SPARC Is a Source of Copper-binding Peptides that Stimulate Angiogenesis

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Abstract. SPARC is a transiently expressed extracellular matrix–binding protein that alters cell shape and regulates endothelial cell proliferation in vitro. In this study, we show that SPARC mRNA and protein are synthesized by endothelial cells during angiogenesis in vivo. SPARC and peptides derived from a cationic region of the protein (amino acids 113–130) stimulated the formation of endothelial cords in vitro; moreover, these peptides stimulated angiogenesis in vivo. Mapping of the active domain demonstrated that the sequence KGHK was responsible for most of the angiogenic activity; substitution of the His residue decreased the effect. We found that proteolysis of SPARC provided a source of KGHK, GHK, and longer peptides that contained these sequences. Although the Cu^{2+}-GHK complex had been identified as a mitogen/morphogen in normal human plasma, we found KGHK and longer peptides to be potent stimulators of angiogenesis. $SPARC_{113-130}$ and KGHK were shown to bind Cu^{2+} with high affinity; however, previous incubation with Cu^{2+} was not required for the stimulatory activity. Since a peptide from a second cationic region of SPARC ($SPARC_{54-73}$) also bound Cu^{2+} but had no effect on angiogenesis, the angiogenic activity appeared to be sequence specific and independent of bound Cu^{2+}. Thus, specific degradation of SPARC, a matrix-associated protein expressed by endothelial cells during vascular remodeling, releases a bioactive peptide or peptides, containing the sequence (K)GHK, that could regulate angiogenesis in vivo.

THE extracellular matrix is a repository for a number of growth-regulatory factors (Flaumenhaft and Rifkin, 1992). In addition, matrix proteins themselves clearly influence the proliferative and migratory status of cells (Ingber and Folkman, 1989*a*, 1989*b*). The promotion of growth in vitro by extracellular matrix components has generally been attributed to shape changes that result from interactions between cells and their substrates (Folkman and Moscona, 1978; Form et al., 1986; Ingber, 1990; D'Amore, 1992). However, several matrix-associated proteins also manifest growth stimulatory activity that is independent of their adhesive properties. For example, peptides from an EGF-like domain of laminin have been shown to stimulate cell replication in vitro (Panayotou et al., 1989). Because such proteins are immobilized on cell surfaces and in the extracellular matrix, they might provide a localized source of growth-regulatory activity (Engel, 1989); moreover, they could be a source of diffusible morphogens through proteolytic processing and turnover. Recently, cationic sequences within a follistatin-like domain of SPARC (see Lane and Sage, 1994) have been shown to regulate the proliferation of endothelial cells in vitro (Funk and Sage, 1991, 1993). Cationic region 1 (amino acids 54–73) was shown to inhibit the proliferation of subconfluent endothelial cells, whereas cationic region 2 (amino acids 113–130) stimulated DNA synthesis, an activity that is masked in the intact protein.

SPARC is a highly conserved, matrix-associated glycoprotein transiently expressed during the embryonic development of vertebrates (Mason et al., 1986*a*, 1986*b*; Holland et al., 1987; Damjanovski et al., 1992). It is a major secretory product of embryonic parietal endoderm (Mason et al., 1986*a*), osteoblasts (Otsuka et al., 1984; Whitson et al., 1984), odontoblasts (Fujisawa and Kuboki, 1989; Sage et al., 1992), and fibroblasts (Wasi et al., 1984). In adults, expression is limited but has been identified in tumors (Dziadek et al., 1986; Wewer et al., 1988; Schulz et al., 1988) and in a variety of tissues undergoing cellular remodeling or renewal (Holland et al., 1987; Sage et al., 1989*a*; Raines et al., 1992; Reed et al., 1993). Overexpression of SPARC in vertebrates and invertebrates results in extensive disruptions of the body axis that are consistent with a role in cellular attachment and morphogenesis of structures derived from mesoderm (Schwarzbauer and Spencer, 1993; Lane and Sage, 1994).

