Chelating Agents Stabilize the Monomeric State of the Zinc Binding Human Papillomavirus 16 E6 Oncoprotein

Roland Degenkolbe,*,‡ Patrick Gilligan,‡ Sanjay Gupta,‡ and Hans U. Bernard§

Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Republic of Singapore, and Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California 92679-3900

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ABSTRACT: The E6 protein of human papillomavirus 16 is known to be difficult and, when overexpressed, insoluble and agglomerated. It has two putative zinc ion binding sites crucial for its function. No metallochaperone has yet been found to deliver zinc ions to the E6 protein. Here, we report that a specific chelating agent, which we think functionally mimics a metallochaperone, stabilized the soluble monomeric form of E6 and inhibited multimerization *in vitro*. This effect seemed to depend on the chelating strength of the agent. While strong chelating agents precipitated the E6 protein and weak chelating agents did not favor the monomeric form of E6, chelating agents of intermediate strength [L-penicillamine and ethylene glycol bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)] effectively support the formation of a monomer. We did not observe formation of a dimer or defined oligomers. Degradation assays imply that the monomer is the biologically active form of the protein. Since EGTA favors the formation of monomeric over agglomerated E6 protein, we propose that chelating agents of appropriate strength could assist zinc delivery to recombinant metalloproteins *in vitro* and may even destabilize existing agglomerates.

While expression of recombinant genes has become routine, overexpression frequently fails to yield a soluble and folded protein (*1*, *2*). Even short polypeptides that do not require any post-translational modifications such as the formation of disulfide bonds or glycosylation and thus presumably have all the information necessary for proper folding encoded in their primary structure are difficult to prepare in suitable amounts, concentrations, and quality for biophysical measurements and three-dimensional structure determination. The protein encoded by the E6 oncogene of human papillomavirus 16 (HPV16) is one of the proteins known to be problematic, although it is small (151 amino acids) and appears not to require any post-translational formation of covalent bonds (*3*−*5*).

HPVs are causative agents for cervical cancer (*6*); virtually every cervical carcinoma was found to contain the DNA of a subgroup of HPV types, named "high-risk" types. The most common types in this group are HPV16 and -18 with HPV16 being present in ∼60% of cervical cancers. HPV16 encodes three oncoproteins (E5−E7) with scientific interest focusing on E6 and E7 as those are the only HPV-encoded proteins consistently expressed in cervical carcinoma. E6 and E7 are small proteins that bind to zinc(II) ions (hereafter, zinc) with two putative zinc binding domains and one putative zinc binding domain, respectively, which forms unusually large (29 amino acids) "zinc fingers" (*7*, *8*). E6 binds cellular factors such as E6AP, E6BP, paxillin, CBP, hMCM7, hDLG, IRF3 (for a review, see ref *9*), and, interestingly, cruciform DNA (*10*). The most thoroughly investigated and understood function of the E6 protein is the interaction with E6AP, a cellular E3 ubiquitin ligase. The E6−E6AP complex binds and ubiquitinates p53 and targets it for degradation via the 26S proteasome (*11*−*13*).

Interestingly, besides a plethora of publications reporting interactions of the E6 protein with cellular targets, little biophysical data have been published and the three-dimensional structure remains unsolved. In several recent biophysical studies (*2*, *10*), it was revealed that despite expression in the presence of chaperones such as GroEl, careful purification, and painstaking mutation of potentially problematic amino acid residues a large proportion of E6 protein is, even when soluble, unfolded and aggregated, with only a subpopulation less aggregated and functional. It has been concluded, accordingly, that not only solubility but also monodispersity should be a criterion for successful preparation of native E6 (*2*, *14*).

