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Identification and Characterization of a Novel Angiostatin-binding Protein by the Display Cloning Method

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Angiostatin is a potent anti-angiogenic protein. To examine the angiostatin-interacting proteins, we used the display-cloning method with a T7 phage library presenting human cDNAs. The specific T7 phage clone that bound to the immobilized angiostatin was isolated, and a novel gene encoding the displayed polypeptide on the isolated T7 phage was identified. The displayed angiostatin-binding sequence was expressed in *E. coli* as a soluble protein and purified to homogeneity. This novel angiostatin-binding region interacted specifically to angiostatin with a dissociation constant of 3.4×10^{-7} M. A sequence analysis showed that the identified sequence was a part of the large ORF of 1,998 amino acids, whose function has not yet been characterized. A Northern analysis indicated that the gene containing the angiostatin-binding sequence was expressed differentially in the developmental stages or cell types.

Keywords: Angiogenesis, Angiostatin, Angiostatin-binding protein, Display cloning, T7 phage.

Introduction

Angiogenesis is the formation of new capillaries from pre-existing vasculature by the migration and proliferation of endothelial cells (Beck and D'Amore, 1997). It is a fundamental process that is required for a number of pathological events and considered a key step in tumor growth, invasion, and metastasis (Gastl *et al*., 1997; Uhr *et al*., 1997). Since the dependence of the growth and metastasis of tumor cells depends on neovascularization, then angiogenesis may serve as a new target for developing anticancer therapies (Folkman, 1971). The cellular mechanism of angiogenesis has been extensively studied (Folkman, 1995; Hanahan, 1996), and several agents that interfere with the angiogenesis have been reported (Brouty and Zetter, 1980; Rastineiad *et al*., 1989; Ingber *et al*., 1990). Among them, angiostatin was identified in the blood serum of cancer patients and shown to possess strong anti-angiogenic activity (O'Reilly *et al*., 1994).

Angiostatin is an internal fragment of plasminogen containing 5 homologous kringle domains, and it exhibits potent and specific inhibitory activity against the growth of endothelial cells (Castellino *et al*., 1998). It selectively inhibits the proliferation and migration of endothelial cells *in vitro*, and therefore inhibits the growth of both primary and metastatic tumors (Castellino *et al*., 1998). Particularly, the plasminogen fragments containing kringle domains 1-3 (Joe *et al*., 1999), 1-4 (Cao *et al*., 1996) and, 1-5 (Gately *et al*., 1997; Cao *et al*., 1999) inhibit angiogenesis and tumor growth in animal models. Possible target proteins of angiostatin have been searched to elucidate the anti-tumor mechanism of angiostatin. The α-subunit of ATP synthase was identified as an angiostatin-binding protein by the affinity purification method (Moser *et al*., 1999). Troyanovsky *et al.* (2001) also identified angiomotin as an angiostatin-interacting protein by using the yeast two-hybrid screening method, and showed that angiomotin regulated the migration of endothelial cells and tube formation. Although these proteins interact with angiostatin, the anti-angiogenic mechanism of angiostatin has not yet been completely revealed.

Recently, the phage display cloning method was successfully used to identify the proteins that interact specifically with bait proteins or small chemical compounds. Random sequences of hepta- or dodecameric peptides (Scott and Smith, 1990; Clackson and Wells, 1994), or cDNA from human tissue (Crameri *et al*., 1994; Jesfer *et al*., 1996), that are displayed on the surface of the phage particle could specifically bind to the immobilized proteins (Zozulya *et al*., 1999) or chemical compounds (Rodi *et al*., 1999; Jin *et al*., 2002). In this report, we isolate a protein that interacts with angiostatin by the display cloning method that uses a phage

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library presenting the human cDNA. The gene containing the angiostatin specific sequence has been identified, and its transcriptional and translational products examined. Furthermore, the angiostatin-binding sequence was expressed, and its interaction with angiostatin was characterized.

Materials and Methods

Materials Human angiostatin, which contained kringle domains 1-4, was kindly provided by Dr. M. J. Park (Korea Cancer Center Hospital) and also purchased from CalBiochem (Darmstadt, Germany). The 96-well polystyrene plate was purchased from Corning (New York, USA), and the T7 phage library that displayed a human liver cDNA library was purchased from Novagen (Madison, USA). The sensor chip CM5 for the surface plasmon resonance analysis was obtained from Biacore AB (Uppsala, Sweden). All of the other reagents were reagent grade. The lysate of the HepG2 and HUVEC cells were kindly provided by Dr. B. M. Kweon (Korea Research Institute of Biotechnology and Bioscience).