SPARC is also expressed by cultured endothelial cells and is increased further when these cells form cords and/or tubes, a type of remodeling termed angiogenesis in vitro

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(Iruela-Arispe et al., 1991*a*, 1991*b*). The purified protein influences endothelial cell shape, protein synthesis, and proliferation (Sage and Bornstein, 1991). SPARC also binds several divalent cations that include copper (Vernon and Sage, 1989); both Cu^{2+} and Cu^{2+}-binding proteins/peptides have been shown to stimulate angiogenesis in vivo (McAuslan et al., 1980; Raju et al., 1982). These and other studies collectively indicate that SPARC might be important in the regulation of endothelial function, although expression has not previously been demonstrated in endothelial cells undergoing angiogenesis in vivo.

In this study, we show that SPARC is transiently expressed in endothelial cells associated with angiogenesis in vivo. In an assay of angiogenesis in vitro, SPARC stimulated the formation of endothelial cords. Mapping of the active domain with synthetic peptides derived from SPARC identified cationic region 2 as a potent source of angiogenic activity. Although SPARC itself appeared to be a marginal stimulator of angiogenesis in vivo, peptides derived from cationic region 2 were active stimulators of capillary growth in vivo and in vitro. The active peptides corresponded to a novel Cu^{2+}-binding domain in SPARC and overlapped the sequence GHK at amino acid 120 of the secreted sequence. *N*-glycyl-L-histidyl-L-lysine-OH (GHK)[1] (also referred to as liver growth factor) is a Cu^{2+}-binding peptide, isolated originally from human plasma, which has been shown to induce angiogenesis in the chicken chorioallantoic membrane (CAM) and to accelerate the closure of dermal wounds (Raju et al., 1982; Pickart, 1983; Pickart and Lovejoy, 1987). The identification of sequences related to GHK as sources of angiogenic activity in SPARC prompted us to investigate the relationship between Cu^{2+}-binding and angiogenic activity, as well as the possibility that SPARC is a source of GHK-containing peptides in vivo. We provide evidence that the binding of Cu^{2+} is not sufficient to stimulate angiogenesis because a second peptide that bound Cu^{2+} was inactive. Thus, stimulation of angiogenesis by Cu^{2+}-binding peptides from cationic region 2 appears to be sequence specific. Finally, SPARC was a substrate for several proteases that have been implicated in angiogenesis. Incubation with plasmin, as well as the serine protease trypsin, resulted in cleavage at several sites and the release of peptides from cationic region 2. SPARC could, therefore, be a source of GHK-containing peptides, released during the degradation of extracellular matrix, in remodeling tissues that exhibit high levels of angiogenesis.

Materials and Methods

Tissue Preparation

Female Swiss-Webster mice were mated and checked for insemination plugs daily. The morning that followed successful mating was considered to be day 1 after conception. Gestation was terminated between days 11 and 18, and embryos were fixed in either 3% paraformaldehyde/0.1 M $NaPO_4$ (pH 7.4) for analysis of RNA transcripts or in methyl-Carnoy's solution (30% CCl_4, 10% CH_3COOH, 60% CH_3OH) for immunohistochemistry. Fixed embryos, staged by morphological criteria (Rafferty, 1970), were embedded in paraffin, and 5-μm sections were mounted on microscope slides (Probe-on™; Fisher Scientific, Pittsburgh, PA).

