate assembly of the shaft domain. Knob domains from several human and animal Ad serotypes have been expressed as recombinant protein fragments in bacteria or insect cells, where in most cases they assemble into biologically active trimers. Structures of these knob trimers (Cusack 2005) indicate that the globular subunits typically have extensive interfacial surfaces that provide a large number of intersubunit hydrogen bonds and hydrophobic interactions (van Raaij et al. 1999a), thus accounting for the extreme resistance of knob trimers to dissociation (Hong and Engler 1996; Awasthi et al. 2004).

The interfacial surfaces that give rise to strong subunit interactions in the knob trimer would be exposed on monomer and dimer assembly intermediates, raising the question whether these highly interactive surfaces require stabilization or shielding from nonspecific interaction with cellular components. While molecular chaperones potentially could provide this function, various small solutes, collectively termed chemical chaperones (Perlmuter 2002), also have been shown to facilitate the folding of proteins in vitro and in vivo, either by promoting solvation of unfolded or native protein conformations (Qu et al. 1998) or by trapping native protein conformations through specific ligand interactions (Wiens et al. 2001). Here we present evidence that thiamine diphosphate (ThDP), the intracellular coenzyme derivative of vitamin B1, functions as a chemical chaperone in the assembly of recombinant

knob trimers in *E. coli*, possibly by stabilizing assembly intermediates through interaction with exposed surfaces on folded subunits.

Results

Ad2 knob is toxic in thi[−] E. coli

In our initial attempt to produce Ad2 knob protein, we observed that *E. coli* strain DH5 α was severely growth inhibited following transformation with pET15b-Ad2Knob, whereas strain BL21DE3 grew at a normal rate after transformation with the same construct. The strain specificity of this toxic effect was surprising, because unlike BL21DE3 cells, which contain an inducible copy of the T7 RNA polymerase gene, DH5 α cells do not produce T7 RNA polymerase and therefore should be unable to transcribe the knob gene from the T7 promoter in pET15b. In control experiments, DH5 α grew normally following transformation with pET15b-based vectors for expression of Ad5 and Ad12 knobs, which, respectively, are 65% and 41% identical in amino acid sequence to Ad2 knob and have nearly super-imposable three-dimensional structures. Similar results were obtained with a different construct where the Ad2 knob gene was expressed from the inducible Trp/Lac hybrid promoter that is efficiently used by *E. coli* RNA polymerase: pTrc-Ad2Knob could not be established in DH5 α but readily transformed BL21DE3. Large colonies did appear at low frequency in some transformations of DH5 α with pTrc-Ad2Knob, but plasmids recovered from these colonies had deletions in the Ad2 knob coding sequence. A frame-shift mutation constructed to disrupt the Ad2 knob translational reading frame also relieved the toxic effect of pTrc-Ad2Knob in DH5 α . Results of these analyses suggested that the toxic effect resulted from expression of the Ad2 knob protein.

To further investigate the specificity of Ad2 knob protein toxicity, a set of 10 different *E. coli* strains was transformed with pTrc-Ad2Knob, or with the Ad5 knob expression construct pTrc-Ad5Knob as control. pTrc-Ad2Knob could not be established in any of the five *thi[−]* strains tested, whereas all five *thi⁺* strains tested formed normal-sized colonies after transformation with this DNA (Table 1). No other host strain genetic marker was strictly correlated with toxicity. All 10 strains grew equally well following transformation with the control plasmid pTrc-Ad5Knob. Addition of excess thiamine to LB agar plates did not enable growth of *thi[−]* strains that were transformed with pTrc-Ad2Knob.

To confirm that toxicity in *thi[−]* strains indeed results from expression of Ad2 knob protein, the Ad2 and Ad5 knob genes were expressed from the Tet promoter in pASK-IBA3. Transcription from this promoter is stringently