Biopanning About 1 μg of angiostatin in 100 μl of a PBS buffer (100 mM Na-phosphate, 150 mM NaCl, pH 7.0) was incubated overnight on a 96-well plate at 4°C. The plates surface was blocked by 5% skim milk for 1 hr at room temperature and washed five times with a PBST buffer (0.1% Tween-20 in PBS). The T7 phage library of the human liver cDNA (6×10^9 pfu) in 100 μl of a PBS buffer was added to the angiostatin-coated well. The plate was incubated overnight at 4°C with gentle shaking. After the plate was washed 20 times with a PBST buffer, the phage particles that were attached to the angiostatin-coated well were eluted by incubation with 0.1% SDS in a PBS buffer for 20 min. The eluted phages were amplified in the *E. coli* strain BLT5615, and the biopanning procedure was repeated three more rounds. At the third and fourth round, the bound phages were eluted with 1.0 mg/ml of angiostatin rather than 0.1% of SDS.

Sequence determination and analysis The DNA of the isolated phage was extracted with a Lambda Mini Kit (Qiagen, Valencia, USA) and the inserted sequence was amplified by a PCR reaction using two primers (T7-up; 5'-ACTTCCAAGCGGACCAGATT-3', T7-down; 5'-CGTTCATATCGTATGAGCGC-3') that spanned 150 bp upstream and downstream of the insertion site in the T7 phage DNA, respectively. The nucleotide sequence of the PCR product was determined by the dideoxy chain termination method and analyzed using the BLAST search program (Altschul *et al*., 1997).

Protein expression and purification The identified angiostatin-binding sequence (ABS) from the phage of the T7 human liver cDNA library was amplified by restriction PCR (Klimkait, 2000). The PCR product was inserted into the *BamH*I and *Hind*III sites of the pET28a vector (Novagen, Madison, USA). The resulting plasmid was designed to produce the ABS with a histidine-tag at the N-terminus. This plasmid was transformed into the *E. coli* strain BL21 (DE3). The transformed cells were grown at 37°C in a LB media. The protein expression was induced at an OD_{600} of 0.6 by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). After incubation of the cells for 5 h at 30°C, the cells were harvested by centrifugation and disrupted by passing the cell suspension twice through a French Press (SLM Instruments, Rochester, USA). The cell extract was applied to a Ni-NTA column (Qiagen, Valencia, USA), and the bound protein was eluted with 250 mM imidazole (pH 8.0). The eluted protein was loaded onto a Q-sepharose column (Amersham Pharmacia, Uppsala, Sweden). The bound proteins were eluted by a linear gradient of 0.1 M-0.35 M NaCl in 50 mM Na-phosphate buffer (pH 7.5) containing 5 mM β-mercaptoethanol.

ELISA A relative amount of the phage particle or the purified ABS protein that was bound to the immobilized angiostatin was measured by ELISA. The T7 phage particles of 1.1×10^{10} pfu or 3-30 μg of the purified ABS protein in the PBS solution were incubated at 4°C overnight in the angiostatin-coated well. The phages or ABS that were bound on the plate were detected with HRP-labeled T7-tag IgG (Novagen, Madison, USA) or anti-His6-IgG (Qiagene, Valencia, USA) /HRP-labeled anti-mouse IgG (Amersham, Piscataway, USA), respectively, as previously described (Jin *et al*., 2002).

Co-immunoprecipitation The direct interaction between the purified ABS and angiostatin was examined by a co-immunoprecipitation analysis. About 10 μg of the angiostatin and purified ABS were mixed and incubated for 2 h at room temperature with gentle agitation. Next, 3 μg of the anti-angiostatin monoclonal antibody (Oncogene, La Jolla, USA) and 50 μl of Protein G-sepharose (Sigma, St. Louis, USA) were added successively, and the mixture was incubated overnight at 4°C. After washing the beads three times, the bound proteins were eluted by incubation at 95°C for 5 min in 2 × SDS loading buffer. The eluted proteins were separated on a 15% SDS-PAGE gel, blotted to a PVDF membrane, and probed with His-tag IgG (Santacruz, Santa Cruz, USA). The His-tag containing proteins were further visualized by the ECL method using HRP-conjugated anti-mouse IgG (Amersham, Piscataway, USA), as described in the instruction manual.

