BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 190–194 (1998)
ARTICLE NO. RC989617

# Zinc-Binding of Endostatin Is Essential for Its Antiangiogenic Activity

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Received October 1, 1998

**Endostatin is a potent angiogenesis inhibitor *in vitro* and *in vivo*. We used the yeast *Pichia pastoris* to express and purify soluble endostatin. It was discovered that metal chelating agents can induce N-terminal degradation of endostatin. We theorized that a metal was removed from endostatin which changed the conformation and allowed a contaminating protease to degrade the N-terminus. Atomic absorption and amino acid analysis of endostatin purified from *Pichia pastoris* and mammalian cells showed a 1:1 molar ratio of $Zn^{2+}$ to protein. Ding *et al.* have shown that histidines 1, 3, 11, and aspartic acid 76 coordinate the $Zn^{2+}$ atom (1). An H1/3A double, an H11A, and a D76A single mutant of endostatin were not able to regress Lewis lung carcinoma. We conclude that the ability of endostatin to bind $Zn^{2+}$ is essential for its antiangiogenic activity.** © 1998 Academic Press

Over the last few decades, cancer research and therapy focused primarily on tumor cells. However, in 1971 it was proposed that a solid tumor cannot grow without recruiting endothelial cells (2). The formation of new capillaries allows the tumor mass to expand. Today, a major research area is directed at the inhibition of proliferation of endothelial cells during tumor growth. If it were possible to control the mitogenic and migratory activity of these cells, this would be a promising form of anti-cancer therapy.

Many molecules with antiangiogenic activity have been described but few are as potent as angiostatin or endostatin. Both molecules can regress large tumors in mice (3–6). We recently reported that endostatin therapy does not induce acquired drug resistance in three tumor models (4). Additionally, after several cycles of growth and regression, the tumors did not regrow although proliferating tumor cells were found at the original site of tumor inoculation. Endostatin converted aggressive, deadly tumor cells into a harmless dormant tumor, usually of microscopic size, composed of proliferating and dying tumor cells, unable to induce angiogenesis and therefore not able to grow. There were no obvious side-effects in mice. Neither inhibitor has yet been tested in clinical trials.

Zinc is the second most abundant trace metal in most higher animals. An average adult human contains about 2.3 g zinc compared to 4 g of iron. The $Zn^{2+}$ cation is a critical component of many proteins and plays a key role in a host of biological processes (7–9). More than 300 $Zn^{2+}$-containing enzymes have been characterized in some detail. In most cases $Zn^{2+}$ is directly involved in the catalytic activity. Although most of the proteins in which $Zn^{2+}$ has a structural role are transcription factors, examples of a structural role for $Zn^{2+}$ in extracellular proteins have been described. Zinc is involved in the dimerization of human growth hormone and increases the affinity of human growth hormone for the prolactin receptor by approximately 8,000 fold (10, 11). Conversely, $Zn^{2+}$ inhibits the biological activities of nerve growth factor and related neurotrophins by blocking binding to their receptors (12).

We report here that endostatin binds zinc at a 1:1 molar ratio. In a paper recently accepted for publication, all four zinc ligands are determined and the structure of the zinc-binding pocket is presented (1). The zinc molecule is coordinated by histidine 1, 3, 11, and

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Abbreviations used: EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline; DMEM, Dulbecco's Modified Eagle's Medium.

0006-291X/98 $25.00

**TABLE 1**

Designation, Growth Medium, and N-Terminal Sequence of Different Endostatin Batches Used in This Study

| | | |
|---|---|---|
| ppES08, ppES10 | F, MM w/o CA | QDF . . . |
| ppES13 | F, MM w/ CA | YVEFHTHQDF . . . |
| ppES16 | F, RM | YVEFHTHQDF . . . |
| ppES17, ppES18 | S, RM | YVEFHTHQDF . . . |
| $ES_{B_{16}F_{10}}$ | DMEM, 2.5% FCS | HTHQDF . . . |

*Note.* pp = Pichia pastoris; ES = endostatin; F = fermentor; S = shaker flasks; MM = minimal medium; RM = rich medium; CA = casamino acids; DMEM = Dulbecco's Modified Eagle's Medium; FCS = fetal calf serum; B16F10 = B16F10 melanoma tumor cell line.

aspartic acid 76. Furthermore, we show here that mutations in the zinc-ligands drastically reduce the biological activity of endostatin. Therefore, we conclude that zinc-binding of endostatin is essential for its antiangiogenic activity.

