PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2014, 41(6): 567~574
www.pibb.ac.cn

Construction and Screening of Truncated Mutants of Recombinant Human Anti-angiogenic Protein proEMAP Ⅱ/p43*

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Abstract proEMAP Ⅱ/p43 was originally described as a scaffolding protein that is a component of the multi-aminoacyl-tRNA synthetase complex. Recently, proEMAP Ⅱ/p43 was found to be a cytokine as well as an endogenic anti-angiogenic protein. The p43 protein is thought to be a precursor of endothelial monocyte-activating polypeptide Ⅱ (EMAP Ⅱ). p43 showed higher biological activity than EMAP Ⅱ, making it a promising anti-angiogenesis inhibitors for cancer therapy. However, the structure of p43 and its function in angiogenesis remain unknown. Here, we constructed p43-like proteins with low molecular mass and high activity. We also determined the functional domains for the anti-angiogenic activity of p43. First, we predicted the secondary structure of p43 using a bioinformatics method, and then constructed 10 p43 truncated mutants. We compared the anti-angiogenic activity of the full-length p43 with the activities of the truncated proteins. We found that all the truncated proteins inhibited the migration of endothelial cells and prevent tubule formation. The deletion of up to 79 amino acids at the N-terminus or 47 amino acids at the C-terminus of p43 increased the activity to 2～3 times that of full-length p43. We identified three p43 truncations with lower molecular mass and higher activity than the full-length p43. These findings will help improve our understanding of the structure and function of p43, which, in turn, will be helpful to the further studies on the possible clinical applications of p43-like drugs.

Key words proEMAP Ⅱ/p43, truncated mutants, anti-angiogenic agents, endothelial cell migration, tubule formation
DOI: 10.3724/SP.J.1206.2013.00206

Angiogenesis, the formation of new blood vessels from existing ones, occurs in a variety of pathological processes, such as tumor growth and metastasis, and various inflammatory disorders. In 1971, Folkman first postulated the importance of angiogenesis in the treatment of tumors [1]. Therefore, anti-angiogenic therapy was established and several angiogenesis inhibitors have been discovered and developed as drug candidates[2–3].

Endothelial monocyte-activating polypeptide Ⅱ (EMAP Ⅱ), isolated originally from the supernatant of cultured methylcholanthrene A-induced fibrosarcoma cells，was expected to have therapeutic potential as an anti-angiogenic factor [4–5]. Its precursor, proEMAP Ⅱ (also known as p43 protein), consists of 312 amino acids and is an auxiliary factor of mammalian multi-aminoacyl-tRNA synthetases[6–7]. The p43 protein is secreted from different types of cells, including cancer, immune, and transfected cells. The C-terminal domain of p43 is equivalent to EMAP Ⅱ, but the structure and function of its N-terminal domain has not yet been understood. Previous studies have shown that p43 has a cytokine function as well as strong antitumor activity *in vitro* and *in vivo* [8–11]. p43 induces various proinflammatory cytokines, such as IL-8, and TNF; it also suppresses angiogenesis by inhibiting the proliferation and inducing the apoptosis of endothelial cells. Interestingly, compared with EMAP Ⅱ, p43 itself

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Received: July 25, 2013 Accepted: October 16, 2013

exerts more potent biological activities[12-13]. Based on these findings, we proposed that p43 could have dual functions as both an active cytokine and an endogenous angiogenesis inhibitor[14].

However, studies on p43 are still in the initial stages, and the structure and function of p43 remain unclear. In this study, we examined the correlation between structure and function of p43 with the aim of finding antitumor candidates with low molecular mass and high activity. We used a bioinformatics method to predict the secondary structure of p43.Then, we constructed a series of p43 domain truncations and determined the anti-angiogenic activities. We found that truncations of 16 or 79 amino acids in the N-terminal domain, or 47 amino acids in the C-terminal of p43, resulted in higher anti- angiogenic activities; we successfully identified three p43 truncations with low molecular mass and high activity. These findings will help improve our understanding of the structure and function of p43 and provide important information on the clinical application of p43 as an anti- angiogenic agent.

