Characterization of Ad5 E3–14.7K, an adenoviral inhibitor of apoptosis: Structure, oligomeric state, and metal binding

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(RECEIVED October 12, 2001; FINAL REVISION January 24, 2002; ACCEPTED February 4, 2002)

Abstract

The adenovirus E3–14.7K protein, expressed early in the life cycle of human adenoviruses to protect the virus from the antiviral response of host cells, inhibits cell death mediated by TNF- α and FasL receptors. To better understand its role in cell death inhibition, we have sought to characterize the biophysical properties of the protein from adenovirus serotype 5 (Ad5 E3–14.7K, or simply 14.7K) through a variety of approaches. To obtain sufficient quantities of recombinantly expressed protein for biophysical characterization, we explored the use of various expression constructs and chaperones; fusion to MBP was by far the most effective at generating soluble protein. Using limited proteolysis, mass spectrometry, and protein-protein interaction assays, we demonstrate that the C-terminal two-thirds of the protein, predicted to be composed of five β -strands and one α -helix, is highly structured and binds its putative cellular receptors. Furthermore, using atomic absorption and ultraviolet/visible spectroscopies, we have studied the metal binding properties of the protein, providing insight into the observation that cysteine/serine mutants of 14.7K lack in vivo antiapoptotic activity. Lastly, results from size exclusion chromatography, dynamic light scattering, sucrose gradient sedimentation, chemical crosslinking, and electron microscopy experiments revealed that 14.7K exists in a stable high-order oligomeric state (nonamer) in solution.

Keywords: Ad5 E3–14.7K; adenovirus; apoptosis; oligomerization; metal binding; proteolysis

Adenoviruses have evolved sophisticated molecular mechanisms for escaping their host's natural defense systems (Gooding and Wold 1990; Wold 1993; Wold et al. 1994,

1999; Krajcsi and Wold 1998; Horwitz 2001). This evasive strategy involves the encoding and expression of highly conserved proteins that interact with specific components of the host's immune system. To short-circuit the immune response early in the infection process, several immunoregulatory genes are encoded in the "early" E3 region of the adenovirus genome. The immunomodulatory properties of these proteins have elicited interest in their use for gene therapy, with some promising results (Harrod et al. 1998; Bruder et al. 2000; Doronin et al. 2001; Horwitz 2001).

An immunomodulatory adenoviral product that has garnered much attention is the highly conserved 14.7K protein. This protein was initially identified as an inhibitor of TNF-mediated inflammation and cytolysis (Gooding et al. 1990; Zilli et al. 1992; Tufariello et al. 1994a,b) and subsequently was shown to be a general inhibitor of cell death (apoptosis)

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Abbreviations: 14.7K, Ad5 E3–14.7K, a protein encoded in the E3 early region of the genome of adenovirus serotype 5 (gil58510); FIPs (1–3), 14.7K-interacting proteins; FLICE/caspase 8, a cysteine protease in the interleukin-1 β converting enzyme family; DED, death-effector domain; MBP, maltose-binding protein; GST, glutathione-S-transferase; TNF, tumor necrosis factor; ESI-MS, electrospray mass spectrometry; CD, circular dichroism; LB, Luria-Bertani; DTT, dithiothreitol; DMS, dimethyl sulfoxide; AEBSF, 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.4180102>.

regulated by the TNF and FasL receptor pathways (Chen et al. 1998; Li et al. 1998). The 14.7K protein is highly conserved across multiple adenovirus serotypes (Horton et al. 1990) (Fig. 1), underscoring its importance for adenoviral viability. Indeed, deletions or mutations in the 14.7K gene result in adenovirus-infected cells that are very susceptible to cytokine-activated cell death (Gooding et al. 1988, 1990; Horton et al. 1991; Ranheim et al. 1993; Sparer et al. 1996).

To elucidate the molecular mechanism for 14.7K-mediated cell death protection, yeast two-hybrid assays using 14.7K from adenovirus serotype 2 as bait have been used to identify possible cellular targets. These experiments have identified as putative sites of viral intervention a series of proteins called FIPs (fourteen-7K-interacting-proteins) (Li et al. 1997, 1998, 1999). The first of these, FIP-1, is a member of the low-molecular-weight GTP-binding protein family (Li et al. 1997), which has been shown to interact with cellular microtubule components (Lukashok et al. 2000). FIP-2 possesses putative leucine-zipper motifs and

when overexpressed, can reverse the protective effects of 14.7K. FIP-3 (NEMO/IKK γ) (Rothwarf et al. 1998; Yamaoka et al. 1998; Li et al. 1999) exhibits some sequence homology to FIP-2 but has been shown to interact with IKK β , RIP, and NIK, affecting NF- κ B activity (Li et al. 1999). The identification of FIP-3 as a modulator of NF- κ B activity and its direct interaction with 14.7K suggest that the adenoviral protein might inhibit cell death by interfering with NF- κ B-mediated transcription of apoptosis-regulatory genes (Li et al. 1999; Ye et al. 2000). How these putative cellular targets tie into the known apoptotic regulatory pathways remains unclear.