## MATERIALS AND METHODS

*Expression and purification of mouse endostatin using the yeast Pichia pastoris.* The endostatin cDNA was amplified using PCR and cloned into the EcoRI/NotI site of the expressing vector pPIC9K (Invitrogen). This cloning created a fusion protein of the alpha factor secretion signal with endostatin. The construct was verified by sequencing. Integration of this plasmid into SMD1168 was performed according to the manufacturer's instructions (Pichia Expression Kit, Invitrogen). Single colonies were screened by Western blotting for endostatin expression. yTB01#2 was used for purification of all the different endostatin batches.

Endostatin was either purified from yeast supernatant grown in shaker flasks or in a fermentor. For shaker flask purification, the recommendations of the manufacturer were followed (Invitrogen). We used rich medium buffered with 0.1 M potassium phosphate at pH 6.0. The cultures were induced with methanol for 48–72 hours. Cells were removed by centrifugation and the supernatant was slowly mixed with a saturated ammonium sulfate solution to a final concentration of 45% ammonium sulfate. The ammonium sulfate solution was prepared by dissolving 767 g of ammonium sulfate (ICN) in 1L of 10 mM Tris, pH 7.0. The pH of the solution was readjusted to pH 7.0 and it was stored at 4°C until use. The ammonium sulfate/supernatant solution was stirred for 2 hours at 4°C and centrifuged at 5000 rpm for 20 min. The precipitated proteins were resuspended in 0.1–0.2 starting volumes of PBS[1]. The solution was dialyzed against PBS and directly applied to a heparin-Sepharose column (Pharmacia). After washing the column with PBS and 0.2 M NaCl, 20 mM Tris, pH 7.4, endostatin was eluted using a NaCl (0.2 to 1.5 M) gradient. Endostatin containing fractions were pooled and dialyzed against PBS. The protein was stored at −20°C.

yTB01#2 was also grown in a fermentor (5 liter bench-top B. Braun fermentor). In this case, rich medium and minimal medium in the presence or absence of 1% casamino acids at pH 6.0 were used. The presence of casamino acids blocked N-terminal degradation of endostatin (Table 1). We followed the fermentation conditions recommended by Invitrogen. The supernatant was dialyzed against PBS. Ammonium sulfate precipitation was not possible and the solution was directly applied onto a heparin-Sepharose column. All the other steps were performed as described above.

*Expression and purification of mouse endostatin using a mammalian tumor cell line.* We used a retroviral vector for the expression of endostatin in mammalian cells. Cloning of the endostatin cDNA into the retroviral vector and virus amplification will be described elsewhere.[2] This vector expresses the green fluorescent protein and endostatin from the same promoter. B16F10 melanoma cells were incubated in 5 ml of virus containing supernatant in the presence of 8 $\mu$g/ml polybrene. After 5 hours normal growth medium (10% fetal calf serum, DMEM, glutamine and antibiotics) was added. After at least 10 days in culture, 10% of the most intensely green cells were isolated by FACS sorting. For protein purification, cells were grown in T162 flasks in 2.5% FCS and the medium was collected every 48 hours for at least 6 days. Cell debris was removed by centrifugation and the supernatant was diluted 1:3 with double distilled water and directly applied onto a DEAE-Sepharose column (Pharmacia) equilibrated in PBS diluted 1:3 with double distilled water. All steps were performed at 4°C. The flowthrough was applied onto a heparin-Sepharose column. The column was washed with PBS and 0.2 M NaCl, 20 mM Tris and bound endostatin eluted with a NaCl (0.2 to 1.5 M) gradient in 20 mM Tris, pH 7.4. Endostatin containing fractions were pooled, dialyzed against PBS and stored at −20°C.

*Amino acid analysis, atomic absorption analysis, and mass spectrometry.* Amino acid analysis was done in duplicates on an ABI 420A. Atomic absorption analysis was performed on a Perkin-Elmer Model 2280 flame atomic absorption spectrophotometer. Mass spectrometry was done on a VG/Fisons TofSpec mass spectrometer.

*Mutational analysis.* Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing analysis.

*Mouse studies.* Animal work was carried out in the animal facility of Children's Hospital in accordance with institutional guidelines. Experiments presented in Figure 3 were performed as described (2, 3).