In Situ Hybridization and Immunohistochemistry

Riboprobes consisted of ^{35}S-UTP-labeled single-stranded transcripts derived from a full-length mouse SPARC cDNA (1,147 bp) inserted into the multiple cloning site of the plasmid pGEM-1 linearized with BamHI or HindIII (Mason et al., 1986*a*). Sense-strand riboprobes were produced in transcription reactions initiated from the T7 promoter of plasmids made linear with HindIII. Antisense riboprobes were produced in reactions initiated from the SP6 promoter of plasmids made linear with BamHI. Riboprobes were hydrolyzed to an average length of 250 bp by alkaline hydrolysis, reprecipitated in ethanol, and resuspended in 10 mM dithiothreitol before use in hybridization experiments. In situ hybridization was performed as described by Iruela-Arispe et al. (1993). Detection of SPARC transcripts required 10–15-d exposures. Immunohistochemistry was performed as described (Reed et al., 1993) with an affinity-purified rabbit antipeptide antiserum (SPARC $_{16-35}$; αM1.2) that recognizes intact SPARC. This IgG has been shown to precipitate SPARC from solution and is completely neutralized by absorption with the immunizing peptide. Serial sections were also reacted with biotinylated B4 lectin from *Bandeiraea simplicifolia* (BSLB4) (Vector Laboratories, Inc., Burlingame, CA), or with a monoclonal antibody (IgG) against proliferating cell nuclear antigen (PCNA) (Boehringer Mannheim Corp., Indianapolis, IN). A biotinylated goat anti-mouse IgG (Vector Laboratories, Inc.) was used as a secondary antibody. Antibody and lectin complexes were detected with an avidin-biotin-horseradish peroxidase system or were visualized by fluorescence microscopy with avidin-FITC or avidin-rhodamine as fluorochromes.

Purification of SPARC and Peptide Synthesis

Murine SPARC was purified from PYS-2 cells as described by Sage et al. (1989*a*), and human SPARC from platelets was obtained from Haematologic Technologies, Inc. (Essex Junction, VT). Peptide synthesis was carried out as described (Lane and Sage, 1990). Peptides were synthesized with N^{α}-9-fluorenylmethyloxycarbonyl (*f*-moc) amino acid derivatives by Dr. K. De Jongh and A. Colvin (Molecular Pharmacology Facility, University of Washington). The location and amino acid sequence of peptides used in this study are shown in Table I. Sequences were derived and numbered based on the predicted amino acid sequence of mature murine SPARC after removal of the signal sequence (Mason et al., 1986*a*). We also synthesized peptides with amino acid substitutions (mutations), which are designated by single letter codes as described in Table I. Molecular mass and charge (pI) were calculated for each peptide with the MacProMass™ program (S. Vemuri and T. D. Lee, Beckman Research Institute, Duarte, CA).

After synthesis, peptides were purified by preparative HPLC on either C-18 (Vydac) or PRP-3 columns (Hamilton; Reno, NV), as described by Lane and Sage (1990). Hydrophilic peptides that were not retained on C-18 or PRP-3 columns were purified by high performance hydrophilic interaction chromatography on polyhydroxyethyl aspartamide columns (polyLC; Columbia, MD). Samples were applied to polyhydroxyethyl aspartamide in 80% CH_3CN/15 mM ammonium formate (pH 2.7) (buffer A), and peptides were eluted with a linear gradient of buffer B (30% CH_3CN in 15 mM ammonium formate at pH 2.7). Peptides purchased from commercial sources were analyzed for purity by HPLC or hydrophilic interaction chromatography and were used as provided by the manufacturer. Sequences of peptides made for this study were verified by amino acid analysis after hydrolysis in 6 N HCl (Dr. S. Kumar, Dept. of Biochemistry, University of Washington) and by mass-spectral analysis. Purified peptides were lyophilized repeatedly to remove salts before use in cell culture. Peptides were soluble in physiological buffers, and the concentrations of stock solutions were determined by amino acid analysis. Concentrations of SPARC protein were calculated based on its absorbance at 280 nm and an extinction coefficient ($E_{0.1\%;\ 1\ cm}$) of 0.82 (Lane and Sage, 1990).

Cell Culture

Bovine aortic endothelial (BAE) cells were cultured in DME with 10% fetal bovine serum, 250 mg/ml amphotericin B, 100 U/ml penicillin G, and 100 U/ml streptomycin sulfate, as previously described (Iruela-Arispe et al., 1991*a*). Cells from cultures predisposed to cord formation (defined herein as angiogenic cultures) were cloned and passaged with trypsin-EDTA. Nonangiogenic BAE cells (i.e., cells that maintained a contact-inhibited monolayer and never formed cords or tubes) cultured under identical conditions were used as controls. Experiments were performed with clones be-

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; Cu-IMAC, copper-immobilized metal affinity chromatography; GHK, *N*-glycyl-L-histidyl-L-lysine-OH; KGHK, *N*-lysyl-L-glycyl-L-histidyl-L-lysine-OH; PCNA, proliferating cell nuclear antigen.