In a different study with 14.7K from adenovirus serotype 5, immunoprecipitation experiments revealed an interaction with the DEDs of FLICE/caspase 8 (Chen et al. 1998), a cysteine protease central to the Fas and TNF-R apoptotic regulatory pathways (Muzio et al. 1996). This result suggested an alternate pathway in which 14.7K employs a strategy similar to that of other viruses that inhibit the activation

Fig. 1. Sequence alignments of 14.7K proteins from various human adenovirus serotypes. The sequence alignment was generated with CLUSTALW (Higgins et al. 1992), the figure with ESPr1.9 (Gouet et al. 1999). Secondary structure prediction was obtained from the Predict Protein Server (<http://cubic.bioc.columbia.edu/predictprotein>) (Rost 1996) and PSIPRED server (<http://insulin.brunel.ac.uk/psipred>) (Jones 1999). Sequences are those from human adenovirus serotypes 5 (gil119065), 1 (gil2230741), 2 (gil119063), 15 (gil6957477), 3 (gil119064), 19 (gil6940697), 11 (gil423753), 9 (gil6940715), 35 (gil984538), 7 (gil119066), 8 (gil6940709), 12 (gil313383), 40 (gil9626583) and 41 (gil93533). *, sites of trypsin cleavage under native conditions; ^, cysteine residues in Ad5–14.7K which when mutated to serine result in loss of cell death protection; •, Cys/Ser mutations with near wild-type phenotype (Ranheim et al. 1993).

of FLICE via DED-containing proteins, termed FLICE inhibitory proteins (FLIPs) (Thome et al. 1997). However, direct inhibition of FLICE activation has not been demonstrated, and 14.7K lacks a DED sequence or other motif known to mediate cell-death signaling.

Confusion as to the relevant cellular target and absence of a significant homology to proteins with known structures leaves its mechanism of action unclear. To obtain structural insight into its mechanism of cell death inhibition, we sought to characterize 14.7K from adenovirus serotype five by biochemical and biophysical methods (Kim 2000). To that end, we have (1) developed an efficient scheme for expression and purification of soluble protein, (2) found that 14.7K contains a proteolytically-resistant, C-terminal domain that retains the capacity to interact with FIP-1 and the DEDs of FLICE, (3) demonstrated that 14.7K is highly oligomerized, and (4) shown that 14.7K binds zinc with a 1:1 stoichiometry.

Results

Unfused 14.7K is insoluble; fusion to MBP yields soluble protein

The 14.7K protein is largely insoluble when overexpressed in *Escherichia coli* in the absence of the solubilizing proteins, thioredoxin or GroEL/GroES, or fusion to MBP (Fig. 2). ESI-MS of recombinantly expressed wild-type 14.7K (Kim 2000) showed that the samples contained a mixture corresponding to full-length protein (Met¹ through Asn¹²⁸; 40%), and three posttranslationally modified forms (Bradshaw et al. 1998): methionine-excised protein (Thr² through Asn¹²⁸; 40%) and N-acetylated species of both proteins

(10% each). Introduction of an additional methionine at the amino terminus effectively eliminated excision of the amino-terminal methionine as well as N-acetylation (Hirel et al. 1989; Kim 2000).

While wild-type 14.7K was highly overexpressed in *E. coli*, to obtain sufficient amounts for biophysical experiments, an elaborate refolding procedure was necessary (see Materials and Methods), raising questions as to whether the observed properties were artifacts of the refolding procedure. The protein obtained in this fashion was soluble to ~1 mg/mL. In parallel with investigations on refolded protein, a method to obtain large quantities of solubly expressed 14.7K was pursued. As shown in Figure 2, wild-type (14.7K), histidine-tagged (H₆-14.7K), and GST-tagged (GST-14.7K) proteins are almost exclusively present in the insoluble pellet (P). Fusion to thioredoxin (Trx-H_p-14.7K) or coexpression of thioredoxin (14.7K/Trx) or GroEL/GroES (14.7K/GroE) slightly increased soluble protein expression, although coexpression reduced the overall amounts of protein produced. Only fusion to MBP (MBP-14.7K) caused a dramatic increase in the amount of protein present in the soluble fraction (S), while retaining a high level of expression (Fig. 2).

Although MBP-fused 14.7K is soluble at high concentrations (>30 mg/mL), upon removal of MBP by thrombin cleavage, the solubility of 14.7K reverts to that of the unfused protein. Fusing MBP to the DED of FLICE similarly increased its solubility, although upon thrombin cleavage the DED solubility decreased dramatically (data not shown). In addition, while the MBP tag is almost three times the molecular weight of 14.7K, as evidenced by gel filtration (Kim 2000), the fusion protein exhibited the same oligomeric state as unfused 14.7K (see below).

Fig. 2. Effect of protein tag and/or coexpression partner on expression of 14.7K protein in *Escherichia coli*. SDS-PAGE analysis of whole-cell uninduced (U), induced (I), or soluble (S) and insoluble (P) fractions of cell lysate. The 14.7K bands are indicated by arrows, while the “*” indicate migration positions of the coexpressed protein chaperones, thioredoxin (Trx), GroEL/GroES (GroE), and MBP. The lanes marked 14.7K correspond to expression of unfused 14.7K from pET21a (Novagen); H₆-14.7K, a histidine-tagged construct expressed in pET28a (Novagen); GST-14.7K, a GST fusion expressed in pGEX-2T (Pharmacia); Trx-H_p-14.7K, a fusion of 14.7K with thioredoxin containing “His-patch” in plasmid pTrcHisA (Invitrogen); MBP-14.7K, a fusion of 14.7K with MBP expressed in vector pIH1119 (New England Biolabs); 14.7K/Trx, coexpression of 14.7K/pET21a with thioredoxin pTRX; 14.7K/GroE, coexpression with GroEL/GroES (Yasukawa et al. 1995).

Bacterially expressed, refolded 14.7K is structured

CD spectroscopy was used to examine whether recombinantly expressed 14.7K had well-defined secondary structure. The CD spectrum of 14.7K showed that it contains a significant portion of α -helix and β -strand features (Fig. 3). Deconvolution analyses using the programs K2D (Andrade et al. 1993) and CDNN (Bohm et al. 1992) indicate an approximate composition of 33% α -helix, 33% β -strand, and 33% coil in agreement with PHDsec prediction (Rost and Sander 1993b) (Fig. 1). Solubly expressed (via thioredoxin coexpression) or refolded 14.7K were indistinguishable by CD spectroscopy (Kim 2000) or other measurements (below), indicating that the native secondary structure is regained upon refolding.