## RESULTS

In previous studies, endostatin was expressed in *E. coli,* purified and injected into mice as an insoluble, aggregated material (3, 4). To show that soluble endostatin has similar activity, the cDNA was cloned into the *Pichia pastoris* expression vector pPIC9K. The cloning created a fusion protein of the alpha factor secretion signal with endostatin which, if correct processing occurs, should be secreted as a protein with the following N-terminus: YVEFHTHQDF (YVEF are from the multiple cloning region of the expression vector). Endostatin was purified from the supernatant using an ammonium sulfate precipitation step, followed by a heparin-Sepharose column. In rich medium, grown either in shaker flasks or using a fermentor, the purified protein had the expected N-terminus (Table 1). When the yeast cells were grown in minimal medium, the N-terminus started with QDF. Addition of casamino acids to the minimal medium restored the expected N-terminus (Table 1).

Because this material was intended for use in nuclear magnetic resonance studies, purified endostatin was incubated at 37°C in the presence of different protease inhibitors. During these experiments it was observed that EDTA induced an SDS-PAGE mobility shift of endostatin (Fig. 1a). Mass spectrometry analysis revealed that 5–7 amino acids were removed during the incubation period.[3] A different endostatin batch,

[2] T. Boehm, unpublished observations.

[3] B. Carter and R. Mulligan, manuscript in preparation.

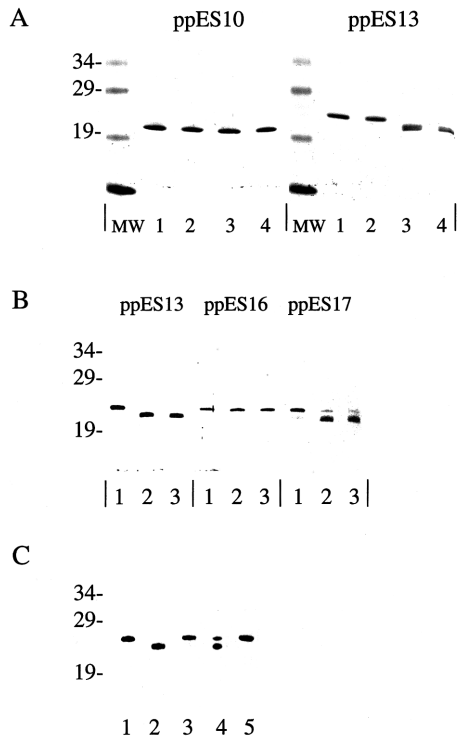


**FIG. 1.** Endostatin degradation is induced by EDTA. (A) EDTA induces degradation of endostatin. 10 $\mu$g of ppES10 and ppES13 were incubated in a total volume of 40 $\mu$l in PBS alone (1), in PBS and 1.25 mM $MgCl_2$ and 1.25 mM $CaCl_2$ (2), in PBS and 5 mM EDTA (3) and in PBS, 1.25 mM $MgCl_2$ and 1.25 mM $CaCl_2$ and 5 mM EDTA (4), for 70 hours at 37°C. An aliquot was boiled in SDS buffer and analyzed by SDS-PAGE and stained with coomassie brilliant blue. (B) EDTA induced degradation is not an intrinsic property of endostatin. ppES13, ppES16 and ppES17 were dialyzed against 150 mM NaCl, 10 mM Tris, pH 7.4 and 10 $\mu$g (total volume 30 $\mu$l) were incubated in Tris buffer (1) plus 5 mM EDTA (2) or 5 mM 1,10-phenanthroline (3). Aliquots were analyzed after 24 hours at 37°C and stained with coomassie brilliant blue. (C) Degradation of endostatin is blocked by serine protease inhibitors. 10 $\mu$g of ppES13 were incubated in 30 $\mu$l Tris buffer (1) plus 5 mM EDTA (2), 5 mM EDTA in the presence of 200 $\mu$M leupeptin (3), 200 $\mu$M pepstatin (4) or 100 $\mu$M PMSF (5). SDS-PAGE was performed after a 44 hour incubation period at room temperature.

ppES10, did not show any shift after incubation with EDTA (Fig. 1a). ppES16 did not migrate faster after incubation with EDTA, whereas ppES17 behaved like ppES13 (Fig. 1b). ppES16 and ppES17 possess the same N-terminus (Table 1). The results with ppES16 and ppES17 suggest that the mobility shift is not an intrinsic property of endostatin.