Table 1. Transformation of *E. coli* strains with pTrc-2K and pTrc-5K

Strain	Genotype	Transformation ^a	
		pTrc-5K	pTrc-2K
ER1821 ^b	F ⁻ <i>glnV44 e14⁻ (McrA⁻) rfbD1?relA1 spoT1? thi-1 Δ(mcrC-mrr)114::IS10</i>	+	—
ER2267 ^b	F ⁻ <i>proA⁺B⁺ lacI^q Δ(lacZ) M15 zzf::mini-Tn10(Kan^R)/Δ(argF-lacZ) U169 glnV44 e14⁻ (McrA⁻) rfbD1? recA1 relA1? endA1 spoT1? thi-1 Δ(mcrC-mrr)114::IS10</i>	+	—
JM101 ^b	F ⁻ <i>traD36 proA⁺B⁺ lacI^q Δ(lacZ) M15/Δ(lac-proAB) glnV thi</i>	+	—
JM109 ^b	F ⁻ <i>traD36 proA⁺B⁺ lacI^q Δ(lacZ) M15/Δ(lac-proAB) glnV44 e14⁻ gyrA96 recA1 relA1 endA1 thi hsdR17</i>	+	—
DH5α ^c	F ⁻ <i>φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r_K⁻, m_K⁺) phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	+	—
Top10 ^c	F ⁻ <i>φ80lacZΔM15ΔlacX74 mcrA Δ(mrr-hsdRMS-mcrBC) deoR recA1 araD139 Δ(ara-leu)7697 galK rpsL(Str^R) endA1 nupG</i>	+	+
BL21(DE3) ^d	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm (λDE3)</i>	+	+
HB101 ^e	F ⁻ <i>Δ(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20(Str^R) xyl-5 mtl-1 recA13</i>	+	+
D1210 ^e	F ⁻ <i>Δ(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacI^q Δ(mcrC-mrr) rpsL20(Str^R) xyl-5 mtl-1 recA13</i>	+	+
DH10B ^e	F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara-leu)7697 galU galK λ⁻ rpsL(Str^R) nupG</i>	+	+

^a Plus symbol (+) indicates growth on selective medium; minus symbol (—), no growth on selective medium. *E. coli* strains obtained from:

^b New England Biolabs;

^c Invitrogen;

^d Novagen;

^e Private collection of Dr. J. Dunn (Biology Department, Brookhaven National Laboratory, Upton, NY).

regulated by the Tet repressor and can be induced by low concentrations of anhydrous tetracycline. In accordance with the model, *thi*⁻ and *thi*⁺ host cells (DH5α and GC10, respectively) both were readily transformed by pASK-Ad2Knob and grew in the absence of inducer. However, in the presence of inducer, the DH5α culture failed to grow, while growth of GC10 transformants was approximately equivalent to that on un-induced control plates. By contrast, both strains transformed by the control pASK-Ad5Knob construct grew well in the presence or absence of inducer (see Fig. 7).

ThDP copurifies with Ad2 knob

To investigate the basis for the selective toxicity of Ad2 knob protein in *thi*⁻ cells, Ad2, Ad5, and Ad12 knob proteins were analyzed for small molecule content by electrospray ionization mass spectrometry (ESI-MS). The proteins were expressed with N-terminal 6-histidine tags in *thi*⁺ (BL21DE3) cells, and were purified by metal affinity chromatography (MAC) and dialyzed exhaustively against distilled water before analysis. The Ad2 knob sample produced a strong signal at *m/z* 423.031 (Fig. 1), within the error range (<4 ppm) of measurement of a standard solution of ThDP (theoretical *m/z* 423.029866 [M-2H]⁻), the intracellular coenzyme derivative of thiamine. A weak signal at this mass also was detected in the Ad5 knob sample (Fig. 1, inset), but was not present in the Ad12 knob sample (data not shown). The measured masses of residual polypeptides in solution were in good agreement with calculated values (Table 2).

MAC-purified Ad2 knob trimers were then further purified by cation-exchange chromatography, to determine if ThDP could be separated from knob protein under these conditions. Before loading onto the column, the protein was treated with trypsin to remove the N-terminal 6-histidine tags, thereby eliminating the large positive charge that would be contributed by the tags at pH 6. Western blot analysis showed that removal of the tags was complete and that the knob protein itself was resistant to trypsin digestion (Fig. 2A). The trypsin-cleaved protein bound quantitatively to the cation exchanger at pH 6, and separated into three major peaks (labeled *a*–*c*) during elution with a linear salt gradient (Fig. 2B). Peaks *a*–*c* were collected separately, individually rechromatographed on the

Figure 1. Mass spectra of knob proteins. Knob proteins were purified by metal affinity chromatography and then dialyzed exhaustively against distilled water before analysis by ESI-MS in negative ion mode. The masses of species associated with Ad2 and Ad5 knobs (*inset*) similar in mass to ThDP are indicated. The mass of a standard sample of ThDP was 423.0319 (data not shown). Measurement errors were <4 ppm.