14.7K contains a protease-resistant C-terminal domain

To delineate structurally distinct domains 14.7K was subjected to limited proteolysis with trypsin or α -chymotrypsin. SDS-PAGE analysis of the products of limited trypsin digestion of solubly expressed (via thioredoxin coexpression) or refolded 14.7K revealed two protease-resistant fragments (Fig. 4). A 12-kDa band appeared after initial digestion with 0.1% trypsin (w/w), and an 11-kDa band appeared as the digestion progressed and/or the percentage of trypsin was increased. No further degradation of the 11-kDa fragment was observed, even when cleavage reactions were performed in the presence of 1–2 M urea (Kim 2000).

Fig. 3. CD spectra of 14.7K and its fragments. Solid line, 14.7K; dotted line, C-terminal tryptic fragment (residues 31–128); dashed line, N-terminal recombinant peptide (2–43). Spectra were acquired in 15 mM sodium borate (pH 8.0), 150 mM NaF, 1 mM DTT.

Fig. 4. Trypsin digestion of 14.7K. Time course for trypsin digestion of 14.7K monitored by SDS-PAGE. The protein was subjected to trypsin digestion for the indicated time using either 0.1 or 1% trypsin (w/w) in 15 mM sodium borate (pH 8.0) containing 0.45 M NaCl and 1 mM DTT. Cleavage is observed at Arg23 followed by slower cleavage at Arg30. Cleavage experiments with refolded or solubly expressed 14.7K (coexpressed with thioredoxin) produced identical results.

ESI MS revealed that the tryptic products corresponded to C-terminal fragments consisting of residues 24–128 (11,815 Da observed, 11,811 expected) and 31–128 (11,059 Da observed, 11,056 expected). The 31–128 fragment was not further digested under the conditions used, although there are 12 additional trypsin cleavage sites (K/R) within residues 31–128. α -Chymotrypsin also did not further digest the C-terminal fragment, although it possesses 13 potential cleavage sites (W/Y/F/L). This proteolytic pattern indicates that the predicted N-terminal helix (~15–42) is disrupted near residue 24, and that residues 31–128 are structured in solution and inaccessible to the enzymes. The CD spectrum of the C-terminal proteolytic fragment showed that the fragment had lost some α -helix content, as monitored by ellipticity at 208 and 222 nm, relative to the full length 14.7K (Fig. 3). The solubility of the trypsin-generated, C-terminal fragment was similar to that of full-length 14.7K (or $\sim$ <1 mg/mL).

On the other hand, a recombinantly expressed N-terminal peptide comprising residues 2–43 was highly soluble (>5 mg/mL) and its CD spectrum of exhibited signals consistent with significant α -helical content (Fig. 3). However, 2D ^1H NOESY NMR spectra (Kim 2000) contained no sequential (i, i+1) backbone amide proton NOEs, indicating the α -helical content of the isolated peptide is probably transient.

Recombinant 14.7K and its C-terminal region are capable of interacting with FIP-1 and FLICE

Binding assays were performed to determine whether the refolding procedure yielded recombinant protein capable of binding its putative cellular targets, FIP-1 and FLICE, and to investigate whether the 14.7K proteolytic fragment retained the same capacity. Our results indicate that refolded 14.7K is capable of specifically interacting with FIP-1 (Fig. 5). Further, 14.7K:31–128 is able to bind both FIP-1 and the N-terminal death-effector-containing domain of FLICE (1–

Fig. 5. In vitro binding of 14.7K to FIP-1 and FLICE. Both full-length 14.7K and the 14.7K:31–128 were able to selectively bind (A) GST-FIP-1 or (B) GST-FLICE:1–271 over equal amounts of GST control. No interaction was observed between the amino-terminal helix (GST-14.7K:2–43 and the C-terminal fragment [Ad5:31–128]).

271), although it also exhibits weak nonspecific binding to GST. In contrast, the amino-terminal domain (residues 2–43) did not bind itself, GST-FIP-1, GST-FLICE:1–271 (Kim 2000), or indeed to 14.7K:31–128 (Fig. 5).

14.7K and its C-terminal fragment (31–128) are highly oligomerized

Size exclusion chromatography was used to test whether extreme line broadening observed in ^1H NMR spectra of 14.7K (not shown) was the result of oligomerization. In size exclusion chromatography, 14.7K (refolded or coexpressed with thioredoxin) eluted as a single symmetrical peak with a Stokes radius of 51 Å (Fig. 6). This radius is much too large for a monomeric globular protein of 14.7 kDa, rather it would correspond to a globular protein with a molecular weight of ~192 kDa. The Stokes radius of the tryptic fragment (14.7K:31–128) was similarly large (37 Å), corresponding to a 73-kDa globular protein. These data suggested that both 14.7K and the C-terminal fragment were highly oligomerized in solution. Assuming globular shapes and uniform hydration, these apparent masses would be consistent with oligomers with 14 monomers (14×14.7 kDa) for the full-length protein and seven monomers (7×11.1 kDa) for the 31–128 fragment.