1 Materials and methods

1.1 Design of truncated mutants of p43

We used the GOR Ⅳ software[15] to predict the secondary structure of p43. To produce soluble proteins and not disrupt the secondary structure of p43, we designed five N-terminal domain truncations and five C-terminal truncations. The truncated p43 genes code amino acids residues were 5～312, 14～312, 17～312, 72～312, 80～312, 1～288, 1～285, 1～277, 1～268, and 1～264 and the products were named p43-1, p43-2, p43-3, p43-4, p43-5, p43-6, p43-7, p43-8, p43-9, and p43-10, respectively.

1.2 Construction of p43 truncations

The truncated DNA fragments were amplified by polymerase chain reaction (PCR) using the pET28a-p43 plasmid as the template. The oligonucleotide primers used for the PCR amplification contained endonucleases restriction sites *Eco*RⅠand *Xho*Ⅰ(NEB, USA) and are shown in Table 1. The amplified DNA fragments were then ligated and subcloned into the pMD18-T vector (TaKaRa, Japan) to create pMD18T/p43-*n* (where n =1～10). Next, pTIG-Trx vector and the pMD18T/p43-*n* vectors were digested with both *Eco*RⅠand *Xho*Ⅰ. The linearized pTIG-Trx vector and each of the p43-*n* fragments were ligated together using T4 DNA Ligase(NEB) to create pTIG-Trx-p43-*n*. The pTIG-Trx expression vector incorporates a C-terminal His-tag for affinity purification. The ligation products were transformed into competent *E. coli* BL21 (DE3) cells (Invitrogen, USA), the recombinant transformants were digested with endonucleases. The sequences of the final recombinants were confirmed to be correct by sequencing.

Table 1 Primers used for the PCR amplifications in this study

Products	Sense primer (5′→3′)	Antisense primer (5′→3′)
p43-1	CGGAATTCTAATGGATGCTGTTCTG	CGCTCGAGTTTGATTCCACT
p43-2	CGGAATTCTAATGAAGGGTGCAGAG	CGCTCGAGTTTGATTCCACT
p43-3	CGGAATTCTAATGGAGGCAGATCAA	CGCTCGAGTTTGATTCCACT
p43-4	CGGAATTCTAATGCAAAATGGAGTG	CGCTCGAGTTTGATTCCACT
p43-5	CGGAATTCTAATGTTTCCATCTGGT	CGCTCGAGTTTGATTCCACT
p43-6	CGGAATTCTAATGGCAAATAATGATGCTG	CGCTCGAGGTATGTAGCCAC
p43-7	CGGAATTCTAATGGCAAATAATGATGCTG	CGCTCGAGCACACACTCATC
p43-8	CGGAATTCTAATGGCAAATAATGATGCTG	CGCTCGAGATCAGGCTGGAT
p43-9	CGGAATTCTAATGGCAAATAATGATGCTG	CGCTCGAGCTTCTTAGGATTCAG
p43-10	CGGAATTCTAATGGCAAATAATGATGCTG	CGCTCGAGCAGCTCCTTGT

1.3 Expression and isolation of the truncated p43 proteins

The recombinant cells were inoculated in LB medium at 37℃ (A_{600} =0.4), then the cultures were induced with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L and shaken at 37℃ for 4 h to induce expression of target proteins. Cells were centrifuged and the pellets were resuspensed in 20 mmol/L Tris solution and lysed by ultrasonication and centrifuged at 13 000 r/min for

30 min. The supernatants were collected and loaded onto a HisTrap FF affinity column, the column was washed with 20 mmol/L Tris, 0.5 mol/L NaCl, 25 mmol/L imidazole buffer to remove protein contaminants, and then with 20 mmol/L Tris, 0.5 mol/L NaCl, 250 mmol/L imidazole buffer to obtain active fraction. The peak fraction was desalted and concentrated with Superdex 75 gel-filtration chromatography (GFC) and further lyophilized.