Table 2. Protein mass from positive ion mode ESI-MS

Protein	Measured mass ^a	Calculated mass
Ad2 knob ^b	23,696.7 ± 4.3	23,682
Ad5 knob ^b	22,445.8 ± 2.3	22,447
Ad12 knob ^b	22,296.3 ± 6.0	22,299
Ad2 knob isoform <i>a</i> ^c	21,936.8 ± 0.6	21,931
Ad2 knob isoform <i>b</i> ^c	21,939.9 ± 2.5	21,931
Ad2 knob isoform <i>c</i> ^c	ND ^d	21,931

^aMean ± standard deviation of triplicate measurements.^bN-terminal 6-histidine tag attached.^cN-terminal 6-histidine tag removed by proteolysis.^dNot determined, insufficient sample.

same column, dialyzed exhaustively against water, and then analyzed by ESI-MS (Fig. 2C). A species with mass equivalent to ThDP was detected in all three samples, but the signal-to-noise ratio was greatest for sample *b*. In positive ion mode, the measured masses of protein in samples *a* and *b* (Table 2) were in good agreement with the calculated mass of knob after hydrolysis at the thrombin cleavage site (Arg-Gly bond) immediately following the 6-His tag. The protein concentration in sample *c* was not sufficient for mass analysis. These results together suggest that the binding of ThDP to native Ad2 knob trimers is essentially irreversible under physiological conditions and that ThDP is released from the complex only under conditions that dissociate knob trimers to their polypeptide subunits (ionization in the mass spectrometer). Incubation of MAC-purified Ad2 knob trimers with ThDP in vitro prior to cation-exchange chromatography did not change the elution profile, (data not shown), indicating that isoforms *a–c* cannot be interconverted by exposure to an excess of ThDP.

Role of shaft peptide in ThDP binding

Separation of Ad2 knob into multiple isoforms by cation-exchange chromatography was observed in an earlier study (van Raaij et al. 1999a) where it was noted that crystals of the isoform mixture diffracted poorly, whereas crystals of purified isoforms had good diffraction quality, allowing the Ad2 knob structure to be determined to high resolution. One striking difference between this structure and the nearly super-imposable structures of Ad5 and Ad12 knobs is that the N-terminal 13 amino acids, which are derived from the adjacent shaft domain of fiber protein, were highly ordered in the Ad2 knob structure (Fig. 3A) but were disordered and thus not visible in the Ad5 and Ad12 knob structures. Furthermore, residues N393, D397, and K398 within the Ad2 knob shaft peptide contributed directly to the tight binding of two sulfate ions per subunit (Fig. 3B,C), which also were well-resolved in the crystal structure (labeled I and II in panels

A–C). Based on these earlier results, we reasoned that the chromatographic heterogeneity of Ad2 knob on the cation-exchange column might result from partial disorder of the shaft peptides with the loss of anion-binding sites associated with the ordered peptide structure. We further hypothesized that these anion-binding sites might interact with the pyrophosphate group of ThDP, contributing to the observed tight binding of ThDP to native knob trimers. To test this hypothesis, the shaft peptide of wild-type Ad2 knob was shortened to three residues (D397 to L399). As observed with the original pTrc-2Knob construct, the resulting expression plasmid for knob with the truncated shaft peptide (pTrc-2KnobSS) could not be established in *thi*– strains but did transform *thi*+ strains. However, in contrast to the long-shafted Ad2 knob protein, the short-shafted protein eluted from the cation-exchange column as a single peak very late in the salt gradient (Fig. 3D), and no ThDP was detected by MS (data not shown). These results suggest that the 13-residue shaft peptides and their associated anion-binding sites are not necessary for ThDP binding to Ad2 knob in vivo but may stabilize knob-ThDP complexes during purification.

To further characterize these anion-binding sites, Ad2 knob protein was purified from a single *E. coli* culture using either the standard protocol with phosphate buffer, or an alternate protocol where Tris buffer was substituted for phosphate. After purification by MAC and treatment with thrombin to remove the 6-His tags, both preparations were purified by anion-exchange chromatography in Tris buffer before final purification by cation-exchange

Figure 2. Heterogeneity of Ad2 knob protein. Removal of N-terminal 6-histidine tags from MAC-purified Ad2 knob by limited trypsinolysis was monitored by Western blotting (A). After quantitative removal of the 6-histidine tags, the protein was applied to a cation-exchange column at pH 6 and eluted with a linear gradient of NaCl (B). The protein separated into three major peaks, *a–c*, which were rechromatographed under identical conditions. (C) Mass spectra of rechromatographed protein samples *a*, *b*, and *c* by ESI-MS (negative ion mode). The masses of species associated with knob isoforms similar in mass to ThDP are indicated.