Because ppES10 did not change its migration behavior after incubation with EDTA, we concluded that the N-terminus of endostatin was degraded by a protease co-purified from the yeast supernatant. This putative protease was also present in the ppES10 batch as shown by mixing experiments.[3] If this hypothesis were correct, protease inhibitors should be able to block degradation. The responsible protease activity could be neutralized using PMSF and leupeptin, two serine protease inhibitors whereas pepstatin, an aspartic protease inhibitor, could not prevent degradation (Fig.1c).

From these data we concluded that chelating agents removed a cofactor in the form of a metal from the N-terminus of endostatin which allowed the removal of several N-terminal amino acids by a serine protease copurified from the yeast supernatant.

The first three amino acids of native mouse endostatin are histidine, threonine and histidine (3). It is well established that histidines can coordinate zinc. Several batches of endostatin were analyzed using atomic absorption for the presence of different metals. Amino acid analysis was used to quantify the amount of protein. A 1:1 molar ratio of $Zn^{2+}$ to endostatin was found only in the endostatin batches where the N-terminus was YVEFHTHQDF (Fig. 2a). No significant amounts of other metals could be measured.[3] ppES08 and ppES10 gave a 0.2:1 $Zn^{2+}$ to protein ratio. Incubation of ppES13 with 1 mM EDTA and 1 mM PMSF (to inhibit degradation during the incubation period) decreased the $Zn^{2+}$ concentration by a factor of 10 but did not change the migration behavior (Fig. 2b).[3] Incubation of ppES10 with EDTA did not change the zinc to protein ratio (Fig. 2b)

To rule out that $Zn^{2+}$-binding was an artifact of the growth conditions for the yeast cells, we also analyzed endostatin purified from B16F10 melanoma cells which were transfected with a retrovirus expressing endostatin. The N-terminus of endostatin isolated from conditioned medium of this mammalian cell line was HTHQDF which is identical to the original N-terminus of endostatin purified from the murine hemangioendothelioma cell line EOMA (3). Also in this case, endostatin and $Zn^{2+}$ showed a 1:1 molar ratio. From all these data, we concluded that endostatin is a $Zn^{2+}$-binding protein.

We then asked whether $Zn^{2+}$-binding is necessary for the full biological activity of endostatin. Ding *et al.* describe the structure of human endostatin in the presence of a metal (1). In the first publication of the structure of mouse endostatin the $Zn^{2+}$-binding capacity of endostatin was not demonstrated (13). Ding *et al.* show that histidines 1, 3, 11, and aspartic acid 76 coordinate the metal ion. Site-directed mutagenesis was used to change the $Zn^{2+}$-ligands to alanines. We made one double (H1/3A) and two single (H11A and D76A) mutants in an *E. coli* expression vector (3, 4). Wild-type endostatin and the three mutants were purified in parallel as previously described (3, 4). The different endostatin preparations were compared on an SDS gel under reducing and non-reducing conditions and no significant difference in the purity and cross-linking pattern was observed.[3] SDS-PAGE of endostatin purified from *E. coli* showed a protein ladder including monomers, dimers, trimers and higher molecular weight complexes caused by cross-