On the basis of these findings, we aimed to assess the factors influencing agglomeration of the E6 protein, and postulated that agglomeration is but a consequence of overexpression and purification. E6 has two potential zinc binding domains comprised of four cysteins each and no disulfide bonds. Therefore, we assumed that reducing conditions with some additional zinc should provide conditions resulting in the purification of the functional E6 protein. Unfortunately, zinc (as with other transition metals) is rather difficult to administer properly. Most proteins use zinc to facilitate electron transfer or to introduce secondary structure with stable noncovalent links. The versatility of zinc is reflected in the large number of zinc binding proteins as well as in the tight control the cell exerts on zinc levels and trafficking and delivery of zinc to the target proteins. Indeed, the level of unbound zinc in the cytoplasm is vanishingly low (*15*, *16*). Even though there is a lack of a general understanding of how the cell dispenses zinc, recent years

* To whom correspondence should be addressed. Telephone: (65) 68743778. Fax: (65) 67791117. E-mail: mcbrd@imcb.nus.edu.sg.
‡ Institute of Molecular and Cell Biology.
§ University of California at Irvine.

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Table 1: Stoichiometric Ratios of Zinc to S-E6 after Individual Steps of Purification and Dialysis with Different Chelating Agents[a]

[Zn]:[S-E6]				
first step (after metal affinity)	second step (after anion exchange)	third step (after dialysis and size exclusion)		
		S-E6 multimer	S-E6 monomer	
(0.6 ± 0.08):1	(0.5 ± 0.09):1	(1.8 ± 0.36):1[b]	(0.9 ± 0.18):1[b]	without zinc or chelator
		(2.2 ± 0.24):1	n/a[c]	with zinc
		n/a[d]	n/a[d]	with zinc and EDTA
		(1.8 ± 0.4):1	(0.9 ± 0.1):1	with zinc and EGTA
			(0.9 ± 0.1):1[e]	
		(1.9 ± 0.26):1	(1 ± 0.15):1	with zinc and L-penicillamine
		(1.8 ± 0.24):1	n/a[c]	with zinc and L-carnosine
		n/a[d]	n/a[d]	with zinc and 1,10-phenanthroline

[a] The amount of zinc bound by S-E6 was measured using the TSQ assay. [b] Some of the protein precipitated during dialysis. [c] No protein eluted from the size-exclusion column at this volume. [d] All of the protein precipitated during dialysis. [e] Additional determination of zinc content with ICP/OES.

have seen spectacular advances in understanding of how copper, another transition metal frequently incorporated into proteins, is delivered to its targets. Several soluble cytosolic proteins, termed metallochaperones, have been found to be required for the delivery of copper ions to their target proteins through direct protein−protein interactions (*17*). This intricate mechanism has been demonstrated for the copper−zinc superoxide dismutase and its metallochaperone (*18*−*20*). It is not known how the E6 protein acquires the zinc vital for its function, whether from a sequestered cellular metallochaperone or by other means.

Here, we present evidence that the proper administration of zinc is crucial for formation of the E6 monomer by stabilizing monodisperse E6 protein with a chelating agent. We suggest that the chelator mimics the function of a metallochaperone *in vitro*. Furthermore, we show that a chelating agent disperses agglomerated E6 and conclude that agglomeration occurs due to uncontrolled zinc delivery during the folding of E6. Our findings lead to a strategy for obtaining monomeric, active, and stable E6 protein and may help in the re-evaluation of preparation protocols for other metalloproteins.

EXPERIMENTAL PROCEDURES

Expression and Purification of SET-HPV16 E6. The HPV16 E6 gene was synthesized using 20 overlapping oligonucleotides with optimal codon usage for high-level expression in *Escherichia coli* with an N-terminal solubility enhancement tag of 56 amino acids of the B1 subdomain of protein G (*21*) and a C-terminal hexahistidine tag. This chimeric gene, SET-HPV16 E6 (hereafter, S-E6), was cloned into the bacterial expression vector pET22b (NOVAGEN, U.S.A.). The construct was transformed into *E. coli* ER2566 (NEB, U.S.A.), cultures grown in LB medium with 100 ng/mL ampicillin, expression induced at an OD_{600} of ∼0.4 with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and cells harvested after incubation for 4 h at 30 °C. Bacterial pellets were stored at −80 °C.