Figure 3. Anion-binding sites associated with shaft peptides. (A) A space-filling model of the Ad2 knob trimer is shown with the knob monomer subunits colored white and light and dark gray. The shaft peptides, corresponding to the last 13 residues of the fiber shaft domain adjacent to knob, are shown in backbone mode and colored blue, green, and yellow. The two sulfate ions bound to each subunit are colored red and are labeled I and II on one subunit. Close-up views of the residues that contribute to the two sulfate-binding sites are shown in panels B and C. Note that site II is formed from residues in two different shaft peptides (N393 from polypeptide chain A, K398 from polypeptide chain C). (D) Cation-exchange chromatography of a variant Ad2 knob protein with shaft peptides consisting of only three residues (D397, K398, and L399). Contrast the single peak with results of same analysis method of knob with 13-residue shaft peptides, shown in Figure 2B. Images in panels A–C were generated from crystallographic coordinates using PyMOL (DeLano Scientific; <http://www.pymol.org>).

chromatography. Both knob preparations eluted from the anion exchange column as single sharp peaks at approximately the same position in the salt gradient (Fig. 4, inset). However, on subsequent purification by cation-exchange chromatography, the knob protein sample exposed to phosphate buffer separated into four major peaks (Fig. 4, solid line, peaks labeled *a–d*), similar to results shown in Figure 3, whereas omission of phosphate in the alternate protocol using Tris buffer led to the disappearance of isoforms *b* and *d* and an apparent reduction in the amount of isoform *c* relative to isoform *a* (Fig. 4, dashed line). These results are consistent with a model where phosphate ions also can bind to the sulfate-binding sites associated with the 13-residue shaft peptides, and that partial occupancy of these sites with

phosphate or other anions accounts for the heterogeneity of Ad2 knob protein during chromatography on the cation exchanger. Knob isoforms *a* and *c* purified from the alternate, phosphate-free preparation were analyzed for ThDP content by liquid chromatography/MS (LC-MS). Contrary to our prediction that isoform *c* would be enriched for bound ThDP based on its early elution from the cation exchanger, isoforms *a* and *c* both contained a UV-absorbing species whose retention times and masses were similar to a ThDP standard (Fig. 5). Bound ThDP therefore does not appear to contribute appreciably to the observed chromatographic heterogeneity of Ad2 knob.

Mutant analysis of potential ThDP binding sites

To further characterize the interaction of ThDP with Ad2 knob, a set of Ad2 knob mutants was constructed with single amino acid substitutions of residues located in several large buried or partially exposed cavities (Fig. 6) that potentially could accommodate binding of all or part of the ThDP molecule, including a large exposed cavity at the base of knob that is formed in part from residues in the shaft peptide (Fig. 6A). Because of the leaky expression of pET15b and pTrc constructs and the selective advantage conferred by acquisition of spontaneous mutations that disrupt production of knob trimers, the mutations were constructed in the Ad2 knob gene cloned in pASK-IBA3, where transcription of the gene is tightly regulated by the Tet repressor and inducible by anhydrous tetracycline. Results of this analysis are summarized in Table 3. The growth phenotype of *thi*[–] and *thi*⁺ strains after transformation with plasmids encoding a representative

Figure 4. Stable binding of phosphate ions contributes to Ad2 knob heterogeneity. Ad2 knob protein was purified by MAC in either phosphate or Tris buffer, and then further purified by sequential chromatography first on an anion exchanger in Tris buffer (inset) and then on a cation-exchanger in MES buffer. Solid lines indicate sample prepared by MAC in phosphate buffer; dashed lines indicate sample prepared by MAC in Tris buffer. The four main isoforms of Ad2 knob from the phosphate preparation that eluted from the cation-exchange column are labeled *a–d*.

Figure 5. LC-MS analysis of Ad2 knob isoforms *a* and *c*. Ad2 knob isoforms *a* and *c* from the phosphate-free Tris preparation shown in Figure 4 were dialyzed exhaustively against distilled water and then analyzed by LC-MS in positive ion mode (panels A and B, respectively). Traces of absorbance at 280 nm vs. elution time are shown, with mass spectra, as insets. (C) Analysis of a standard sample of ThDP.