Surface plasmon resonance analysis A quantitative analysis of the interaction between ABS and angiostatin was performed by surface plasmon resonance using a BIAcore 3000 (BIACORE, Uppsala, Sweden) instrument as described (Kim, 2001). Angiostatin was covalently attached on a CM5 sensor chip using an amine-coupling kit, as described in the instruction manual. The purified ABS of different concentrations in the running buffer (PBS, pH 7.4) was injected at a flow rate 25 μl/min, and the association and dissociation curves were obtained. The surface of the sensor chip was regenerated by an injection of the regeneration buffer (30 mM NaCl, 1.7 mM NaOH). The SRP response curves were analyzed with BIACORE EVALUATIONS software, version 3.1.

Northern analysis and RT-PCR The mRNA levels that could hybridize with the isolated DNA sequence were measured by Northern analysis. A membrane blot, which consisted of 2 μg of poly A^+ RNA from 8 different human tissues (Clontech, Palo Alto, USA), was hybridized with a 500 bp of ABS region. The membrane was washed twice at room temperature with 2 ×

standard saline citrate (SSC)/0.01% SDS for 40 min, followed by a high-stringency wash in 0.1 × SSC/0.1% SDS at 50°C for 20 min. The hybridized probe was visualized by FUJIX BAS2000 phosphoimager, as described in the instruction manual. The cDNA level that contained ABS in various human tissues was examined by PCR using two primers that were specific to ABS (primer 1; 5'-CACTCTTCCAGCCTTCCTTCC-3', primer 2; 5'-CGGACTCGT CATACTCCTGCTT-3'). The PCR reaction with primers that were specific to the constitutively-expressed β-actin was performed as the control (Mangalam *et al*., 2001).

ABS-specific antibody production and Western blot analysis Polyclonal antibodies against the purified ABS were generated from mice (KOMA Biotech, Seoul, Korea). The lysate from the HepG2 and HUVEC cells was prepared by sonication with a lysis buffer (20 mM Tris-HCl pH 8, 137 mM NaCl, 1% Nonidet P40, 10% glycerol, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 20 μg/ml leupeptin). The proteins of the cell lysate were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The proteins that were specific to the anti-ABS antibodies were visualized by the ECL method using HRP-conjugated anti-mouse IgG (Amersham), according to the manufacturers instructions.

Results

Screening and identification of angiostatin specific clone The T7 phage particles that bind specifically to angiostatin were isolated after 4 rounds of biopanning using an angiostatin-coated plate. The phage titer of the elution fraction was significantly increased at the fourth round (Fig. 1A). The enrichment of specific phage particles was further confirmed by a PCR analysis with primers that flanked the library construction sites of the T7 phage DNA. The PCR product sizes from the T7 library were randomly distributed (Fig. 1B, lane 1). However, distinct bands of the PCR products appeared after the first round of biopanning. The 700 and 900 bp products appeared after the third round of biopanning. Their intensities increased at the fourth round of biopanning (Fig. 1B, lanes 4 and 5). This indicates that the T7 phages that generate 700 and 900 bp PCR products were selectively amplified. The phages that generate the 300 and 560 bp PCR products were not examined since the intensity of these PCR products were not significantly increased, or they disappeared during the biopanning process (Fig. 1B). The phage clone that produced the 900 bp PCR product has an open-reading frame of 21 amino acids, so the sequence was not further characterized. A phage clone that produced the 700 bp PCR product had an open-reading frame of 133 amino acids. The DNA fragment from the selected phage clone was hybridized with the 700 bp PCR product (Fig. 1C), which indicates that the isolated DNA fragment represents the 700 bp product. The isolated T7 phage specifically binds to surface-coated angiostatin (Fig. 1D), which indicates that this T7 phage clone has a propensity to interact with angiostatin.