*Table I. Sequences of Synthetic Peptides**

Name‡	Sequence§	Mass‖	Activity¶
4-23	QTEVAEEIVEEETVVEETGV	2,219.3	−
54-73	CQNHHCKHGKVCELDESNTP	2,279.5	−
55-64$_{C59-S}$	QNHHsKHGKV	1,170.6	ND
64-73	VCELDESNTP	1,105.5	ND
113-130	TLEGTKKGHKLHLDYIG	1,910.2	++++
113-128	TLEGTKKGHKLHLDY	1,740.0	++++
113-130$_{H121-R}$	TLEGTKKGrKLHLDYIG	1,938.2	ND
113-130$_{K119-A}$	TLEGTKaGHKLHLDYIG	1,853.1	ND
119-122	KGHK	469.3	++++
119-122$_{H121-R}$	KGrK	488.3	+
119-122$_{H121-A}$	KGaK	403.3	+
120-122	GHK	341.2	+
154-173	KNVLVTLYERDEGNNLLTEK	2,348.6	−
184-203	NEKRLEAGDHPVELLARDFE	2,466.7	ND
254-273	TCDLDNDKYIALEEWAGCFG	2,263.5	−

* Peptides were synthesized based on the published amino acid sequence of murine SPARC, as described in Materials and Methods.
‡ Peptide names are derived from their position within the wild-type sequence of SPARC after removal of the signal sequence.
§ Amino acid residues are indicated by their single letter codes, and Cys (C) residues are highlighted in bold face type. The sequence GHK is highlighted with a double underline. An inversion of this sequence, KHG, is found in peptide SPARC$_{54-73}$ and has been highlighted with a single underline. Alterations of wild-type sequences were synthesized and served as controls. Substituted residues are shown as lower case letters in the sequence, and the substitution is indicated as a subscript in the name.
‖ Mass (in atomic mass units, D) was calculated by the MacProMass™ program.
¶ Angiogenic activity was assessed by bioassays in vitro as described in Materials and Methods. Peptides were added in serum-free DME (final concentration was 125 μM), and cord formation was measured after 4 d. Results are summarized as active (+) or inactive (−). ND, not determined.

tween passages 9 and 17. As noted in the text, some experiments were carried out with DME supplemented with $CuSO_4$ and BSA. The BSA (a gift from E. Raines, Department of Biochemistry, University of Washington) was tested before use in these experiments, and was found to be free of platelet-derived growth factor, transforming growth factor-beta, and other mitogens that display activity in a 3T3 cell proliferation assay.

Quantitation of Cord Formation

The use of cloned BAE cells that form cords and tubes (a process termed angiogenesis in vitro) for the analysis of potentially angiogenic proteins has been previously described (Iruela-Arispe et al., 1991*c*). Angiogenic BAE cells were passaged, plated in 15.5-mm diameter tissue culture wells, and cultured in DME with 10% fetal bovine serum. Experiments were initiated after cords were evident (7–10 d). Cultures were preincubated in serum-free DME for 24 h and subsequently with DME with or without peptides for the times indicated. Cells were treated for 1–4 d with increasing doses of the test substance. Medium and fresh reagents were changed every 24 h. At 24-h intervals, photomicrographs that represented the projected image of these cultures were taken from randomly selected fields (500 μm^2) of each plate with an inverted phase-contrast photomicroscope. The number of cords was assessed by counts of cord intersections in a microscopic field, as described by Iruela-Arispe et al. (1991*c*). Changes in cord number were assessed in the same field of each well on successive days of culture. The number of cords present at the beginning of the experiment was taken as 100%. Experiments were performed in triplicate. Upon completion of the experiment, cultures were fixed and embedded for further morphometric analysis.