The bacteria were resuspended in buffer A [300 mM NaCl, 10% glycerol, 0.1% Triton X-100, and 50 mM CO_3^{2-}/HCO_3^- (pH 10.5)] with 2 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and protease inhibitor cocktail Complete, EDTA free (Roche), and lysed with a sonicator. The supernatant after centrifugation (100000*g* for 1 h) was loaded on a Ni−NTA−agarose (Qiagen) column equilibrated with buffer A. The column was washed with 30 column volumes of 20 mM CO_3^{2-}/HCO_3^- (pH 10.5), 200 mM NaCl, and 20 mM imidazole and the protein eluted with 3 column volumes of 20 mM CO_3^{2-}/HCO_3^- (pH 10.5), 75 mM NaCl, and 200 mM imidazole. The eluate was loaded on an anion-exchange column (SOURCE 15Q, Amersham) equilibrated with 100 mM NaCl and 20 mM CO_3^{2-}/HCO_3^- (pH 10.5); the column was then washed with 30 column volumes of the same buffer and the bound protein eluted with a linear gradient to 1 M NaCl and 20 mM CO_3^{2-}/HCO_3^- (pH 10.5). Typically, S-E6[1] eluted at a concentration of ∼250 mM NaCl. The pooled peak fractions were gently dialyzed against 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.8), 250 mM NaCl, 2 mM chelating agent, 20 μM $ZnCl_2$, and 4 mM DTT. After dialysis, the protein solution underwent size-exclusion chromatography on a Superdex 75, 26/60 column (Amersham). The peak fractions were pooled and concentrated in the presence of 2 mM DTT and a chelating agent, respectively, with 10 kDa cutoff ultrafiltration spin columns (Amicon). This method usually yielded 1−2 mg of S-E6 per liter of bacterial culture.

Determination of the Zinc Content of the S-E6 Protein (TSQ Assay). In this assay, we ejected the zinc incorporated by the S-E6 protein by oxidation of the coordinating amino acid residues and then determined the concentration of the ejected zinc in the solution by measuring the fluorescence of a complex of zinc and the fluorescent indicator *N*-(6-methoxy-8-quinolyl)-*p*-toluene sulfonamide [TSQ (Molecular Probes, Eugene, OR)] at 465 nm (excitation wavelength of 360 nm). Usually, 1−20 μL of protein solution was mixed with 5 μL of H_2O_2 and buffer T [10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) and 10% glycerol] to a final volume of 100 μL on a 96-well plate and incubated at 25 °C for 30 min. One hundred microliters of buffer TS [10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 10% glycerol, and 200 μM TSQ] was added and the fluorescence measured. Samples were measured in triplicate and zinc concentrations calculated relative to zinc standards of 10, 20, 50, and 100 pmol. The amounts determined were usually <100 pmol, corresponding to 10 μL of a 10 μM solution. The standard errors, combined with an average error of 10% for determination of protein concentrations (see Protein Quantitation), were between 11 and 24% as indicated in Table 1.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; TSQ, *N*-(6-methoxy-8-quinolyl)-*p*-toluene sulfonamide; S-E6, solubility enhancement tag-tagged human papillomavirus 16 early protein 6.

Determination of Zinc Content by Inductively Coupled Plasma/Optical Emission Spectroscopy (ICP/OES). For validation of the zinc concentrations obtained with the fluorescence-based TSQ assay, we submitted the purified, monomeric S-E6 protein to ICP/OES. In this method, the liquid sample is nebulized into a plasma whose temperature is sufficiently high to transform elements present into the gaseous atomic state. In this state, the zinc atoms emit at a characteristic wavelength with an intensity proportional to their concentration. The spectroscope was calibrated with standards at 0.1, 0.5, 1, and 2 ppm (BDH). The sample included 10 mL of a 0.16 mg/mL solution of monomeric S-E6 (after dialysis in the presence of EGTA and size-exclusion chromatography).