To ascertain whether oligomerization of 14.7K is intrinsic or nonspecific, the effects of protein and salt concentration on elution from a size exclusion column were tested. The multimeric state was independent of salt concentration in the range of 0.08 to 1M NaCl, or protein concentration in the range of 1 to 50 μM . Furthermore, solubly expressed MBP-14.7K (~55 kDa) eluted from a gel filtration column near thyroglobulin (680 kDa) while thrombin cleavage of MBP-14.7K produced monomeric MBP and oligomeric 14.7K, indicating that the latter drives oligomerization of the fusion protein (Kim 2000). However, denaturants had a clear effect on oligomerization, as pretreatment of 14.7K with 1–5 M urea prior to elution on a size exclusion column

demonstrated the multimers could be 50% dissociated in ~1.5 M urea (Fig. 7a). In contrast, CD spectra and protease resistance data (above) indicate the protein is still folded under these conditions. Urea-induced unfolding of 14.7K, monitored from ellipticity at 220 nm (Fig. 7b) revealed a half-denatured urea concentration, $[D]^{50\%}$ of ~3.8 M, with the protein remaining >90% folded in 0–2.5 M urea; salt modestly increased the cooperativity of unfolding (m_{D-N}) by 50% over the range of 0.08–0.45 M NaCl. Thus, urea-in-

Fig. 6. Gel filtration of 14.7K and its C-terminal fragment. Solid line, 14.7K; dotted line, 14.7K:31–128. Elution positions of protein standards are indicated by filled triangles in the order: Blue Dextran 2000 (V_0), ferritin, aldolase, BSA, ovalbumin, and chymotrypsinogen. (Inset) Stokes radii of 14.7K and 14.7K:31–128 (squares) from calibrations with the protein standards.

Fig. 7. Effect of denaturants on 14.7K. (A) Fraction oligomeric versus urea concentration indicates a $[D_{\text{olig}}]^{50\%}$ of ~ 1.4 M; data were fit assuming a two-state model. (Inset) size exclusion chromatography (A_{280}) after pretreatment of 14.7K with various concentrations of urea (0, 1, 1.5, 2, 5 M; circles, squares, diamonds, crosses, and triangles, respectively). (B) CD urea denaturation curves. Open circles, 0.08 M NaCl; open squares, 0.15 M NaCl; filled circles, 0.3 M NaCl; filled squares, 0.45 M NaCl. Secondary structure (θ_{220}) denaturation $[D_{\text{str}}]^{50\%}$ occurs at ~ 3.8 M urea.

duced dissolution of the oligomer appears to precede unfolding.

Oligomerization of 14.7K and its C-terminal fragment (14.7K:31–128) was further examined by chemical crosslinking using DMS, a nonspecific reagent that targets primary amines. SDS-PAGE of 14.7K reacted with 14 mM DMS for different lengths of time showed the presence of

multimeric states of at least a heptamer (Fig. 8a). Similarly, DMS crosslinking of 14.7K:31–128 indicates the presence of multimeric states above a hexamer (Fig. 8b). However, the crosslinking data did not enable the unambiguous determination of the native oligomeric state of either protein. Intermolecular disulfide bonds were not found to be responsible for the stability of oligomeric 14.7K complexes, as the absence or presence of 100 mM of the reducing agent DTT had no effect on migration in size exclusion or SDS-PAGE (Kim 2000).

To obtain a more accurate estimate of the oligomeric state of 14.7K and 14.7K:31–128, diffusion and sedimentation coefficients were measured by dynamic light scattering and sucrose gradient sedimentation, respectively. (Analytical ultracentrifugation experiments also were performed to obtain more precise mass measurements, but absence of a strong chromophore and low solubility rendered those results unreliable.) The diffusion coefficients obtained from light-scattering measurements were consistent with the large differences in their Stokes radii, i.e., diffusion through the size exclusion matrix (Table 1). Sedimentation coefficients obtained from 5–20% and 10–40% sucrose gradients were reproducibly similar for both 14.7K and 14.7K:31–128 (Table 1). When combined via the Svedberg equation ($s = M(1 - \bar{v}_2\rho)/N_0f$), the hydrodynamic data indicate that the native molecular weights of 14.7K and 14.7K:31–128 are in the range of 129 and 95 kDa, respectively, corresponding to oligomeric states of ~ 9 for both proteins. These data suggest that the large decrease in Stokes radius upon deletion of the amino-terminal 30 residues results from a significant change in shape and/or hydration (i.e., becoming more compact), rather than from a two-fold change in oligomeric state.

14.7K particles are visible by transmission electron microscopy

Because hydrodynamic experiments indicated an aggregate molecular weight of ~ 130 kDa and an effective diameter of ~ 10 nm (14.7K $R_s \sim 5$ nm), transmission electron microscopic images were obtained to visualize 14.7K. The two-dimensional images of 14.7K on the stained grid revealed regular globular particles (Fig. 9). The resolution of the images was not sufficiently high to observe the subunits of the oligomeric protein; however, measurements of 100 particles yielded a mean diameter of 11.7 ± 1.3 nm, consistent with the hydrodynamic data.

14.7K is a zinc-binding protein

The 14.7K contains several cysteine and histidine residues that are highly conserved among adenovirus serotypes (Fig. 1). Atomic absorption spectroscopy of MBP-14.7K (and MBP as a control) produced from rich (LB) media and

Fig. 8. Chemical crosslinking of (A) 14.7K and (B) 14.7K:31–128 with DMS. Crosslinking experiments were carried out for the indicated time periods in 20 mM sodium borate buffer (pH 8.34) containing 0.45 M NaCl and 2 mM MgCl_2 .

purified without added zinc indicated that the protein binds zinc with a 1:1 stoichiometry (1.23 ± 0.01 mol zinc/mol protein). To further probe the metal-binding properties of 14.7K, the fusion protein was expressed in minimal media supplemented with cobalt, a useful spectroscopic probe of metal coordination centers (Maret and Vallee 1993). MBP-14.7K isolated from cultures supplemented with 100 mM cobalt (but purified without added metal) was blue and exhibited ultraviolet (UV)/visible spectra (Fig. 10) characteristic of a Co(II)-substituted protein with four thiolate ligands (CoS_4) (Maret and Vallee 1993). In addition, the protein exhibited metal-dependent expression, as bacterial cultures expressed and induced in minimal media in the

absence of Zn^{2+} or Co^{2+} produced negligible amounts of protein (data not shown).