CAM Angiogenesis Assays

Methylcellulose, a sustained drug-release matrix, was polymerized with solutions of SPARC, various synthetic peptides, or other test compounds. Disks were prepared by combination of equal volumes of test solution (in PBS) with 0.5% methylcellulose (400 centipoises; Sigma Chemical Co., St. Louis, MO). The mixture was placed on Teflon and dried slowly overnight at room temperature under sterile conditions. Fertilized white Leghorn chicken eggs (Biological Supply, Lynnwood, WA) were incubated at 37°C in 60% relative humidity. On day 3, eggs were opened carefully into petri dishes. Embryos with intact CAMs and yolks were cultured in an humidified tissue culture incubator at 37°C, 1–3% CO_2 under sterile conditions; no culture medium was used. The culture method was essentially as described by Auerbach et al. (1974). After 8 d of culture (embryonic day 11; stage 28–30 [Hamburger and Hamilton, 1951]), methylcellulose disks were placed on the periphery of the exposed surface of the CAMs; the area (nearly one fourth of the CAM) nearest to the embryo was not used. Disks were then incubated for various times before the CAMs were photographed under a dissecting photomicroscope. All treatments were performed in duplicate on at least four different CAMs. Substances with known activity in this assay were applied to each CAM as controls: PBS buffer (a neutral compound), basic fibroblast growth factor (bFGF; a stimulator of blood vessel formation), and protamine (an inhibitor of blood vessel formation) (Taylor and Folkman, 1982).

Copper-immobilized Metal Affinity Chromatography (Cu-IMAC)

SPARC protein and peptides were solubilized in buffer A (0.02 M NaH_2PO_3 at pH 7.0, 0.5 M NaCl) and applied to a 75 × 8-mm HPLC column (Chelate-5 PW; TosoHAAS, Philadelphia, PA) previously charged with $CuSO_4$ and equilibrated in 40% buffer B (0.1 M NaH_2PO_3 at pH 2.8, 0.5 M NaCl) at a flow rate of 1 ml/min. The pH of the eluate at 40% buffer B was 6.0. Eluate was monitored at an absorbance of 214 nm. As a control, peptides were applied to the column in the absence of bound metal. Under these conditions, all peptides eluted within the void volume of the column; no binding was detected. The column was routinely stripped with EDTA to remove residual contaminants and was recharged with fresh $CuSO_4$.

Electrospray Mass Spectrometry

The molecular weights and amino acid sequences of peptides purified from SPARC digests or of synthetic peptides used in these experiments were determined by electrospray-mass spectrometry by continuous infusion of sample into a triple quadrapole instrument (SCIEX API III; SCIEX, Thornhill, Ontario, Canada) equipped with a nebulization-assisted electrospray ion source (Covey et al., 1988; Fenn et al., 1989). Ions of interest were further characterized by tandem mass spectrometry, a technique in which peptides from a complex mixture can be isolated as individual precursor ions by the first of two mass analyzers and subsequently fragmented by collision with argon gas. The resulting fragments represent an overlapping series of ionic dipeptides, tripeptides, etc., which are then identified by a second mass analyzer. The amino acid sequence can be deduced from the observed fragmentation patterns (Hunt et al., 1986). With additional information, in this case, the known amino acid sequence of SPARC and synthetic peptides used as controls, the derived amino acid sequence data are unambiguous. Polypropylene glycol and whale myoglobin served as calibration standards. Masses of parent and product ions deviated by <0.5 D from their theoretical values.

Copper-binding Studies

Water for binding studies was filtered and deionized with a multicartridge deionizing unit; final resistance was >15 $M\Omega \cdot cm^{-1}$ at 25°C (Milli Q™;

Millipore Corp., Bedford, MA). Stock solutions of buffers and monovalent ions were treated with resin (Chelex™ 100; Bio Rad Laboratories, Richmond, CA), prepared as described (Waisman and Rasmussen, 1983), to reduce trace metals. Calibrated stocks of divalent cations were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Binding studies were carried out in 20 mM $NaHCO_4$ (pH 7.4) at room temperature (22–25°C). Copper and magnesium stocks were diluted as indicated in the figure legends. Copper:peptide complexes were analyzed by electrospray and tandem mass spectrometry. The sequences of peptides present in peptide:Cu^{2+} complexes were verified by analysis of daughter ions generated by fragmentation of the parent complex as described above. Before analysis of Cu^{2+}-binding peptides, the silica sample introduction line was flushed with EDTA to remove contaminating divalent ions.