subset of mutants and controls is shown in Figure 7. In the absence of inducer, *thi*[−] cells transformed with pASK-Ad2Knob were able to grow as pinpoint-sized colonies (panel A, sector 5). The heavier growth near the perimeter of the plate, where a higher concentration of inoculum was streaked, probably resulted from early destruction of ampicillin by β -lactamase leached from the cells, enabling outgrowth of cells that had lost the Ad2 knob expression plasmid. Under the same conditions, cells transformed with the control pASK-Ad5Knob grew out as large, isolated colonies (sector 6). The mutants exhibited a range of toxicity either equal to or greater than that of wild-type Ad2 knob. In the example shown in panel A, the toxicity of mutant proteins varied in the order: S408E (sector 3) > P409A/S408E (sector 4) > P409A (sector 1) > S408A (sector 2) = wild type (sector 5). None of the *thi*[−] cells that expressed these or any other mutants shown in Table 3 or wild-type Ad2 knob could grow in the presence of inducer (panel C); however, under these same conditions, the cells tolerated expression of Ad5 knob (panel C, sector 6). In the *thi*⁺ strain GC10, cells that expressed basal levels of these mutants (and all others shown in Table 3), wild-type Ad2 knob, or wild-type Ad5 knob all grew at approximately equal rates (panel B). Similarly, growth of all strains in the presence of inducer (panel D) was approximately equivalent, except for cells that expressed the mutant

S408E, which were unable to grow as isolated colonies. Together the results do not strongly implicate any single, unique binding site for ThDP on Ad2 knob, although the hyper-toxicity of mutants with changes mapping to the subunit-1 cavity (Fig. 6C) suggest a possible direct role for this feature in ThDP binding. The results also are consistent with an alternate model where ThDP interacts nonspecifically with knob, possibly at multiple sites.

Discussion

Here we provide evidence that the recombinant knob domain of Ad2 fiber protein physically associates with ThDP during knob synthesis in *E. coli*. Expression plasmids for Ad2 knob were toxic in *thi*[−] strains but not in *thi*⁺ strains, and a small molecule of mass equivalent to ThDP co-purified with Ad2 knob through several steps of chromatography and dialysis. These results support our conclusion that toxicity results from sequestering of ThDP in knob trimers.

Initially, we suspected that the isoforms of knob that are separated by cation-exchange chromatography (Fig. 2B) might result from binding of one, two, or three molecules of ThDP per knob trimer. In their characterization of the Ad2 knob crystal structure, Cusack and

Figure 6. Surface-exposed and buried cavities of Ad2 knob trimers. Backbone-mode renderings of Ad2 knob trimers showing cavities determined by the program CASTp (Binkowski et al. 2003) that were targeted by mutagenesis. The cavities are rendered in space-filling mode and are colored blue. (A) Shaft junction cavity (1099 Å³) lined by residues K394-D396, L399, and T426-G429. (B) Interfacial cavity-1 (303 Å³), lined by residues D406, P407, K420, T422, A437-L439, A570, and T571 from subunit A, and L502, A503, P505, and T511-N514 from subunit C. (C) Subunit cavity-1 (149 Å³), lined by residues T403, P407-N410, L473, K474, H476, Y477, and W478. (D) Interfacial cavity-2 (1259 Å³, buried), lined by residues V436, A437, I516-Y521, M530, L532, W557, and T571-F576 from each subunit.

Table 3. Mutant analysis of ThDP-Ad2 knob binding

Mutation	Cavity location ^a	Growth on <i>thi</i> – ^b
D397A	shaft junction	–
G429N	shaft junction	–
G429Y	shaft junction	–
K398E	shaft junction	–
T511E	interfacial –1	–
K513A	interfacial –1	–
N514S	interfacial –1	–
D406A	interfacial –1	–
A438V	interfacial –1	–
Q510K	interfacial –1	–
T403A	subunit –1	–
P407A	subunit –1	–
H476E	subunit –1	–
Y477A	subunit –1	–
W478A	subunit –1	–
P409A	subunit –1	–
S408A	subunit –1	–
S408E	subunit –1	–
S408E/P409A	subunit –1	–
Y574A	interfacial –2	–
T575A	interfacial –2	–
F576A	interfacial –2	–

^aLocation of cavities on the Ad2 knob trimer are shown in Figure 6, and residues lining the walls of each cavity are listed in the legend to Figure 6.

^bToxicity test was performed as described in the legend to Figure 7.

colleagues also observed that knob protein could be separated into three major isoforms by cation-exchange chromatography, and they determined the structures of two of these isoforms, neither of which contained unassigned electron density that could correspond to ThDP (van Raaij et al. 1999a). Investigating this further, we found that exposure of knob protein to phosphate increased the number of isoforms compared with the sample prepared in Tris (Fig. 4). It is likely that phosphate ions bind to the same sites as the sulfate ions that were observed in the crystal structure. Expression of Ad2 knob with a truncated, three-residue long shaft peptide was still toxic in *thi*– strains, but the isolated knob trimers did not separate into isoforms during cation-exchange chromatography and did not contain ThDP that was detected by MS. The shaft peptide therefore is not required for ThDP binding in vivo, but it may stabilize knob-ThDP complexes, possibly through interaction with the pyrophosphate moiety of ThDP.