Discussion

Solubility of recombinantly expressed proteins is essential for their biophysical characterization and represents a major obstacle to the progress of structural genomics (Edwards et al. 2000). MBP previously has been shown to be remarkably effective at solubilizing recalcitrant proteins (Kapust and Waugh 1999), a result we corroborate here, with both 14.7K

Table 1. Hydrodynamic parameters for 14.7K and 14.7K:31–128

	14.7K	14.7K:31–128
M_r (monomer)	14,598 Da	11,059 Da
R_s (nm) ^a	5.00 ± 0.19	3.67 ± 0.19
$D_{20,w}$ ($\times 10^{-7}$ cm ² sec ⁻¹) ^b	4.15 ± 0.69	5.9 ± 0.16
$S_{20,w}$ ($\times 10^{-13}$ sec) ^c	6.03 ± 0.69	6.16 ± 0.61
$\bar{v}_2$ (mL g ⁻¹) ^d	0.726	0.731
M_r (oligomer) ^e	129 ± 24 kDa (8.8 ± 1.6 -mer)	94.5 ± 4.8 kDa (8.5 ± 0.4 -mer)

^a Measured by size exclusion chromatography.

^b Determined from dynamic light scattering.

^c Determined from sucrose gradient. Error estimates are the standard deviations of three experiments.

^d Partial specific volume, $\bar{v}_2$, estimated from amino-acid composition (Cantor and Schimmel 1980; McMeekin and Marshal 1952; Perkins 1986).

^e $M_r = sRT/[D(1 - \bar{v}_2\rho)]$; $R = 8.31 \times 10^7$ erg mole⁻¹ deg⁻¹; erg = g cm² s⁻². ρ , density of solvent is taken to be 1 g/mL.

Fig. 9. Negatively stained transmission electron micrograph of 14.7K; mean particle diameter is 11.7 ± 1.3 nm.

Fig. 10. Ultraviolet/visible spectrum of Co^{2+} -MBP-14.7K acquired at a protein concentration of 1.4 mg/mL (25 μM). This profile is consistent with Co(II) chelated by four thiolate ligands (Maret and Vallee 1993).

and the DED of FLICE. Although fusion to MBP effectively produced soluble proteins without affecting the oligomeric state of 14.7K or its ability to bind its protein targets, the enhanced solubility was lost upon proteolytic removal of the 42-kDa tag. This suggests that the utility of fusion to MBP for NMR-based structural genomics will continue to be limited by the inherent properties of the construct. However, by use of a short linker between MBP and a human T-cell leukemia virus type 1 gp21 ectodomain fragment, the feasibility of crystallizing intact MBP-chimeras has been demonstrated (Center et al. 1998). We predict that MBP-fusions will continue to facilitate mechanistic/functional experiments and may provide an effective route to expanding the suitability of proteins for crystallography; we currently are pursuing such an approach for 14.7K.

The biochemical and biophysical insights reported here represent an important step toward understanding the structure and function of 14.7K. The proteolysis data demonstrate that residues 31–128 are well protected from cleavage, but that Arg^{24} in the amino terminus is accessible. Secondary structure predictions (PHDSec and PSI-PRED) (Rost and Sander 1993a; Jones 1999) and CD spectra of 14.7K:2–43 suggest a helix extends from Ile^{13} through His^{42} . However, from a lower PSI-PRED confidence level between Arg^{23} and Ala^{26} , together with the observed trypsin cleavage at Arg^{23} (generating 24–128), we infer that the N-terminal helix is disrupted at this point. Because slow cleavage at Arg^{30} takes place only after cleavage at Arg^{23} , we propose the first cleavage somewhat destabilizes the remaining helix, allowing the protease to access an otherwise structured residue. None of 12 remaining trypsin sites or five chymotrypsin sites is protease accessible, suggesting

that with the exception of a few loops, the entire protein adopts a compact, folded structure.

The protein-binding experiments have shown that the determinants for recognition of FIP-1 and FLICE are retained within residues 31–128. The primary sequence of this highly conserved region of the protein is predicted to be composed of β -strands connected by short coil segments, with an α -helix at the extreme C terminus (Fig. 1); CD spectra are consistent with this prediction. Thus, recognition of FIP-1 and FLICE appears to be mediated by the core β -strands and/or C-terminal helix. However, because *in vivo* data suggest that the entire protein is required for antiapoptotic activity (Ranheim et al. 1993), it appears that the interaction of 14.7K with FIP-1 and/or FLICE is not sufficient for its antiapoptotic effects. Rather, the amino-terminal helical segment has an important function unrelated to overall protein structure or FIP-1/FLICE binding.

Our discovery that 14.7K binds divalent metals (Zn^{2+} , Co^{2+}) may help explain the observation that the antiapoptotic activity of 14.7K is lost upon mutating to serine the three invariant cysteine residues (Ranheim et al. 1993), which are located in the C-terminal protease-resistant domain of 14.7K (Cys^{44} , Cys^{50} , Cys^{119} ; Fig. 1). These mutants, presumed to have compromised metal-binding ability, are poorly expressed in mammalian cells (Ranheim et al. 1993) (M. Horwitz, pers. comm.), consistent with the observed metal-dependent expression in *E. coli*. While these observations implicate these residues in metal binding, more work is needed to fully characterize the metal-binding site in detail and to investigate the possible structural and/or functional role of the bound metal.