Sequence analysis and expression of angiostatin-binding sequence The phage clone that generated the 700 bp PCR product had a cDNA sequence of 401 bp. The 133 amino acids that were encoded from the 401 bp insert were expressed at the C-terminus of the T7 phage coat protein, gene 10. This identified amino acid sequence was matched with part of an ORF that was derived from human genomic DNA

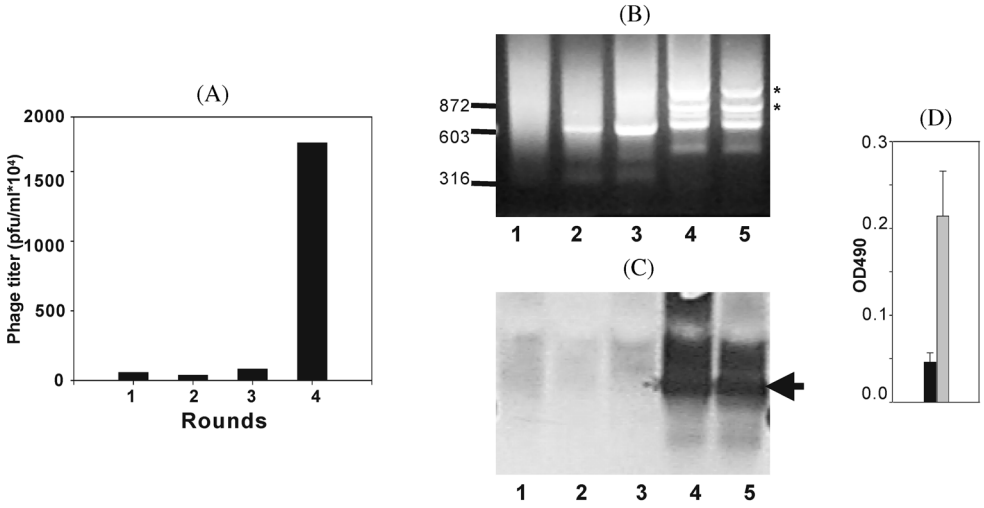

Fig. 1. Analysis of phages eluted from angiostatin-coated plate. (A) The phage titer of the eluted solution was determined after each round of biopanning. (B) The inserted DNAs of the T7 phage library (lane 1) or eluted phages from the first to fourth rounds of biopanning (lane 2 to 5) were amplified by PCR and analyzed by agarose gel electrophoresis. The 700 and 900 bp PCR products are indicated as asterisks. (C) Southern analysis of the PCR products using a DNA fragment representing the ABS sequence. The arrow indicates the 700 bp product. (D) The amount of phage particle attached on the angiostatin-coated plate was measured by the ELISA method with HRP-labeled T7 tag IgG. The gray box represents the screened T7 phage containing ABS and the black box represents unamplified T7 phage library.

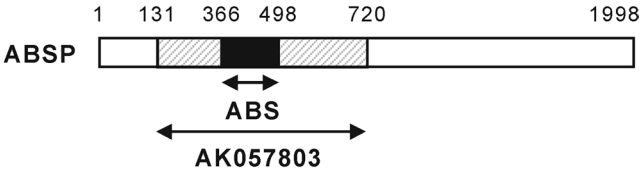

Fig. 2. Schematic representation of the angiostatin-binding sequence (ABS) in the ORF of angiostatin-binding sequence containing protein (ABSP). The black box (■) indicates ABS, and the gray box (▨) indicates an identical region with AK057803 cDNA clone.

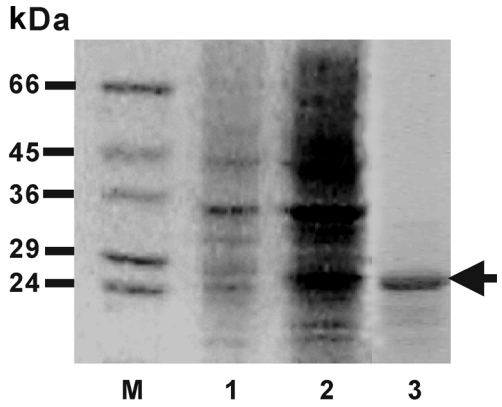

Fig. 3. Expression and purification of the ABS. Cell extracts before (lane 1) and after induction (lane 2), and the purified His-labeled ABS protein (lane 3) were analyzed by SDS-PAGE. The purified protein band is indicated as an arrow.