Mutant analysis failed to strongly implicate any single site on the knob surface as a binding site for ThDP; however, the hyper-toxicity of some mutants suggested that ThDP may interact with a surface-exposed cavity present on each subunit (Fig. 6C). The absence of specific sites on the trimer surface that can reversibly bind ThDP would suggest that ThDP might interact with knob during the trimer assembly reaction rather than at a post-assembly stage. Consistent with this model, we observed

in a preliminary study that refolding and assembly of Ad2 knob trimers in vitro was enhanced by addition of 10 mM ThDP to the reaction (Fig. 8). Further studies will be required to determine the optimum conditions for this effect; however, the observed enhancement occurred at a much lower concentration of ThDP than typically is required for other additives that improve refolding, such as arginine at 0.5 M. ThDP in mammalian cells, such as those typically used for laboratory propagation of adenoviruses, and in bacteria vary in the range of ~50–200 μ M (Makarchikov et al. 2003).

The observation of knob-ThDP complexes was unexpected, because ThDP has no role in fiber protein function and has never been observed in crystal structures of knob domains from several Ad serotypes. Furthermore, MS analysis of a sample of intact fiber protein purified from virus-infected HeLa cells failed to detect ThDP (data not shown). These results do not rule out a possible role for ThDP in the assembly of fiber protein during virus infection of human cells. Molecular chaperones appear to assist in the biogenesis of intact fiber (Macejak and Luftig 1991), possibly to facilitate simultaneous folding and assembly of subunits in the interlocking shaft domain, and under these conditions, dissociation of ThDP may be complete. The interaction of ThDP with recombinant Ad2 knob in *E. coli* described here nonetheless underscores the fact that protein folding and oligomer assembly in vivo occur in the presence of cytoplasmic

Figure 7. Growth phenotype of *thi*– and *thi*+ strains expressing mutant and control knob proteins. Early log phase broth cultures of DH5 α (*thi*–) or GC10 (*thi*+) freshly transformed to express Ad2 knob mutants P409A, S408A, S408E, or P409A/S408E (sectors 1–4, respectively) or wild-type Ad2 or Ad5 knob control proteins (sectors 5 and 6, respectively) were streaked onto selective (ampicillin) agar plates that either were supplemented with anhydrous tetracycline to induce knob expression (+Tet) or did not contain inducer (–Tet). Plates were photographed after incubation for 20 h at 37°C.

Figure 8. ThDP promotes folding and assembly of knob trimers in vitro. Ad2 knob polypeptides in insoluble aggregates (inclusion bodies) were dissolved in 6 M guanidine hydrochloride, and duplicate samples were chromatographed in parallel on gel filtration columns equilibrated in buffer alone or in buffer containing 10 mM ThDP. Protein concentrations in the column excluded volumes were approximately equivalent, as judged by Coomassie Blue staining (data not shown). Equal volumes of each sample were analyzed immediately and again after incubation for 48 h at room temperature by electrophoresis in nondenaturing polyacrylamide gels. Proteins were electro-transferred to a PVDF membrane and probed with anti-Ad2 knob polyclonal antibodies that were affinity-purified on native Ad2 knob trimers. The arrow indicates the position of Ad2 knob trimer.

solutes, which can have important influences on these processes. While solute-driven protein folding can be readily observed in *in vitro* reactions, the difficulty in controlling concentrations of cytoplasmic solutes is an obstacle to characterizing their effects on protein folding and oligomer assembly *in vivo*. Assembly of Ad2 knob trimers therefore provides a rare opportunity to observe solute–protein interaction *in vivo*.

Chemical chaperone-assisted folding has been reported for several proteins (Perlmutter 2002) and is thought to occur by one of two alternate mechanisms. In one mechanism, the chemical chaperones function as osmolytes, promoting solvation of unfolded states or of the native conformation (Qu et al. 1998). In the other mechanism, chemical chaperones function as ligands that are specific for the native protein conformation, as, for example, in hapten-induced folding of antibodies (Wiens et al. 2001). ThDP is likely to interact with knob through the second mechanism for the following reasons: The total concentration of ThDP in the *E. coli* cytoplasm was estimated at ~0.15 mM (Makarchikov et al. 2003), but most of these molecules are present in complexes with ThDP-dependent enzymes. The concentration of free ThDP therefore, would be much lower than the concentrations typically required for solute-driven protein folding. Furthermore, the ligand-induced conformational trapping mechanism would be more likely to generate stable (long-lived) complexes of the ligand with folded monomeric and dimeric intermediates, increasing the chances of trapping ThDP in the final steps of trimer assembly.