Finally, we have found that 14.7K adopts a stable multimeric structure in solution. Size exclusion chromatography revealed Stokes radii corresponding to oligomers of ~ 14 and 7 monomers for the full-length protein and C-terminal proteolytic fragment, respectively. This observation led to the preliminary hypothesis that the amino-terminal helical segment was responsible for dimerizing two heptameric assemblies. However, diffusion and sedimentation measurements together suggested that in fact both the full-length and C-terminal fragments adopt nonameric complexes (9-mers) with different overall shapes/degrees of hydration. The 14.7K oligomers were large enough to be observed on negatively stained electron micrographs, with individual particles having an apparent radius of ~ 5 nm. The multimeric state of the protein is independent of whether the protein is isolated in a soluble form through coexpression of or fusion to a protein “chaperone,” or refolded after resolubilizing from inclusion bodies with denaturants. Oligomerization of 14.7K has been detected in mammalian cells by cotransfection with vectors expressing wild-type and epitope-tagged 14.7K (J. Bruder, pers. comm.), but the stoichiometry of these oligomers remained unknown. The stability of the 14.7K oligomer and its persistence through a range of

protein concentrations, buffers, salts, and reducing agents suggest the oligomeric state identified here is the same as in adenovirus-infected cells.

Although the physiological effects of 14.7K have been recognized for over a decade (Tollefson and Wold 1988; Gooding et al. 1990; Horton et al. 1991) and its potential for use in gene therapy applications is being actively explored (Harrod et al. 1998; Bruder et al. 2000; Doronin et al. 2001; Horwitz 2001), progress toward understanding its mechanism of action has been slow. While several putative cellular targets have been proposed, poor solution characteristics and the lack of a recognizable structural motif that might provide clues to its mechanism of action have contributed to a paucity of structure/function information. Because 14.7K represents a novel class of antiapoptotic proteins and may represent a novel structural fold, continued structure/function analyses are warranted. This work provides a critical methodological and conceptual foundation for a detailed understanding of the structure and function of this interesting protein.

Materials and methods

Plasmids

Several plasmid vectors were constructed to overexpress 14.7K in *E. coli*. The gene encoding 14.7K from the E3 region of the adenovirus genome was amplified using PCR with plasmid pMT2 (generously provided by W.S.M. Wold, St. Louis University) as the template and the resulting PCR products were subcloned into *Nde* I and *Eco*R I sites of pET21 or pET28a (Novagen) for expression of wild-type or His-tagged protein, respectively. The mutant, 14M2 was constructed by inserting an extra methionine codon (ATG) at the start of the 14.7K gene. Fusions of 14.7K and 14.7K:2–43 were obtained by subcloning the appropriate PCR products into *Bam*H I and *Eco*R I sites of pGEX-2T (Pharmacia). The fusion of 14.7K with MBP was constructed by subcloning into the multiple cloning region of a plasmid derived from pIH1119 (generously provided by P. Riggs, New England Biolabs). Thioredoxin-fused 14.7K (pTrxHisA, Invitrogen) was provided by J. Bruder (Genvec, Inc.).

Fusions of the N-terminal death-effector-containing domain of FLICE with GST and MBP were constructed by first eliminating an internal *Eco*R I site from the vector pThioAnFLICE (J. Bruder, GenVec) followed by subcloning into *Bam*H I and *Eco*R I sites of pGEX-2T and the modified pIH1119 vector, respectively. A plasmid expressing GST-fused FIP-1 was obtained from M. Horwitz (Albert Einstein University) and used without modification (Li et al. 1997).

The sequences of the inserts in all the plasmid constructs were verified by automated DNA sequencing.

Expression and purification

Recombinant 14.7K was expressed in *E. coli* BL21(DE3) cells (Novagen), grown in a shaker incubator at 37°C in LB media supplemented with the appropriate antibiotics, and induced for 3 h by addition of 1 mM IPTG at an OD₆₀₀ of 0.6–0.8. To examine expression yield and solubility, cells were sonicated in lysis buffer

(100 mM Tris-HCl, pH 8.0 at 4°C, 0.1 M NaCl, 1 mM DTT, 0.2 mM AEBSF, and 0.5 µg/mL leupeptin). Soluble and insoluble fractions were separated by centrifugation and were boiled in Laemmli buffer, alongside equal amounts (from OD₆₀₀) of uninduced and induced whole-cell pellets, loaded onto 10–20% gradient SDS-PAGE (BioRad) and visualized by staining with Coomassie Brilliant Blue R-250 (Fisher Scientific).

Refolding 14.7K

Cell pellets containing insoluble 14.7K were resuspended in lysis buffer containing 10 mM EDTA and lysed by sonication. Subsequently, inclusion bodies were collected by centrifugation at 12,000g for 30 min at 4°C and solubilized by stirring at 4°C in denaturing buffer (20 mM sodium phosphate, pH 6.4, 6 M urea, 5 mM DTT). The urea-solubilized fraction was clarified by centrifugation (20,000g for 1 h, 4°C) and eluted from an SP-Sepharose column (Pharmacia) by a linear NaCl gradient (0.05–1 M).

Fractions containing 14.7K were refolded as follows: first, denatured 14.7K was diluted and adjusted to pH 8.5 (20 mM Tris-HCl, 140 mM NaCl, 20 mM sodium phosphate, 2 mM MgCl₂, and 25 µM ZnSO₄), then the urea concentration was decreased slowly (3 h, 4°C) to 2 M by drop-wise addition of dilution buffer (20 mM Tris-HCl [pH 8.5], 140 mM NaCl, 2 mM MgCl₂, 25 µM ZnCl₂, 2 mM oxidized glutathione, and 4 mM reduced glutathione) (Noel et al. 1991). Next, the sample was brought to room temperature and gently stirred for 8 h. Remaining urea in the sample was removed by sequential dialysis (at 4°C) in 10–12 volumes of dialysis buffer containing decreasing concentrations of urea (1, 0.5, 0 M). Precipitates were removed by centrifugation and the protein was further purified to >95% by elution from a Q-Sepharose column (Pharmacia) with a linear gradient of 0.1–1 M NaCl. Protein concentrations were estimated from extinction coefficients calculated for the denatured state ($\epsilon_{276} = 2900 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{280} = 2560 \text{ M}^{-1} \text{ cm}^{-1}$).