1.4 Endothelial cell migration assay

Human microvascular endothelial cells-1(HMEC-1) were cultured in MCDB131 medium with 10% FBS at 37℃ and 5% CO_2. Cells (2×10^4 cells per well) were seeded into the upper chamber of fibronectin pre-coated transwell filter (8 μm pores, Costar) with MCDB131 medium containing 0.2% FBS. MCDB131 medium containing 10% FBS were added to the lower chamber. Full-length p43 or truncated p43 proteins (1.5 μmol/L)were added into the upper and lower chambers of the well, with PBS as the negative control. The endothelial cells were allowed to migrate for 16～18 h at 37℃ and 5% CO_2. Cells that did not migrate to the lower chamber were gently wiped off using a cotton swab. Then the cells that had migrated completely through the filter to the lower chamber were stained with 0.1% crystal violet at room temperature for 10 min, washed, and extracted with 10% acetic acid. Then the cells were counted in a microplate reader at A_{595} and the inhibition rate was calculated as $(A_{control}-A_{sample})/A_{control}\times100\%$.

1.5 Endothelial cell tubule formation assay

The tubule formation assay was carried out as previously described [16]. Briefly, the HMEC-1 cells were cultured in MCDB131 medium with 10% FBS at 37℃ and 5% CO_2. After thawing on ice, ECM matrigel (60 μl/well) (Sigma, USA) was added into 96-well plates and the cells were incubated at 37℃ for 30 min. The HMEC-1 cells were seeded at 3.0×10^4 cells per well. Full-length p43 (Sine Pharmaceutical, Shanghai) and truncated p43 mutants were added at a final concentration of 1.5 μmol/L, and on this matrix, the endothelial cells migrated and formed tubules within 4 h. Wells were then stained with Calcein AM and the cells were visualized under a fluorescent microscope (at 100×magnification). Tubule formation was assessed in five randomly selected fields of view per well using the image analysis package.

1.6 Statistical analysis

Data are represented as $\bar{x} \pm s$, and error bars indicate the standard deviations. Experiments were performed three or more times independently. P values were calculated using a by Student's t test with a paired, one-tailed distribution. For all the analyses, SAS software version 8.0 was used. $P < 0.05$ indicates statistical significance.

2 Results

2.1 Construction of truncated mutants of p43

The p43 protein consists of two domians: an N-terminal domain and C-terminal domain. The C-terminal domain (EMAP Ⅱ) consists of 11 β-strands that form a structural core and 3 flanking α-helices (Figure 1a). But the structure of N-terminal domain is unknown. Here, we predicted the secondary structure

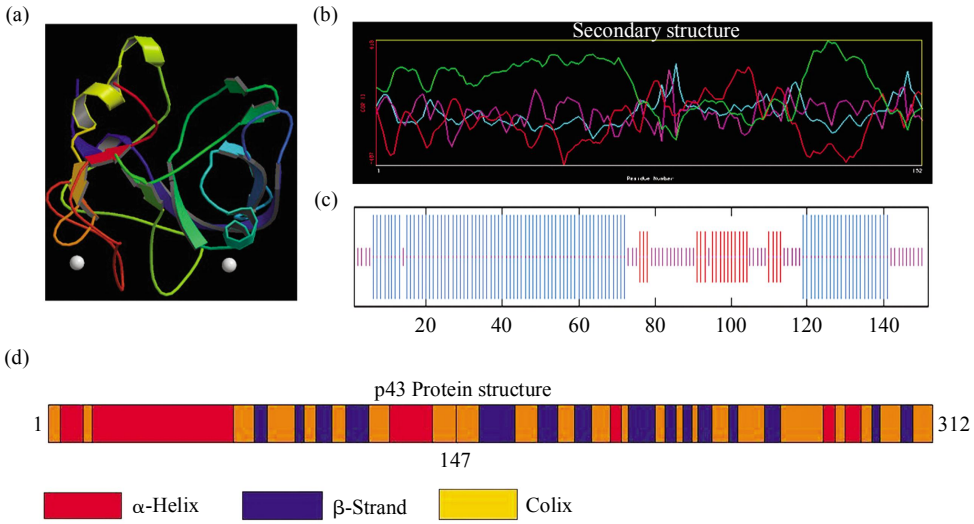

Fig. 1 Overall structure of the p43 protein

(a) Stereo view of the C-terminal domain (EMAP Ⅱ) structure of p43. (b)and(c) GOR Ⅳ predicted secondary structure of N-terminal domain of p43. (d) Secondary structure of full-length p43; the α-helices are shown in red, the β-strands in blue, and the coils in yellow.

of N-terminal domain of p43 using the GOR Ⅳ software. The results showed that N-terminal domain consists of 4β-strands and 3 flanking α-helices (Figure 1b and 1c). The overall secondary structure of full-length p43 is shown in Figure 1d. We used this information to produce soluble proteins in which the secondary structure of p43 was maintained; five N-terminal truncations (residues 5～312, 14～312, 17～312, 72～312 and 80～312)and five C-terminal truncations(residues 1～288, 1～285, 1～277, 1～268 and 1～264), and named them p43-1～p43-10, respectively(Figure 2).