The assembly of knob and other oligomeric viral and cellular proteins often is difficult to characterize, owing

in part to the difficulty in isolating stable assembly intermediates. It seems reasonable, however, that in many cases the subunits would need to be stabilized to block nonspecific side reactions of exposed interfacial surfaces. While solutes with moderate affinity for interfacial surfaces could rapidly cycle on and off the interface and thus have a chaperone-like effect on oligomer assembly, solutes that bind with high affinity could potentially inhibit oligomer assembly through the formation of stable complexes with assembly intermediates. Like knob, many viral proteins must oligomerize to acquire their biological function; therefore, inhibition of oligomer assembly by small molecules could be an attractive approach for antiviral therapy. The tet-inducible system for Ad2 knob expression described here could be exploited to screen for compounds that block knob trimer assembly, thereby relieving the toxicity to *thi*[−] strains that otherwise would result from sequestration of ThDP in the knob trimer subunit interface.

Materials and Methods

Plasmids and strains

Gene fragments encoding the knob domain plus short regions of the adjacent shaft domain of fiber proteins from adenovirus serotypes 2, 5, and 12 were amplified from viral DNA by polymerase chain reaction (PCR). The forward (sense) and reverse (anti-sense) primers were tailed with restriction sites for cloning the PCR products between the *Nde*I and *Xho*I or *Bam*HI sites of pET15b (EMD Biosciences, Inc.), the *Bam*HI and *Hind*III sites of pTrc-HisB (Invitrogen Corp.), or the *Sac*II and *Xho*I sites of pASK-IBA-3 (IBA). The primer sequences used are shown in Supplemental Table 1. The genotypes and sources of *E. coli* strains used for transformation and expression experiments are listed in Table 1. A frame-shift mutation was constructed in pTrc-2Knob by digesting the DNA with *Bam*HI, “filling in” the cohesive ends by DNA synthesis using the Klenow fragment of DNA polymerase, and recircularization of the blunt-ended DNA by T4 ligase. DNA manipulations and cell transformation were performed using standard methods (Maniatis et al. 1989).

Protein expression, purification, and analysis

Cultures in LB broth (Sigma-Aldrich Co.) were inoculated with BL21DE3 colonies freshly transformed with pET-2Knob, or with similar pET-based vectors for expression of Ad5 or Ad12 knob, and were grown in shaking flasks at 37°C. When cultures reached mid-log phase, the temperature was reduced to 20°C, and expression of knob protein was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cultures were harvested after overnight incubation at 20°C. Cells were lysed with BugBuster reagent (EMD Biosciences, Inc.) following the method provided by the manufacturer. After centrifugation for 20 min at 10,000g, the supernatant containing soluble 6His-tagged knob protein was passed through a column of Ni-NTA agarose beads (Qiagen). The beads were then washed and bound proteins eluted

with 250 mM imidazole in 10 mM sodium phosphate buffer containing 0.3 M NaCl, according to the manufacturer's instructions.

His-tags were removed from knob protein prior to cation-exchange chromatography, by digestion with either trypsin or thrombin. Proteins eluted from the Ni-NTA column were dialyzed against 10 mM Tris (pH 8) containing 100 mM NaCl, prior to incubation at room temperature with trypsin-agarose beads (Sigma-Aldrich; 1.5 unit per μ mole knob protein). Reaction progress was monitored by SDS-PAGE and Western blotting using monoclonal anti-polyhistidine-peroxidase conjugate (Sigma-Aldrich). Digestion was usually complete by 3–5 h. For thrombin digestion, proteins were dialyzed against 10 mM Tris (pH 8) containing 150 mM NaCl and further adjusted by addition of 0.1 volume of 10 $\times$ reaction buffer (200 mM Tris pH 8.4, 1.5 M NaCl, 25 mM CaCl_2), prior to digestion for 3 h at room temperature with biotinylated thrombin (Novagen, 120 units per μ mole knob protein). Thrombin was removed by batch adsorption to avidin-agarose (Sigma-Aldrich). Digested proteins were rechromatographed on Ni-NTA agarose to remove undigested species prior to cation-exchange chromatography.