Solubilizing 14.7K by coexpression with TRX or GroES

E. coli BL21(DE3) cells were doubly transformed with plasmids expressing genes encoding 14.7K (pET21-Ad5 14.7K) and thioredoxin or GroES/L (Yasukawa et al. 1995) and cells cultured with the antibiotics appropriate to each vector. The purification procedure was adapted from a method developed for extracting Ad2–14K from HeLa cells (Persson et al. 1978).

Soluble MBP-14.7K

E. coli cells containing overexpressed MBP-14.7K were lysed by sonication and clarified by centrifugation. The supernatant was adsorbed onto amylose-Sepharose resin (New England Biolabs), packed into a gravity column and bound MBP-14.7K was eluted with 20 mM maltose. To remove the MBP tag, thrombin cleavage of MBP-14.7K was carried out with 1% (w/w) thrombin (Sigma) in cleavage buffer (50 mM Tris-HCl (pH 8.4) 0.15 M NaCl, 2.5 mM CaCl₂, and 1 mM DTT) and terminated by addition of AEBSF (1 mM).

GST-14.7K:2–43 (N-terminal fragment)

Cell pellets containing GST-14.7K:2–43 were lysed in NETN buffer (50 mM Tris-HCl [pH 8 at 4°C], 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5 mM DTT, 0.2 mM AEBSF, and 0.5 µg/mL

Leupeptin) and adsorbed onto a glutathione-Sepharose 4B column (Pharmacia), cleaved by thrombin, eluted from the column and further purified by gel filtration (TSK G2000SW, 21.5 × 300 mm, 3 mL/min, TosoHaas) in 20 mM sodium phosphate (pH 7.0, 0.1 M NaCl).

Co²⁺-14.7K

Co²⁺-MBP-14.7K was obtained by culturing cells in M9 minimal media supplemented with vitamins (GIBCO Eagle Basal Vitamin Mix) and 100 μM CoSO₄. Cells were induced and harvested as above and the protein purified as for MBP-14.7K obtained from rich media, with the exception that DTT was excluded from the buffers. UV/visible spectra of the purified protein were recorded at 1.4 mg/mL (25 μM) on an HP-8452A diode array spectrophotometer in a 1-cm path length cell. The water used for these experiments was deionized with a Milli-Q water system (Millipore).

Trypsin proteolysis

Aliquots of 14.7K (0.5 mg/mL, in 20 mM Tris-HCl pH 8.4, or 15 mM sodium borate pH 8.0, containing 0.15–0.45 M NaCl and 1 mM DTT) were mixed at room temperature with 0.1–2% (w/w) trypsin (Sigma) previously treated with 100 μg/mL TCPK (Sigma). Aliquots of proteolyzed 14.7K were subjected to SDS-PAGE or to liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) for mass analysis of trypsin-produced fragments. For detailed biophysical analysis of 14.7K:31–128, the tryptic fragment was further purified on a Superdex-200 HR column (16 × 600 mm; Pharmacia).

CD measurements

Purified proteins were dialyzed overnight at 4°C in 15 mM sodium borate buffer (pH 8.0, 0.15 M NaF, 1 mM DTT). CD spectra were recorded in the range of 190–260 nm at 20°C on an AVIV Model 62A DS. Urea denaturation was monitored by recording ellipticity at 220 nm, 20°C, over a urea concentration of 0–8 M on a Jasco J-500A spectropolarimeter. Urea-induced unfolding thermodynamics were analyzed as a function of NaCl concentration (0.08–0.45 M) by assuming a two-state unfolding model (Clarke and Fersht 1993; Fersht 1999).

Protein-binding assay

Cell pellets containing GST, GST-FIP1, or GST-FLICE:1–271 were lysed in NETN buffer, clarified by centrifugation, and nuted with glutathione-Sepharose 4B resin for 3 h at 4°C; unbound proteins were eluted with NETN buffer. Purified 14.7K, 14.7K:31–128 or 14.7K:1–43 were mixed with aliquots of immobilized GST-FIP1, GST-FLICE:1–271, or GST for 3 h at 4°C. Unbound proteins were removed by extensive washing with NETN buffer containing 0.15 M NaCl (3×), 0.45 M NaCl (2×) and 0.15 M NaCl (2×). Bound proteins were visualized by boiling the resin in Laemmli buffer, separating by SDS-PAGE and staining with Coomassie Brilliant Blue R-250.

Zinc atomic absorption spectroscopy

MBP-14.7K and MBP samples obtained from growth in LB but purified without added zinc were dialyzed overnight in buffer con-

taining 20 mM Tris-HCl (pH 8.4 at 4°C), 0.2 M NaCl, and 1 mM DTT. Using a Perkin Elmer Z5000 graphite furnace atomic absorption spectrometer, zinc atomic absorption of a protein sample was measured at 213.9 nm in the peak area mode during 5 sec after 20 μL injections. Zinc concentrations in serial dilutions of the protein samples were calculated by comparison to solutions of known concentrations (0–40 μg/L) prepared by diluting a zinc atomic absorption standard solution (Perkin Elmer; Cat. No. 766). The zinc content in MBP-14.7K and MBP was measured eight and three times, respectively.