Competition Assay for the Relative Strength of Chelating Agents (TSQ). We modified the TSQ assay to compare the chelating strengths of different agents indirectly by measuring the fluorescence of the TSQ−Zn(II) complex at different chelating agent concentrations. Solutions (190 μL) containing different concentrations of EDTA, EGTA, or L-penicillamine in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 10% glycerol, and 100 μM TSQ were added to 10 μL of a 10 μM $ZnCl_2$ solution on a 96-well plate. The fluorescence of the TSQ−Zn(II) complex was measured at 465 nm (excitation wavelength of 360 nm) until equilibrium was reached (usually after 5−10 min).

Protein Quantitation. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's recommendation using bovine serum albumin as a standard. Additionally, we measured the absorbance at 280 nm and determined the concentration with a molar extinction coefficient of 29 700 M^{-1} cm^{-1} for the S-E6 protein, calculated with PROTEAN, part of the DNAstar software bundle. Usually, the differences in calculated and measured protein concentrations obtained with the two methods were smaller than 10% of the total concentration.

p53 Degradation Assay. [^{35}S]Methionine-labeled p53 was expressed using the TnT-Quick coupled transcription/translation system (Promega, Madison, WI) according to the manufacturer's recommendation and incubated with 5 nmol of the purified S-E6 protein or buffer without S-E6 (control) and ribonuclease A (to suppress further expression of p53) at 30 °C. Samples were taken at the indicated time points, and the reaction was stopped with sodium dodecyl sulfate (SDS) loading buffer. The samples were run under reducing conditions on SDS−polyacrylamide (SDS−PAGE) gels (*22*), the gels dried, and the resulting bands after autoradiography integrated using the Bio-Rad densitometer software.

RESULTS

To achieve high levels of expression of the HPV16 E6 protein in *E. coli*, we redesigned the entire E6 coding sequence with codons that are expressed at high levels in *E. coli*. To facilitate purification, we attached a C-terminal hexahistidine tag. To increase the solubility of the E6 protein, we attached an N-terminal SE tag (*21*). This chimeric gene, S-E6, was efficiently expressed in *E. coli*, but we realized that the bulk of the protein was insoluble at physiological pH. However, under nonphysiological alkaline conditions (pH 10.5), a substantial proportion of the overexpressed protein was soluble and could be purified without major obstacles, resulting in pure S-E6 after metal-affinity and anion-exchange chromatography (Figure 1).

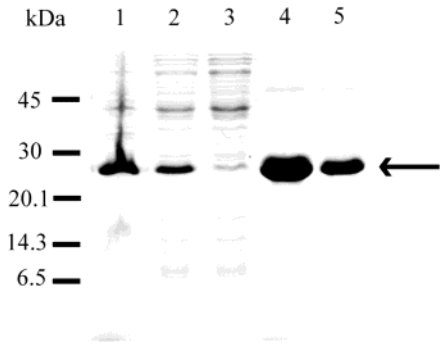

FIGURE 1: Purification of S-E6 expressed in *E. coli*. The molecular mass markers are indicated on the left. The arrow indicates the position of the S-E6 protein: lane 1, lysate after sonication; lane 2, supernatant after centrifugation; lane 3, flow-through of the metal-affinity column; lane 4, eluate of the metal-affinity column; and lane 5, eluate of the anion-exchange column.