After His-tag removal, Ad2 knob was further purified by cation-exchange chromatography according to a method described earlier (van Raaij et al. 1999a). Briefly, the protein was dialyzed into MED buffer (10 mM 4-morpholineethanesulfonic acid [MES] pH 6, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM dithiothreitol [DTT]; all from Sigma-Aldrich), injected onto a HiTrap SP-HP column attached to an AKTA *prime* liquid chromatograph (GE Healthcare Bio-Sciences Corp.), and eluted with a linear gradient of NaCl (0–0.6 M) in MED buffer. Where noted, the protein was first purified by anion-exchange chromatography on a HiTrap Q-HP column in Tris buffer (pH 8), prior to cation-exchange chromatography.

Electrophoresis in sodium dodecylsulfate-containing 12% polyacrylamide gels was performed according to standard methods (Maniatis et al. 1989). Electrophoresis in nondenaturing polyacrylamide gels was performed as described (van Raaij et al. 1999a). For Western blotting, proteins were transferred from either SDS gels or nondenaturing gels to Hybond-P membranes (GE Bio-Sciences) using a semi-dry transfer apparatus (C.B.S. Scientific Co.) and the discontinuous Tris/CAPS buffer system (omitting SDS) described by Bio-Rad (Bio-Rad Laboratories, Inc.). Membranes were blocked in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 0.05% Tween20 (Sigma), and probed in PBS containing 0.3% BSA and 0.05% Tween20. Chemiluminescent development of membranes was performed using SuperSignal West-Dura substrate (Pierce). Antibodies specific for native Ad2 knob trimers were affinity purified from rabbit anti-serum by passage over a column of Ad2 knob trimers coupled to CNBr-activated Sepharose beads (Sigma-Aldrich) in bicarbonate buffer, using the method provided by the manufacturer. Bound antibodies were eluted in 0.1 M glycine buffer (pH 2.5) and neutralized immediately by addition of 0.1 volume of 1 M Tris base, essentially as described (Harlow and Lane 1988).

Mass spectrometry

Protein samples were dialyzed exhaustively against distilled water and then mixed with an equal volume of methanol containing 5% acetic acid immediately before analysis. Precipitates formed upon addition of methanol/acetic acid were removed by centrifugation. Samples were then injected into a

Bruker Apex 9.4 Tesla Apex-Qe Hybrid Qq-Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer (Bruker BioSciences Corp.). The sample was directly infused into the FT-ICR MS by nanoESI with a 30 μ m id fuse silica tip (New Objective, Inc.) at a 15 μ L/h flow rate. Bruker Daltonics Data Analysis software (v. 3.4) was used to analyze the data.

For LC-MS analysis, an HP 1100 series II LC system (Agilent Technologies) with a multi-wavelength photodiode array detector was coupled to a Bruker Esquire ion-trap mass spectrometer (MSD trap XCT system) equipped with an ESI. A reverse phase C18 (5 μ m, 4.6 $\times$ 250 mm) column (Phenomenex) was used to resolve the samples. The temperature of the column was kept at 25°C. The sample was eluted in 0.2% (v/v) formic acid with an increasing gradient of acetonitrile (0–8 min, 1%–5% [v/v]; 8–10 min, 5%–100% [v/v]) at a flow rate of 1 mL/min. Fifty percent of the eluant was spiked into MSD trap through a passive valve, and the positive-ion mass spectra were acquired.

Refolding Ad2 knob trimers *in vitro*

Ad2 knob was expressed without N-terminal His-tags, in BL21DE3 cells. Cells were disrupted by sonication, and insoluble inclusion bodies containing knob protein were isolated by centrifugation and washed as described (De Bernardez Clark et al. 1999). Twenty micrograms of wet weight of washed inclusion bodies was dissolved in 520 μ L of 6 M guanidine-HCl/10 mM DTT and incubated for 30 min at room temperature. The solution was then acidified to pH $\sim$ 3 by addition of 32 μ L of 1 M HCl. Insoluble material was removed by centrifugation for 10 min at 10,000g, and the supernatant was then divided in two equal parts. One part was applied to a 0.7 $\times$ 12 cm column of BioGel P30 medium (Bio-Rad Laboratories, Inc.) equilibrated in 50 mM sodium phosphate buffer (pH 7) containing 10 mM ThDP, 1 mM EDTA, and 1 mM DTT. The other part was loaded onto an identical column equilibrated in the same buffer without ThDP; 0.5 mL fractions were collected. The bulk of protein eluted in the void volume, in fractions 7–9. Knob trimer content in eluates was detected by immunoblot analysis of proteins separated by nondenaturing gel electrophoresis, using rabbit antibodies specific for native Ad2 knob trimers that were purified as described above.

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