Size exclusion chromatography

The 14.7K, its tryptic fragment, MBP, MBP-14.7K, and thrombin-treated MBP-14.7K (15 mM sodium borate, pH 8.0, 0.45 M NaCl, 2 mM MgCl₂, and 1 mM DTT) were subjected to size exclusion chromatography on Superdex-200 or Superdex-75 gel filtration columns (Pharmacia). Diffusion coefficients ($D_{20,w}$) and Stokes radii (R_s) were determined from regression analysis of K_{av} versus the hydrodynamic parameters of protein standards (Siegel and Monty 1966; Cantor and Schimmel 1980). The protein standards used were ferritin (450 kDa, R_s 61.0), catalase (240 kDa, R_s 52.2, $D_{20,w}$ 4.1), aldolase (158 kDa, R_s 48.1, $D_{20,w}$ 4.7), BSA (67 kDa, R_s 35.5, $D_{20,w}$ 6.1), ovalbumin (44 kDa, R_s 30.5, $D_{20,w}$ 7.4), and chymotrypsinogen (25 kDa, R_s 20.9, $D_{20,w}$ 9.5) (Pharmacia). The effect of denaturants on oligomerization was assayed by pretreating aliquots of 14.7K (~10 μM) with urea (0–5 M) prior to injection on a Superdex-75 column (7.8 × 300 mm; 1 mL/min flow rate).

Chemical crosslinking

Crosslinking experiments with 14.7K or 14.7K:31–128 were performed in 14 mM DMS (dimethyl sulfoxide-2 HCl, Pierce) (20 mM sodium borate, pH 8.99, 0.45 M NaCl, 2 mM MgCl₂, 1 mM DTT). Aliquots of 20 μL of protein (~65 μM) and 5 μL of DMS (70 mM) were nuted at room temperature for 15, 30, 60, 90, or 120 min and analyzed by SDS-PAGE (Fig. 8).

Dynamic light scattering

Right-angle light scattering was performed using a DynaPro-801 dynamic light scattering/molecular sizing instrument (Protein Solutions). The scattering of 14.7K (37 μM in 20 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 2 mM MgCl₂, 1 mM DTT) and 14.7K:31–128 (70 μM in 15 mM sodium borate, pH 8.0, 0.45 M NaCl, 2 mM MgCl₂, 1 mM DTT) were measured 11 to 18 times at 23°C. Light scattering data were analyzed using the Dynamics and DynaLS software programs (Protein Solutions) and were found to be consistent with monodisperse solutions; diffusion coefficients (D) were corrected to 20°C by $D_{20,w} = D \cdot (293/T) \cdot (\eta_{T,w}/\eta_{20,w})$, where η is solvent viscosity (η).

Sucrose gradient sedimentation

The sedimentation coefficients ($s_{20,w}$) of 14.7K and 14.7K:31–128 were determined by using linear gradients of 5–20% or 10–40% sucrose (v/v) (20 mM Tris-HCl, pH 8.0 at 4°C, 0.45 M NaCl, 2 mM MgCl₂, 1 mM DTT). One milliliter of a sample mixture containing protein standards and 14.7K (or trypsin-treated 14.7K) was layered on each sucrose gradient. After sedimentation at 160,000g (L7–55 Ultracentrifuge, Beckman) for 19 h at 4°C, mi-

gration distances were obtained by a Gaussian fit of SDS-PAGE band intensities. Sedimentation coefficients of 14.7K and 14.7K:31–128 were obtained by fitting the $s_{20,w}$ values of the standards versus their migration distance (Table 1) (Martin and Ames 1961). Measurements were performed in triplicate.

Oligomeric masses

Oligomeric masses (M) of 14.7K and 14.7K:31–128 were calculated with the Svedberg equation ($M = sRT/[D(1 - \bar{v}_2\rho)]$) combining the diffusion coefficients (D) obtained from light scattering and sedimentation coefficients (s) from sucrose-gradient sedimentation (Table 1); protein partial specific volumes ($\bar{v}_2$) were estimated from amino-acid compositions (McMeekin and Marshall 1952; Siegel and Monty 1966; Cantor and Schimmel 1980; Perkins 1986), and ρ (density of medium) was taken to be that of water.

Transmission electron microscopy

Transmission electron microscopy of 14.7K (0.6 mg/mL in 20 mM Tris pH 8.0, 0.33 M NaCl, 2 mM $MgCl_2$, and 1 mM DTT) was performed by sequentially floating FORMVAR-coated copper grids (400 mesh/inch, Ted Tella, Inc.) on a drop of the wetting reagent, bacitracin (50 μ g/mL; 1 min), a drop of 14.7K solution (3 min), and then negatively stained with 2% phosphotungstic acid (PTA, pH 7.4, 2 min). After air drying, the sample was observed in a Phillips CM 12 at a magnification of 100,000 and accelerating voltage of 60 kV. The TEM image was photographed with 2.5-fold enlargement and scanned for analysis (Fig. 9).

Acknowledgments

We thank W. Wold (St. Louis Univ.), M. Horwitz (Albert Einstein College of Medicine), J. Bruder (Genvec, Inc.), J. Riggs (NEB), and R. Kriwacki (St. Jude Children's Research Hospital) for generously providing plasmids used in this work; K. Greenchurch and The Ohio State University (OSU) CCIC for mass spectrometry support; G. Renkes (OSU) for atomic absorption; the OSU CCIC Biopolymer Facility and Neurobiotech Center for DNA Sequencing; K. Wolken and B. Kemmenoe of the Campus Microscopy and Imaging Facility for EM; and V. Gopalan and J. Kuret for helpful discussions. This work was supported by grants from the American Cancer Society and Milheim Foundation for Cancer Research.

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