A large proportion of this S-E6 preparation precipitated during dialysis against buffer at pH 7.8. The protein in the supernatant eluted off a size-exclusion column in two peaks, one at a high molecular mass close to the void volume of the column, indicating a multimer, and another peak at a volume close to one of the size standards, carbonic anhydrase (molecular mass of 29 kDa), indicating an S-E6 monomer (calculated mass of 25.4 kDa) (Figure 2A). Since S-E6 does not fully retain its ability to bind zinc at pH 10.5 and might precipitate because of a lack of zinc, we added zinc to the dialysis buffer. Concentrations of zinc of >0.5 mM precipitated S-E6 quantitatively (data not shown), as they would most other proteins (*23*). However, a concentration of 20 μM yielded a soluble but multimeric protein (Figure 2B). We concluded that zinc was necessary for the solubility of the S-E6 protein but probably coordinated too indiscriminately for selective formation of a monomer or a defined oligomer. To overcome this, we added a competitor for zinc during dialysis. Ideally, such a competitor would be a sufficiently strong chelator to compete with nonspecific, but not native, coordination of zinc by S-E6, thus mimicking the action of a metallochaperone *in vitro*. We investigated the effects of some chelating agents (*24*, *25*) on S-E6 agglomeration by size-exclusion chromatography after dialysis. In our experiments, L-carnosine did not seem to have any effect on the oligomerization of S-E6 as no precipitate was formed but the protein was completely multimeric (Figure 2G); ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline precipitated the protein almost completely (Figure 2C,D), whereas EGTA and L-penicillamine stabilized monomeric S-E6 to different extents (Figure 2E,F). Dialysis against a buffer containing EGTA but no additional zinc yielded S-E6 largely in a monomeric state, similar to the result observed in the presence of EGTA and zinc. However, the protein was not stable and precipitated more rapidly than in the presence of both EGTA and zinc. The presence of 20 μM $ZnCl_2$, 2 mM EGTA (or L-penicillamine), and 2 mM DTT stabilized the monomer (1−2 days at 4 °C) up to concentrations of 0.1−0.2 mM.

We were intrigued by structurally related chelating agents such as EDTA and EGTA having such different effects on the oligomerization of S-E6 and indirectly compared the