from the sequence analysis of the Human Genome Resource in the NCBI database. The gene that contained this ORF was located at the human chromosome 16q24.3 and was previously assigned as Hs16_10699_31_5_2. The ORF of this gene encodes a large polypeptide of 1,998 amino acid residues. The cellular function of this gene has not yet been characterized. The displayed sequence at the surface of the isolated T7 phage corresponded to amino acids 366-498 of ORF (Fig. 2). In this study, this region was tentatively named the "angiostatin-binding sequence" or "ABS", and the full length ORF was tentatively named the "angiostatin-binding sequence containing protein" or "ABSP". A homology search of ABS in the cDNA database revealed that it has an identical sequence to part of AK057803, an mRNA sequence from human cerebellum. This indicates that AK057803 is part of Hs16_10699_31_5_2 (Fig. 2). The ABS containing a His-tag sequence at the N-terminus was expressed in *E. coli* as a soluble protein and purified homogeneity using Ni-affinity and ion exchange column (Fig. 3). The ABS expression as a soluble protein suggested that ABS might form a structural domain.

Characterization of the interaction between ABS and angiostatin The interaction between angiostatin and the purified ABS was characterized using ELISA, co-immunoprecipitation, and a surface plasmon resonance analysis. When the purified ABS was incubated in an angiostatin-coated plate, it bound to angiostatin in a dose-dependent manner (Fig. 4A). The physical interaction between ABS and angiostatin in solution was also examined by a co-immunoprecipitation analysis. The antibodies that are specific to angiostatin could not cross-react with ABS (Fig. 4B, lane 1). However, it could precipitate the purified ABS in the presence of angiostatin (Fig. 4B, lane 2), indicating that the ABS directly binds to angiostatin. The kinetic property of the association and dissociation of ABS to angiostatin was

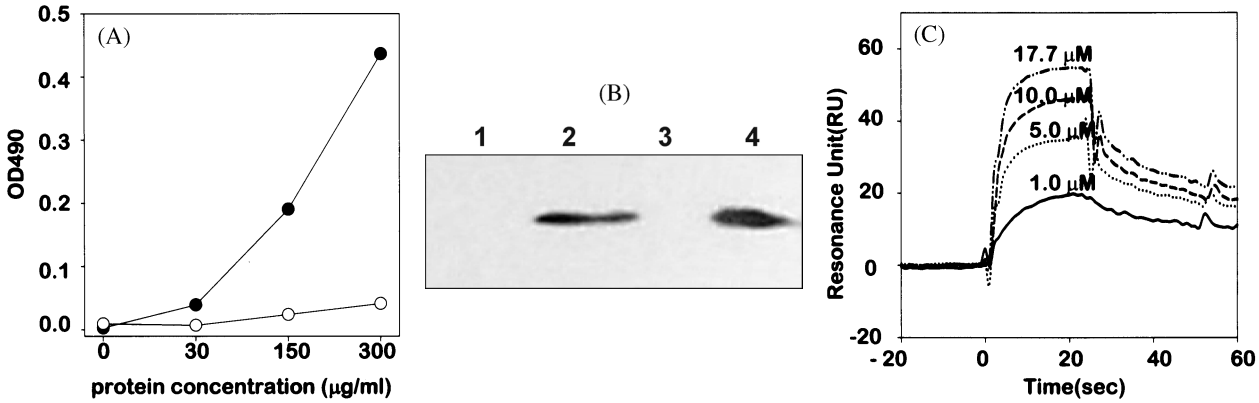

Fig. 4. Specific interaction between ABS and angiostatin. (A) The amount of purified His-labeled ABS attached on the angiostatin-coated plate was measured by the ELISA method. The filled circle represents the ABS and the empty circle represents His-labeled thioredoxin that was used as a negative control. (B) Co-immunoprecipitation of ABS. Angiostatin-specific antibodies were incubated with angiostatin (lane 1), angiostatin, and His-labeled ABS (lane 2) or His-labeled ABS (lane 3). After the precipitation of the antibodies with Protein G -sepharose, the extracted proteins were separated in SDS-gel, and His-labeled ABS was detected using His-tag antibodies (Santacruz, USA). His-labeled ABS was used as positive control for the His-tag antibodies (lane 4). (C) The binding sensorgram of the ABS on angiostatin. Sensorgrams were obtained for 1.0 (solid line), 5.0 (dotted line), 10 (long-dashed line), 17.7 (dashed-dot line) μM of ABS against angiostatin that was immobilized on the sensor chip.