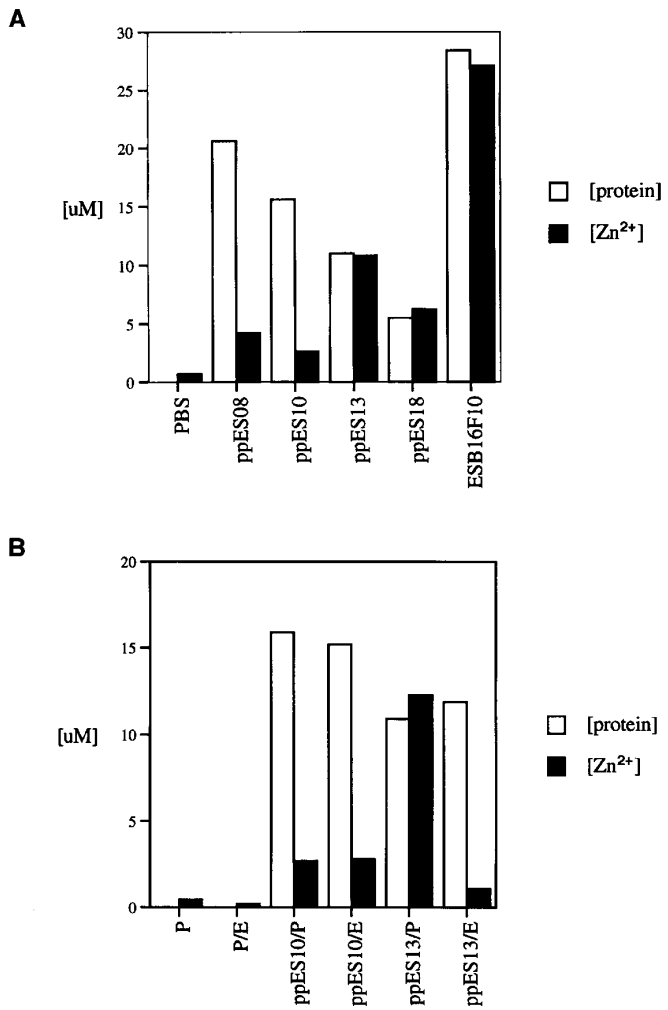


**FIG. 2.** Endostatin is a $Zn^{2+}$-binding protein. (A) Full length endostatin binds zinc in a 1:1 molar ratio. Several batches of endostatin expressed in *Pichia pastoris* (ppES08, 10, 13 and 18) and in the B16F10 melanoma cell line were analyzed for zinc content by atomic absorption. The amount of protein was quantified by amino acid analysis. The graph represents the amount of protein and $Zn^{2+}$ in $\mu$M. The N-termini of the different batches are listed in Table 1. (B) Effect of EDTA on the protein to $Zn^{2+}$ ratio. Endostatin batch ppES10 and ppES13 were dialyzed in PBS containing 100 $\mu$M PMSF for 48 hours at room temperature in the presence or absence of 1 mM EDTA. The amount of protein and $Zn^{2+}$ were measured and expressed in $\mu$M. P=PBS, E=EDTA.

linking of endostatin to other endostatin molecules via disulfide bond formation.[3]

The different endostatin preparations were tested in parallel in our standard Lewis lung carcinoma model for biological activity. As can be seen in Figure 3, none of the three mutants of endostatin could regress Lewis lung carcinoma whereas the wild-type protein behaved as described (3, 4). The double mutant was completely inactive. Each single mutant showed some activity but as can be seen in Figure 3, both mutants could only slow tumor growth by about 50%.

## DISCUSSION

Endostatin is one of the most potent angiogenesis inhibitors reported to date. It can regress large tumors in animals and does not induce acquired drug resistance after repeated treatment cycles (3, 4). Although the structure of mouse endostatin has been recently published, no structure-function studies were included in the paper (13). Here we report that endostatin needs a cofactor for full biological activity. It forms a 1:1 molar complex with zinc and this interaction is essential for its strong antiangiogenic activity.

Mutations in the zinc-ligands of endostatin significantly reduced the antiangiogenic activity. The H1/3A double mutant shows only a minor inhibitory activity in the Lewis lung carcinoma model. Two possible explanations could account for this loss of functionality. First, zinc may no longer be bound to endostatin. Ding *et al.* (1) and we have shown that the first two histidines of human and mouse endostatin are essential for zinc-binding. Second, $Zn^{2+}$ -binding could be restored by using two histidines from the His-tag but this "modified" conformation decreases the biological activity. The N-terminal sequence of the H1/3A double mutant is ARRASVGTDHHHHHHMATAQDF. We predict the first to be true because the crystal structure shows that the N-terminal loop induced by zinc-binding is very compact and it does not seem likely that there is enough flexibility to allow to use two histidines from the His-tag (1).

As described by Ding *et al.*, $Zn^{2+}$-binding causes the formation of a loop. This N-terminal loop is fixed to the region between the E and F $\beta$-strands by the fourth zinc ligand, D76 (1). The D76A endostatin mutant may