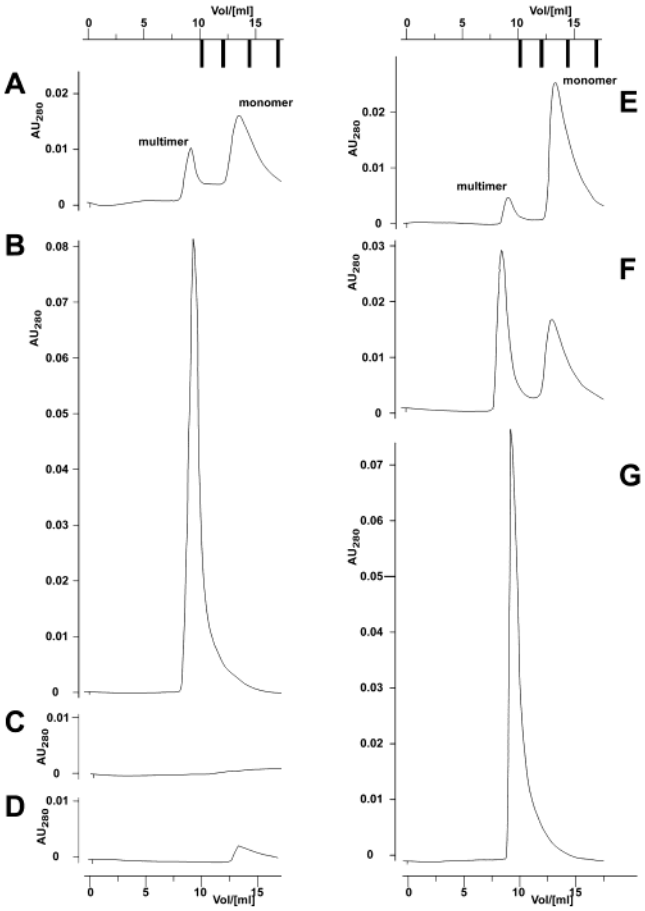

FIGURE 2: Differences in the proportion of multimeric to monomeric S-E6 after dialysis in the presence of various chelating agents. Purified S-E6 (after anion-exchange chromatography) was dialyzed and analyzed with size-exclusion chromatography. The dialysis buffer contained 20 mM phosphate (pH 7.8), 250 mM NaCl, 4 mM DTT, and (A) no chelating agent and no zinc, (B) no chelating agent and 20 μM $ZnCl_2$, (C) 2 mM 1,10-phenanthroline and 20 μM $ZnCl_2$, (D) 2 mM EDTA and 20 μM $ZnCl_2$, (E) 2 mM EGTA and 20 μM $ZnCl_2$, (F) 2 mM L-penicillamine and 20 μM $ZnCl_2$, and (G) 2 mM L-carnosine and 20 μM $ZnCl_2$. The black bars in the upper right corners indicate the elution volumes of the molecular size markers (Sigma): bovine albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.9 kDa), and aprotinin (6.5 kDa) (starting from the left).

chelating strength of three agents (EDTA, EGTA, and L-penicillamine) in a competition assay against a constant concentration of zinc and the fluorescent indicator TSQ. As expected, EDTA appears to chelate zinc ~10 times more effectively than EGTA, which seems to be ~100 times stronger than L-penicillamine (Figure 3). A chelator of intermediate strength, like EGTA, appears to be most efficient at stabilizing the monomeric state of S-E6.

We monitored the stoichiometry of zinc bound by S-E6 during purification and after dialysis in the presence of chelating agents with the TSQ assay and, for the monomeric protein after dialysis, also by inductively coupled plasma/optical emission spectroscopy (ICP/OES). Since S-E6 has two putative zinc-binding domains, we expected to find a stoichiometric ratio of 2:1 (Zn:S-E6) in a biologically active protein. Surprisingly, we found Zn:S-E6 ratios closer to 1:1 for the monomeric protein and a ratio of ~2:1 for the multimeric protein only (Table 1). For the case of the two strongest chelating agents, EDTA and 1,10-phenanthroline,

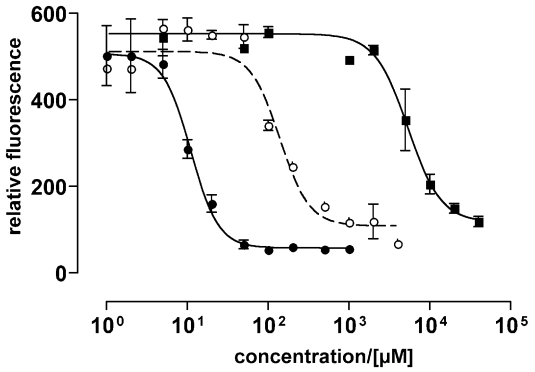

FIGURE 3: Competition for zinc among three different chelating agents. $ZnCl_2$ (100 pmol) was added to a mixture of 100 μM TSQ and the indicated concentrations of EDTA, EGTA, or L-penicillamine. The residual fluorescence of the TSQ−zinc complex was measured at 465 nm: (●) EDTA, (○) EGTA, and (■) L-penicillamine.

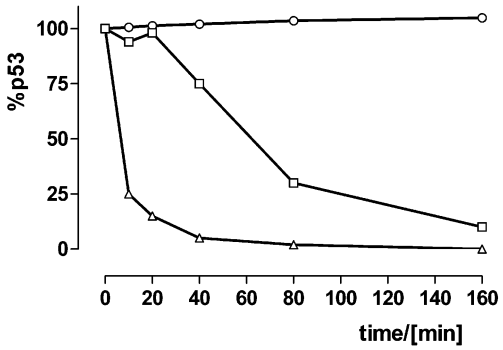

FIGURE 4: Monomeric S-E6 is more active biologically than its multimeric form in catalysis of p53 degradation. [^{35}S]Methionine-labeled p53 was incubated with S-E6 eluting via size-exclusion chromatography at volumes corresponding to the exclusion limit (multimeric S-E6) (□), ~29 kDa (monomeric S-E6) (△), and buffer without S-E6 (○).

we could not detect any zinc in the precipitated S-E6 (data not shown).