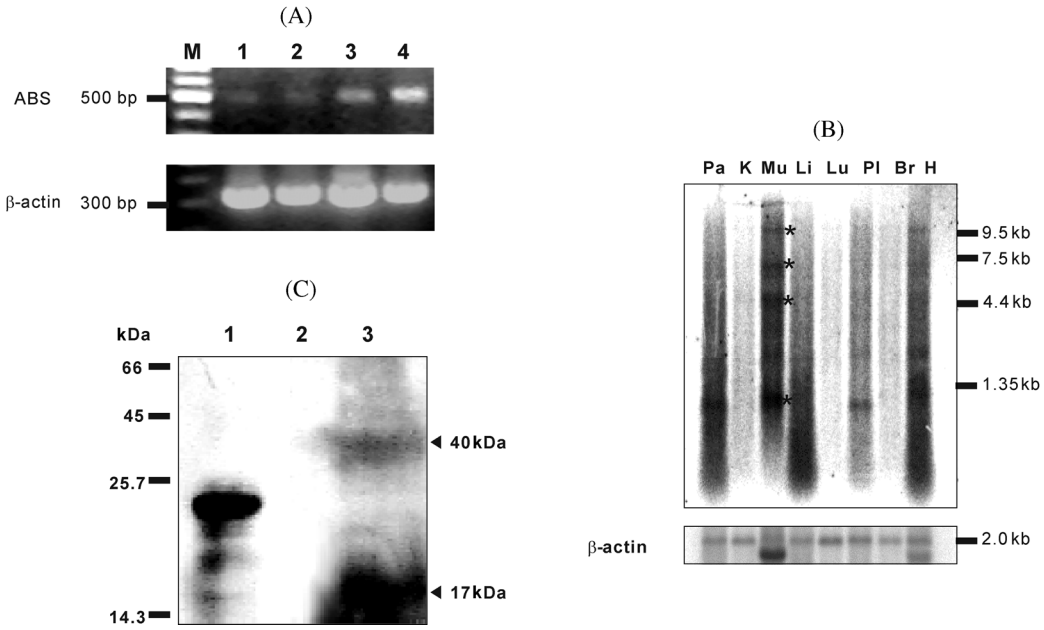

Fig. 5. Analysis of the ABSP gene expression. (A) The cDNAs from the human fetal liver (lane 1), brain (lane 2), lymphocyte (lane 3), and placenta (lane 4) were used as a template for PCR. Different transcriptional levels of cDNA were adjusted by β-actin as a house keeping gene. (B) Northern blot analysis of ABSP expression in adult tissues. A nylon membrane containing 2 μg of poly A^+ RNA was analyzed using ABS fragment as a probe. Pa, pancrease; Ki, kidney; Mu, muscle; Li, liver; Lu, lung; PL, placenta; Br, brain; H, heart. The β-actin probe was used for the analysis for the control of the constitutively-expressed gene. The mRNA bands of 1.3, 5.0, 6.5, and 9.0 kb are indicated as asterisks. (C) Western analysis of ABS. The protein extract from the HepG2 cell (lane 2) and HUVEC (lane 3) was separated in 12% SDS-PAGE, and the ABS containing proteins were detected using antibodies specific to the purified ABS. The purified ABS was used as a positive control (lane 1). Two detected proteins of 17 and 40 kDa are indicated as arrows.

further examined by a surface plasmon resonance analysis using a BIAcore instrument. The purified ABS showed a typical association and dissociation curve against angiostatin in a dose-dependent manner (Fig. 4C). The apparent dissociation constant (K_d) was calculated as 3.4×10^{-7} M from the association and dissociation kinetic parameters. The strength of this interaction is comparable or slightly less than those from the interaction between receptors and ligands (Cochrane *et al*., 2000), or antigen and antibody (Daugherty *et al*., 1998). The strength of this interaction, however, belongs to the general protein-protein interactions that have K_d values in the range of 10^{-6}-10^{-7} M (Causey *et al*, 1996).