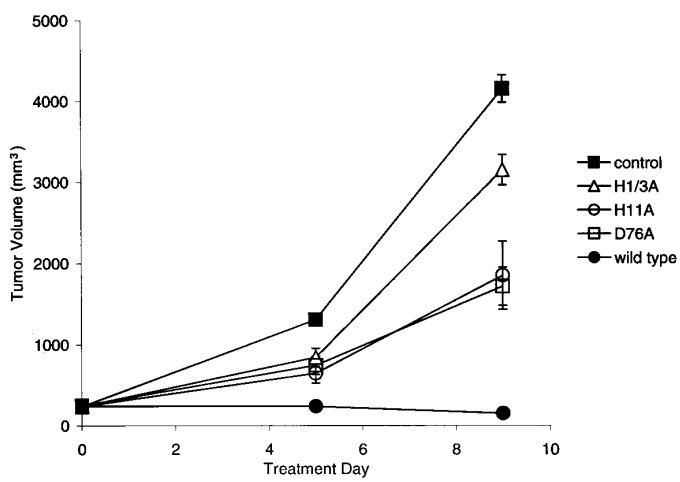


**FIG. 3.** $Zn^{2+}$-binding of endostatin is essential for its antiangiogenic activity. The subcutaneous dorsa of mice were implanted with Lewis lung carcinoma and systemic therapy with recombinant mouse wild-type endostatin and three $Zn^{2+}$-binding mutants (20 mg/kg/day) was initiated when tumors were $\cong$200 mm$^3$. Each point represents mean $\pm$ s.e.m. (n=5 in each group).

lose most of its wild-type activity by its inability to bind $Zn^{2+}$. Alternatively, it could still bind zinc by using the three histidines, but the N-terminal loop would now be released and flexible. The N-terminus may block binding of endostatin to its receptor. We have recently reported that a $His_6$-Tag on the C-terminus completely inactivated endostatin (3). The N- and C-terminus of endostatin are in close proximity. It is possible that both the N- and C-terminal region are critical for receptor binding. Expression of soluble D76A endostatin mutant will be necessary to decide whether reduced activity is caused by loss of zinc-binding or a misshaped conformation.

The single H11A mutation may also lose most of its wild-type activity by its inability to bind zinc. Alternatively, we may speculate that the N-terminal loop is not stabilized anymore despite binding zinc using the three remaining ligands. Ding *et al.* show that glutamic acid 175 interacts with histidine 11 (1). It is possible that this interaction is essential for the N-terminal loop stability. It could also be that the C-terminus is destabilized because the histidine 11/glutamic acid 175 interaction is lost. We have shown in a previous report that the C-terminus is critically involved in endostatin activity. A $His_6$-tag on the C-terminus completely inactivated endostatin whereas it is tolerated on the N-terminus (3).

We also expressed the endostatin corresponding region of mouse collagen XV in our *E. coli* expression system and found no activity.[3] This region of mouse collagen XV is 64% identical to mouse endostatin. A possible explanation for this finding could be that H1, H3 and D76 are not conserved in either human or mouse collagen XV and therefore zinc-binding is not possible.

We conclude that $Zn^{2+}$-binding is essential for the full biological activity of endostatin. The capacity of endostatin to bind zinc may serve two functions. First, zinc-binding protects the N-terminus from proteolytic degradation and subsequently from inactivation. Although we do not yet know which proteases can generate endostatin from collagen XVIII, it is evident from our data that removing the $Zn^{2+}$ with EDTA makes the N-terminal amino acids susceptible to proteases. Second, the conformation induced by zinc-binding might be important for the interaction of endostatin with a putative endothelial cell receptor. Mutations in the zinc-binding amino acids greatly reduce the antiangiogenic activity of endostatin. It is not clear yet whether all three endostatin mutants are inactive because zinc-binding is disrupted. It is also possible that additional significant structural changes are involved in the loss of activity. These alterations could occur in the N-terminal loop but could also destabilize the C-terminus. Clearly, more structural analysis and mutational studies are necessary to gain a better understanding of how the N- and C-terminus of endostatin interact and how this interaction is important for the antiangiogenic activity of endostatin.

## ACKNOWLEDGMENTS

We would like to thank Richard Peters for suggesting incubation of endostatin at 37°C with different protease inhibitors, which subsequently led to the discovery of $Zn^{2+}$-binding. The mouse collagen XV sequence and a cDNA clone were obtained from Taina Pihlajaniemi, University of Oulu, Oulu, Finnland. T.B. would like to thank C. Fernandez and M. Moses for introducing to me the *Pichia pastoris* expression system. Mass spectrometry and N-terminal sequencing analysis was performed at the Biopolymer Facilities, Howard Hughes Medical Institute, Harvard Medical School (J. Rush, M.Obenauer and E. Piro). We gratefully acknowledge N-terminal sequencing, DNA sequencing, and amino acid analysis done at the Dana Farber Cancer Institute Core Facility. A. Flint at the Howard Hughes Medical Institute, Children's Hospital, did FACS sorting on the mammalian cell line. This work was supported by NIH grants to J.F and a grant to Children's Hospital from EntreMed (Rockville, Maryland). T.B. was supported by an Erwin-Schroedinger-Stipendium (Fond zur Foerderung der Wissenschaftlichen Forschung, Austria).

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