We wondered if the monomer of S-E6 is the biologically active form, as reported for one of the multiple functions of E6 (*26*), or alternatively a "multimeric" complex of specifically interacting S-E6 monomers. To address this question, we performed a p53 degradation assay with multimeric and monomeric S-E6 preparations. Figure 4 shows that the S-E6 monomer is markedly more active than its multimeric counterpart in this assay.

As we had studied the effect of chelating agents during dialysis of S-E6 from a high pH to a physiological pH, we asked what effect a chelating agent might have if added after the protein had completely multimerized. To study this question, we took a preparation of the completely multimeric S-E6 protein and added EGTA to a final concentration of 2 mM. Size-exclusion chromatography of the protein preparation before and 1 h after addition of EGTA showed that the majority of the protein was monomeric after EGTA treatment (Figure 5). From this, we conclude not only that the presence of the proper chelating agent stabilizes the monomeric, biologically active form of S-E6 when present during refolding but also that an appropriate chelating agent promotes the formation of monomeric S-E6 even after it had multimerized completely.

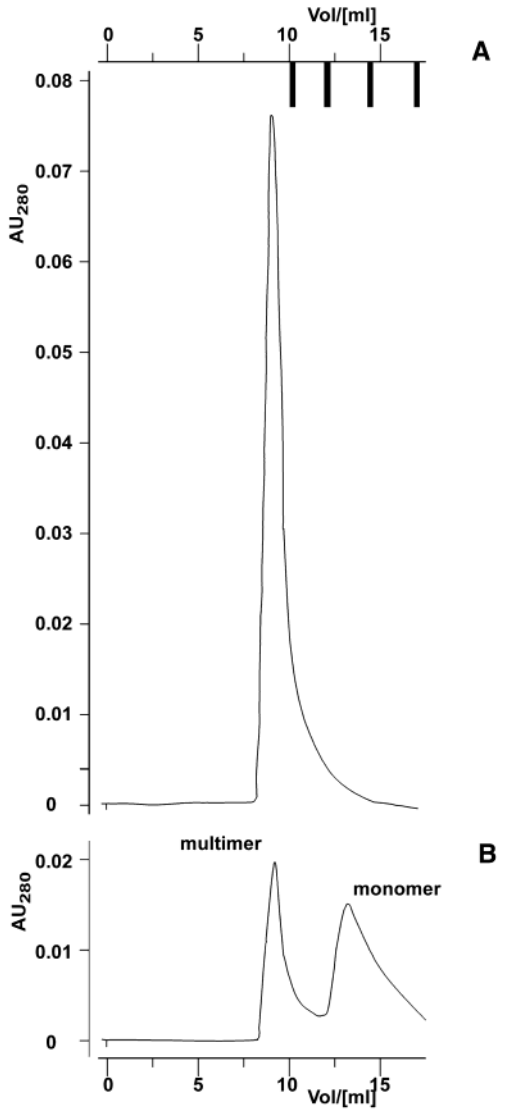

FIGURE 5: Agglomerated protein is destabilized by addition of a chelating agent. (A) Size-exclusion chromatography of purified S-E6 after dialysis in the absence of a chelating agent. (B) The same sample 1 h after addition of 2 mM EGTA. The black bars in the upper right corner indicate the elution volumes of the molecular size markers (Sigma): bovine albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.9 kDa), and aprotinin (6.5 kDa) (starting from the left).