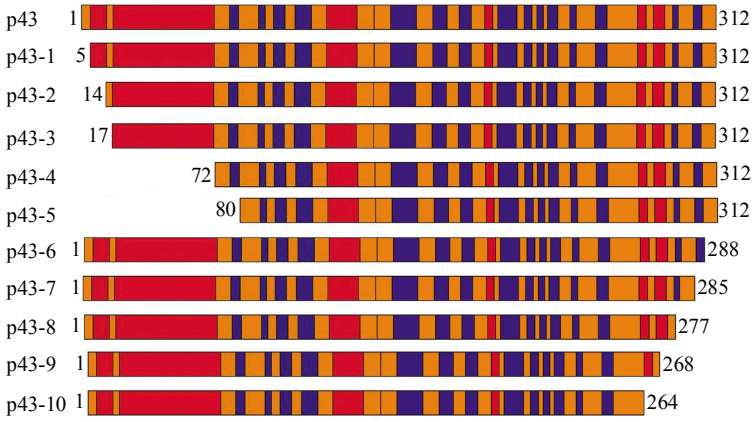

Fig. 2 Secondary structure of the p43 truncated proteins used in this study

The α-helices are shown in red, the β-strands in blue, and the coils in yellow. The five N-terminal truncated proteins are p43-1 (5～312), p43-2(14～312), p43-3(17～312), p43-4(72～312), p43-5(80～312) and five C-terminal truncations are p43-6(1～288), p43-7(1～285), p43-8(1～277), p43-9(1～268), p43-10(1～264).

2.2 Cloning and expression of the p43 truncated genes

The p43 truncated genes was PCR amplified with pET28a-p43 DNA as the template with the initiation codon "ATG" added ahead of each forward primer. After agarose gel electrophoresis, the desired bands were obtained, as shown in Figure 3. DNA sequence analysis of the PCR products confirmed that the gene sequences were correct. Bands with objective sizes were detected when digested by *Eco*R Ⅰ and *Xho* Ⅰ. The PCR products were cloned into the pTIG-Trx plasmid.

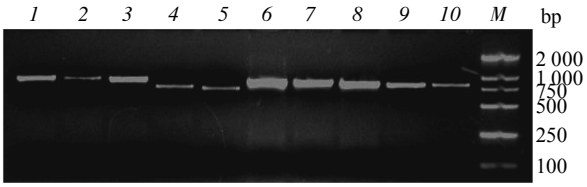

Fig. 3 Agarose gel electrophoresis of truncated mutants of p43 produced by PCR

All the desired bands for truncated genes were obtained. Lane *1*～*10*: p43-1～p43-10; *M*: Marker DL2000.

2.3 Expression and purification of p43 truncated proteins

The recombinant pTIG-Trx-p43-*n* expression vectors were transformed into *E. coli* BL21 (DE3) cells. At the optimal optical density, the cells were induced using IPTG to a concentration of 1 mmol/L to obtain the aimed-for expression of the proteins. SDS-PAGE gel electrophoresis of the lysates of *E. coli* cells revealed significant bands at ～34 ku. All the proteins were soluble. The fusion proteins were purified using a Ni^{2+}-affinity chromatography column. The purity of the proteins was over 90%, as identified by SDS-PAGE(Figure 4). Endotoxin from the purified proteins was removed using a polymyxin affinity column.