Expression pattern of ABS The cellular function, as well as the expression profile of the newly identified ABSP, has not yet been reported. Although the *in vivo* expression of ABSP could be inferred from the occurrence of the ABS sequence in the T7 cDNA display library or EST database, the tissue specific expression pattern of mRNA or polypeptide have not been examined. When cDNAs from 4 different human tissues were used as templates for PCR, the cDNAs from the placenta and lymphocyte generated more PCR products than those from the brain or fetal liver. All of the cDNA samples generated equal amounts of the PCR product for the β-actin gene (Fig. 5A). When the ABS was hybridized with the polyA^+ RNAs from various human tissues, then the RNA bands of 1.3, 5.0, 6.5, and 9.6 kb were detected in the muscle tissue (Fig. 5B). These results indicate that the ABSP gene is rather preferentially expressed in the early stages of tissues development, such as blood cells or muscles. In addition, the presence of multiple transcripts suggested that the ABSP gene has multiple transcription initiation sites or several alternative slicing patterns, depending on the tissue types or developmental stages.

The size of ABSP that is expressed in human cell lines was further examined using polyclonal antibodies that were raised against the purified ABS. When the cell extracts from the HUVEC and HepG2 cells were cross-reacted with the anti-ABS antibodies, two protein bands of 17 and 40 kDa were detected only in the extract of the HUVEC cells (Fig. 5C). These results suggest that the ABSP gene was expressed as multiple forms of polypeptides, which are much smaller than the calculated size of ORF. Also, these proteins are preferentially expressed in certain cell types, such as the HUVEC cell.

Discussion

The identification of the binding partners to specific protein or ligands provides essential information for understanding their cellular function as well as for developing therapeutic