DISCUSSION

There has been considerable interest in the E6 protein ever since its importance as one of the transforming proteins of HPV16, the main causative agent for cervical cancer, emerged. However, the HPV16 E6 protein is notorious for its awkward biophysical properties (*14*, *27*). Routinely, tags (GST, MBP, His_6, FLAG, etc.) have been used to increase the solubility or ease the purification of E6 without impairing its function (*10*, *13*, *28*, *29*). We decided to tag E6 with six histidines and the protein G subdomain B1, which is soluble, does not interact with its fusion partner, and is small enough to allow structural studies with NMR, while attached (*21*). The resulting fusion protein, S-E6, is more soluble than native E6 but appears to be otherwise unchanged. Still, 14 of its amino acids are cysteines (the tags do not contribute here), of which eight are highly conserved and constitute the two putative zinc binding domains, while the remaining six cysteines are not conserved and might simply require reducing conditions (*10*, *14*). Mutational studies indicated that the cysteine residues of the zinc-binding motif are crucial for all but a few biological functions (*30*). It has been postulated that E6 exists as a dimer, mainly due to anecdotal evidence obtained while purifying the E6 protein. The biological relevance of a dimer or an even larger homo-oligomer remained elusive, and a study of the interaction with E6AP presented evidence for the function of monomeric E6 in this mechanism (*26*). In our project, we postulated that the oligomerization properties might be the root of the problematic behavior of the S-E6 protein *in vitro*. This postulation was based on unsuccessful cross-linking experiments and on mass spectrometry, which yielded molecule peaks of the monomeric protein (excluding zinc) under denaturing conditions and no signal under native conditions, presumably because the complex is too large to vaporize and ionize (data not shown). Since the S-E6 protein has two putative zinc-binding domains and zinc itself agglomerates proteins, we investigated the effects of zinc on the oligomerization of S-E6. We found that high concentrations of zinc precipitate S-E6 and low concentrations agglomerate it. To keep the S-E6 protein monomeric and soluble, a molecule was needed that mimicked a metallochaperone to traffic and deliver zinc ions more specifically to the zinc-binding domains of S-E6. Since no natural metallochaperone has been identified for E6, we investigated the effect of different chelating agents on the multimerization status of our S-E6 fusion protein. Our findings suggest four different agglomeration states for S-E6, depending on the chelating agent and therefore the amount of zinc bound. (i) S-E6 devoid of any zinc due to the presence of a strong chelating agent is insoluble and precipitated. (ii) S-E6 with one zinc ion per molecule, delivered with a chelating agent like EGTA (or, to a lesser extent, L-penicillamine), is soluble, biologically active, and monomeric. (iii) S-E6 with two zinc ions per molecule leads to a protein multimerized in a large complex. (iv) S-E6 with a large excess of bound zinc is insoluble and precipitated. The 2:1 (Zn:S-E6) stoichiometry for preparations of agglomerated S-E6 would imply specific zinc binding, as two putative zinc-binding sites are conserved throughout all HPVs. However, we did not find evidence for biological activity of an S-E6 multimer. A ratio of one zinc ion per S-E6 monomer indicates that only one zinc-binding site is occupied, a finding suggested by earlier mutational studies (*30*) but not confirmed more recently (*31*). Possibly the affinity of the two zinc-binding sites is sufficiently distinct that our chelating agents remove zinc from one site but not the other. Since the stoichiometric ratio of 1:1 did not negatively affect the S-E6-dependent degradation of p53, but rather influenced the oligomerization behavior of the protein, one may consider that one of the two zinc-binding sites could coordinate zinc inter- rather than intramolecularly, promoting switches between monomeric and multimeric forms depending on the zinc concentration (*32*).

The *in vitro*, zinc-dependent, agglomeration of the S-E6 protein is likely to be seen with other metalloproteins. If it is, then our strategy of screening diverse chelating agents to identify those with the desired effect on the protein might be generally useful. Since the chelating agents varied greatly in their ability to favor monomeric over agglomerated or precipitated S-E6 protein, screening chelating agents for their

ability to dissolve other problematic proteins might be promising (*33*). This is reminiscent of the current attempts to dissolve plaques of Alzheimer's A beta, which are rapidly induced by zinc (*34*), with a suitable transition metal chelator (*35*).

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