2.4 Screening and identification of p43 truncated proteins with anti-angiogenic activity

We investigated whether the p43 truncated proteins have any effect on anti-angiogenic avtivity in endothelial cells *in vitro*, and compared this with the effect of the full-length p43 protein. Cell migration was measured using a transwell migration assay[17]. We observed that the migration of HMEC-1 was inhibited

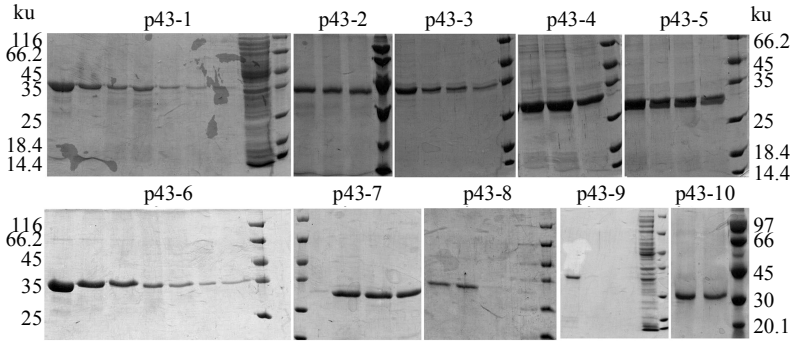

Fig. 4　SDS-PAGE electrophoresis of purified truncated p43 proteins

The proteins were purified by affinity chromatography and the purity of the proteins was over 90%. The protein bands are at ～34 ku.

by the full-length p43 protein and by all the p43 truncated proteins. However, p43-3, p43-5, and p43-10 had significantly higher inhibitory effects (increases of 310%, 308%, and 208%, respectively) on endothelial cell migration than the full-length p43 protein(Figure 5).

A concordant result was obtained in the tubule formation assay. HMEC-1 cells were seeded on matrigel to assess their ability to form tubule networks. As expected,, the full-length p43 protein and all the p43 truncated proteins inhibited tubule formation (Figure 6). In all cases, the inhibition of tubule formation was considered to be significant because, compared with tubule formation in the no-drug treated control, the inhibitory effect was almost double. We also noted that p43-3, p43-5, and p43-10 were better at

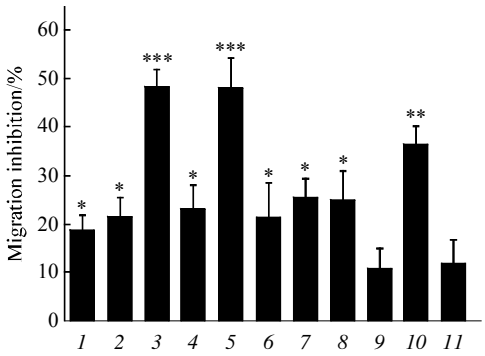

Fig. 5　Effects of p43 and its truncated mutants on human microvascular endothelial cell-1 migration

The cells were treated with 1.5 μmol/L full-length p43 or its truncated mutants for 16 h, the data are representative of the results from three independent experiments. Error bars indicate standard deviations. *P < 0.05, **P < 0.01, ***P < 0.001. *1*～*10*: p43-1～p43-10; *11*: Full-length.

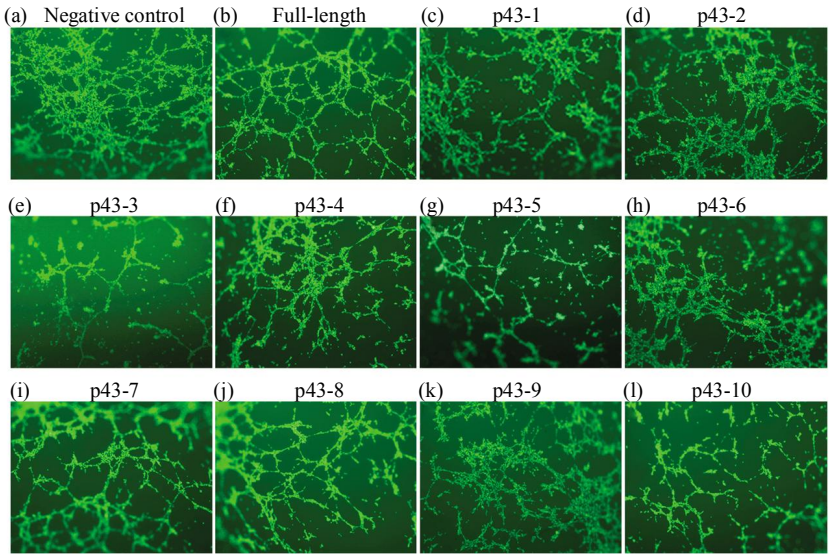

Fig. 6　Effects of full-length and truncated p43 proteins on human microvascular endothelial cells-1 tubule formation