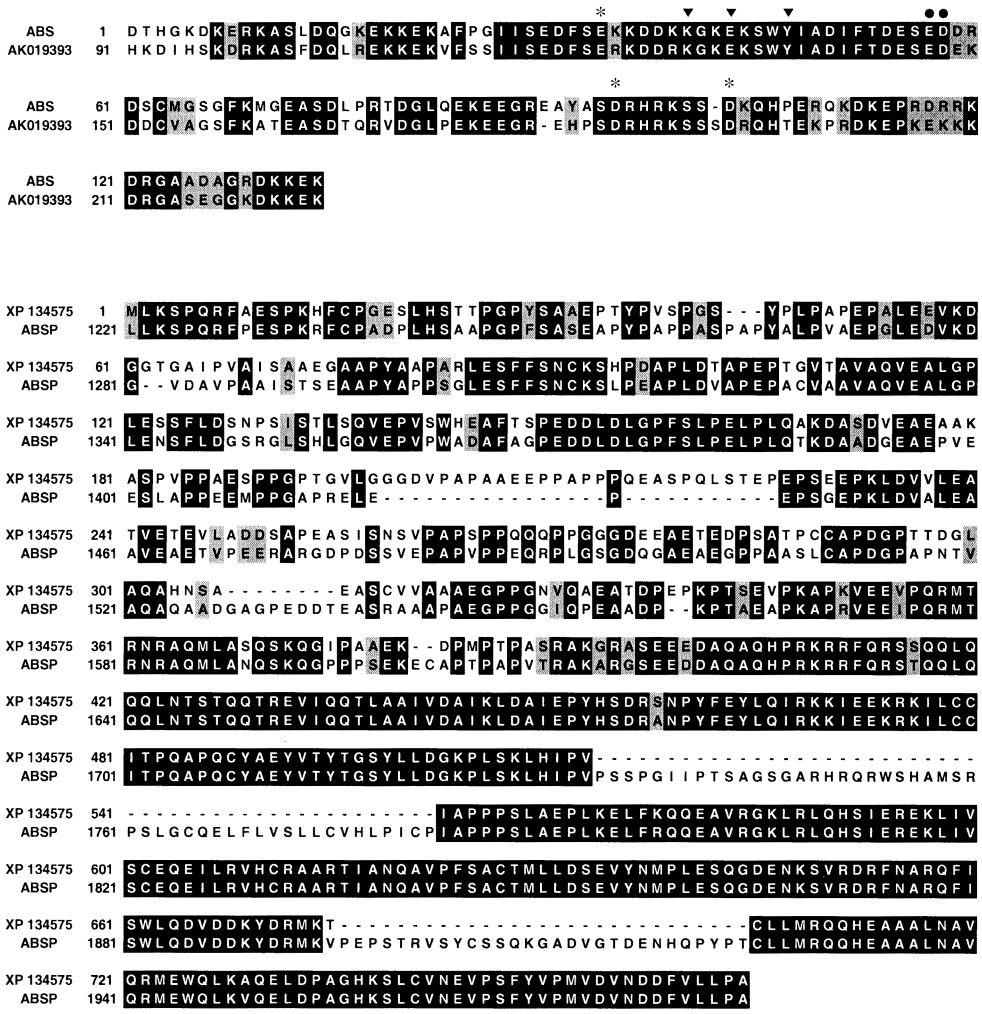

Fig. 6. (A) Alignment of the ABS with AK019393. The two sequences showed a 64% identity. The potential phosphorylation sites in ABS by protein kinase C (*), tyrosine kinase (●), and casein kinase II (▼) are indicated. (B) Alignment of the C-terminal region of the identified ABSP with Riken cDNA 2410104C19. The two sequences showed a 65% identity.

methods in case the target molecules are related to the disease process. Yeast two-hybrid or affinity selection methods have been used for this purpose, although low abundant proteins, or proteins that are targeted to organelles other than nucleus, are hardly applicable for these approaches. In contrast, there are advantages to the biopanning method with a T7 phage system that displays foreign proteins from a cDNA library. These advantages include rapid growth and amplification, detection of low-level expressed protein, and convenience of *in vitro* selection. With the T7 phage display system displaying human cDNA, we identified a specific sequence (ABS) against angiostatin by biopanning. Previously, the α-subunit of ATP-synthase and angiomotin were known to interact with angiostatin (Moser *et al*., 1999; Troyanovsky *et al*., 2001). It is noticeable that these proteins were not screened by the display cloning method in this study. On the other hand, the ABS that was identified was not previously observed from the affinity selection or yeast two-hybrid screening. These observations indicate that the currently used methods for the detection of protein-interacting partners have certain limitations, and different screening methods should be applied to identify the interaction partners of the target protein.

The inhibitory mechanism of angiogenesis by angiostatin has been inferred from its interaction with the α-subunit of ATP-synthase and angiomotin (Moser *et al*., 1999; Troyanovsky *et al*., 2001). Angiostatin may perturb the energy metabolism of the endothelial cell by inhibiting the biosynthesis of ATP or preventing the growth of endothelial cells via the interaction with angiomotin (Moser *et al*. 2001; Moser *et al*., 2002). The newly identified angiostatin-binding sequence may provide another inhibitory pathway of angiogenesis. A Northern analysis, RT-PCR, and Western analysis of the newly identified ABS indicated that the identified sequence was expressed in various tissues with different levels. Also, the smaller size of the transcripts and polypeptides products from the ABSP gene, in contrast to the

expected size of the single largest ORF, suggest that the nascent transcripts or polypeptides undergo extensive processing in order to form mature proteins. From the Western analysis, we failed to detect the large polypeptides that were bigger than 200 kDa. However, it cannot be ruled out that the ABS-containing gene is expressed as a large polypeptide since proteins that were bigger than 100 kDa are often difficult to be transferred to the PVDF membrane during Western blotting.

A sequence analysis of ABS failed to find any homologous genes whose functions were characterized. Instead, it showed a homology to a mouse cDNA, AK019393 (GI 12859577) (Fig. 6A). Furthermore, the C-terminus region of ASBP showed a sequence similarity to another mouse cDNA (Riken cDNA 2410104C19; XP 134575, GI 20887359) (Fig. 6B) whose functions are also currently unknown. The functions of these mouse genes are currently unknown, which is similar to ASBP. An analysis of the structural and functional motives indicates that ABSP has no significant transmembrane regions and signal peptide sequence (data not shown), which suggests that it may not be a surface-expressed receptor. On the contrary, it has possible nuclear localization signal and potential phosphorylation sites (Fig. 2) that are specific to protein kinase C, casein kinase II, or tyrosine kinase (Gattiker *et al*., 2002). This indicates that it may locate at the cytosol or nucleus and be involved in signaling pathway. These observations, along with the cell-type specific expression pattern, suggest that the ABS-containing gene may be involved in the cellular proliferation process. The cellular function of ABSP and its interaction with angiostatin *in vivo* will be the objectives of future study.

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