HMEC-1 cells were treated with (a) PBS(Negative control), (b)1.5 μmol/L full-length p43, or (c～l) truncated p43 proteins. Tubule formation was measured at 4 h. Images from five random fields per well of three independent experiments were obtained. Wells were stained with calcein AM and the cells were visualized under a fluorescent microscope at 100× magnification.

inhibiting tubule formation than the full-length p43 protein. In conclusion, these findings show that the p43-3, p43-5, and p43-10 truncated proteins have better anti-angiogenic activities than the full-length p43, as demonstrated by their abilities to significantly inhibit the migration of endothelial cells and prevent tubule formation.

3 Discussion

Angiogenesis is an important component of the physiological process that occurs during wound healing, fetal development, and tumorigenesis [18]. Disrupting tumor angiogenesis is a novel approach to the treatment of cancer, and multiple targets in the tumor angiogenesis process may be found for therapeutic use[19-20]. proEMAP Ⅱ/p43 is a cofactor in the mammalian multi-aminoacyl-tRNA synthetase (ARS) complex, and its ability to function as a proinflammatory cytokine has been reported previously. Recently, it was reported that p43 can directly target endothelial cells to prevent angiogenesis, suggesting that p43 may be a potent inhibitor of angiogenesis.

Previous studies showed that EMAP Ⅱ, the C-terminal domain of p43, was released in apoptotic cells by the proteolysis of p43, where it acted as a functional cytokine with antivascular effects [21-22]. Recent studies showed that the full-length p43 protein was more active than EMAP Ⅱ, suggesting that the N-terminal domain of p43 may be responsible for its increased biological activity [23-25]. However, the structure of the N-terminal domain of p43 does not share homology with any known protein. The p43 protein consists of 312 amino acids, and has a molecular mass of 34 ku. Protein drugs with smaller molecular mass usually have low immunogenicity and weak side effects. Therefore, we generated smaller p43 proteins and screened them for higher activity. First, we predicted the secondary structure of the N-terminal domain of p43(Figure 1) and found that it consisted of four β-strands and three flanking α-helices. To reduce the length of the p43 sequence without disrupting the secondary structure and to obtain proteins that were soluble, we constructed ten truncated mutants based on the p43 secondary structure. All the truncated p43s were expressed as soluble His-tag fusion proteins in *E. coli*, and purified to high purity by nickel affinity chromatography.

Many experimental models and evaluation criteria for *in vivo* and *in vitro* trials have been established, including endothelial cell proliferation assay, cell migration assay, tubule formation assay, chick chorioallantoic membrane assay, and corneal microcapsule assay [26]. In a previous study, we established methods to evaluate recombinant human p43 activity in inhibiting angiogenesis *in vitro* using endothelial cell migration and tubule formation assay. A Boyden chamber model is often used in cell migration assay in which the numbers of cells that pass through a cell membrane are counted to observe the *in vitro* impact of drugs on angiogenesis. Cells are counted using colorimetric, fluorescent, or radio-labeled technologies; therefore, the method is simple and quantifiable. Tubule formation is an important step in angiogenesis, and it is the most complete index of endothelial cell function *in vitro*. Tubule formation is also simple, and easy to observe. Endothelial cells form tubes *in vitro* under specific conditions, such as matrigel or collagen culture. Inhibition of tubule formation or the cracking of formed lumens by drugs can inhibit angiogenesis. *In vitro* anti-angiogenic assay, we found that the full-length p43 and the truncated p43 proteins inhibited the migration of endothelial cells and prevented tubule formation. Moreover, three of the low-molecular-mass truncated mutants of p43, namely p43-3, p43-5 and p43-10, showed much higher activities in inhibiting angiogenesis than the full-length p43.

Our results suggest that the deletion of up to 79 amino acids at the N-terminus or of 47 amino acids at the C-terminus did not interfere with the anti-angiogenic activity of p43. Rather, the activity increased to 2～3 times the activity of full-length p43. Therefore, the N-terminal domain of p43 (residues 1～16 and 1～79) and the C-terminal domain of p43 (residues 265～312) do not seem to be responsible for the anti-angiogenic activity of the p43 protein, and, indeed, the deletion of these domains may expose active sites or motifs, allowing the protein to better perform its biology activities.

Ko *et al*. [12] reported that, at the same molar concentration, p43 had better anti-angiogenic activity than EMAP Ⅱ and showed a good dose-response relationship. These researchers also found that full-length p43, N-terminal domains containing residues 1～146 and 1～108, and a p43 protein (residues 92～256), which was truncated at both the

N- and C- terminal ends, had better cytokine activities than EMAPⅡ (equivalent to residues 147～312 of the full-length p43). Further, some of the p43 derivatives tested in their study could activate tumor necrosis factor (TNF) and interleukin 8 (IL-8) expressions, with a highly truncated p43 (residues 92～146) showing the highest cytokine activity. Other truncated p43s (residues 218～312 and 257～312) were unable to activate TNF and IL-8 expressions. Our results correspond to the findings of this previous study. These data indicated that the N-terminus of p43 as a whole, or at least some amino acids within this region, can promote the anti-angiogenic activity of p43.

Recently, three functional domains at the N-terminus of p43 were identified in a deletion mapping experiment [9]: (1) residues 101～114 that promote endothelial cell death and caspase-3 activation; (2) residues 6～46 that are responsible for fibroblast proliferation; and (3) residues 114～192 that promote endothelial migration. Interestingly, the role of p43 in angiogenesis was reported to be biphasic and dose-dependent; low concentrations were pro-angiogenic while high concentrations were anti-angiogenic[27]. For example, cell migration increased by about four-fold at 1 nmol/L p43, but decreased at concentrations of p43 higher than 1 nmol/L. In our study, we found that 1.5 μmol/L p43 inhibited endothelial cell migration. Further, using a gene microarray technique, we screened 132 genes, including IL-8 and signal transducer and activator of transcription 1 (STAT1), which were differentially expressed in endothelial cells incubated with p43(data not shown). These studies will help reveal the molecular mechanisms that underlie the effects of p43 truncations.

Thus, we have successfully identified three truncated mutants of p43 with low molecular mass and high activity. We found that residues 1～16, 1～79, and 265～312 were not required for the anti-angiogenic activity of p43. Further studies to construct and screen other truncated p43s (residues 17～264 and 80～264) are now being conducted. Our results will provide an important basis for studying the correlation between structure and function of p43 and the molecular mechanism involved, which may also provide new small molecular drug candidates for tumor therapy.

Acknowledgments We greatly thank Profs DING Jian and ZHAO Zhi-Hu for providing the cells and plasmid.

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抗血管生成活性的重组人 proEMAPⅡ/p43 蛋白缺失突变体的构建和筛选 *

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摘要 proEMAPⅡ (又称 p43 蛋白)是哺乳动物氨酰 tRNA 合成酶的辅助因子，近年来发现，p43 具有细胞因子活性以及抗新生血管生成的作用，具有潜在的抗肿瘤疗效．p43 的 C 端结构域即为 EMAPⅡ，其结构和功能都已知，具有细胞因子和抗血管生成的双重功能．但是 N 端的结构还不清楚，研究表明全长的 p43 比 EMAPⅡ具有更高的生物学活性，但是 p43 的结构和作用机制尚不明确．此外，作为蛋白质药物，更小的分子能够减少免疫原性和副作用，从而发挥更好的活性．本文利用生物信息学方法，对 p43 N 端的二级结构进行预测，并且在不破坏二级结构的前提下，构建了 10 个 p43 的缺失突变体，在体外对 10 个缺失突变体与全长的 p43 蛋白进行抗新生血管生成活性验证比较，最终我们获得了 3 个活性高的 p43 缺失突变体，并且发现 N 端的 1～16，1～79 位氨基酸和 C 端的 264～312 位氨基酸不是 p43 发挥抗血管生成功能所必需的，而且它们的删除使得活性位点更好地呈现并发挥活性．通过我们的研究，有助于揭示 p43 蛋白结构和功能的关系以及其作用机制，同时为临床筛选肿瘤治疗候选药物奠定基础．

关键词 proEMAPⅡ/p43，缺失突变体，抗血管生成剂，内皮细胞迁移，管腔形成

学科分类号 Q71，Q936 **DOI**: 10.3724/SP.J.1206.2013.00206

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收稿日期：2013-07-25，接受日期：2013-10-16