Proteolytic Cleavage and Analysis of Released Peptides

SPARC or synthetic peptides were solubilized in TS buffer (50 mM Tris HCl at pH 7.5, 150 mM NaCl) and digested with enzymes for the times indicated. After proteolysis, samples were lyophilized and analyzed by hydrophilic interaction chromatography, as described above, or by PAGE. For SDS-PAGE, peptides were solubilized in sample buffer, heated at 95°C for 3 min with or without 5 mM dithiothreitol, and separated on 10–20% gradient minigels (Daiichi Pure Chemicals, Tokyo, Japan) with a Tricine-SDS buffer system (Schägger and von Jagow, 1987). Gels were fixed for 5 min in 1% glutaraldehyde, stained with Coomassie brilliant blue, destained, and dried. Dried gels were photographed directly or exposed to autoradiographic film (X-OMAT, AR; Kodak, Rochester, NY). For hydrophilic interaction chromatography, the absorbance of the eluate was monitored at 214 nm, and peaks of absorbance were lyophilized for mass spectral analysis. Controls included incubation of enzyme in the absence of substrate. Chromatography of synthetic peptides was used to calibrate the system, and blank runs were performed to insure that carryover of peptide fragments did not occur between chromatography runs. For PAGE analysis of SPARC, native protein was labeled with Na[^{125}I] and chloramine-T as an oxidant (final specific activity was ~200 cpm/fmol SPARC) (Hunter and Greenwood, 1962). Enzymes for analysis of protein cleavage patterns were of sequencing grade and included bacterial collagenase, form III (Advance Biofactures Corp., Lynbrook, NY), bovine pancreatic elastase (Cooper Biomedical, Malvern, PA), prostromelysin (gift of Drs. A. Strongin and G. Goldberg, Washington University, St. Louis, MO), human thrombin (gift of Dr. W. Kisiel, University of New Mexico, Albuquerque, NM), and bovine pancreatic trypsin-TPCK (*N*-tosyl-L-phenylalanine chloromethylketone) (Worthington Biochemical Corp., Freehold, NJ). Human plasmin, human Factor X, and Russell's viper venom, used for the activation of Factor X, were the kind gifts of Drs. J. C. M. Meijers (University of Utrecht, The Netherlands) and K. Fujikawa (University of Washington). Human Factor X was activated with Russell's viper venom as described (Meijers et al., 1987), and prostromelysin was activated with *p*-aminophenyl-mercuric acetate as described (Cawston and Murphy, 1981).

Sequence Analysis

Sequence analysis and searches for specific sequence motifs were performed with the SwissProt data base (Release 22.0), with the aid of the FIND program contained within the University of Wisconsin Genetics Computer Group software package (version 7.1) (Devereux et al., 1984). The software was maintained and operated through the Locke computer center at the University of Washington.

Results

Expression of SPARC in Endothelial Cells Exhibiting Angiogenesis In Vivo

Analysis of SPARC expression in endothelial cells is complicated by the small amounts of cytoplasm in these cells and the potential for expression by adjacent cells. Therefore, we looked for expression in the cerebral cortex of embryonic mice and in adult dermis after wounding; both brain and skin characteristically exhibit high levels of angiogenesis and low levels of endogenous SPARC expression (Holland et al., 1987; Reed et al., 1993). Fig. 1 *A* shows the expression of SPARC mRNA in the vasculature of the developing brain of a 17-d-old mouse embryo. Probes from the sense strand of DNA did not hybridize to cellular mRNA under identical conditions (Fig. 1 *B*). The distribution of positive cells was identical to, but more restricted than that of cells stained with the B4 lectin from *B. simplicifolia* (BSLB4) (Fig. 1 *D*), a marker of murine endothelial cells (Coffin et al., 1991). To determine whether the capillary endothelial cells were proliferating, we stained serial sections from the same embryo with antisera against PCNA and the B4 lectin (Fig. 1, *E* and *F*, respectively). PCNA antisera reacted with a subset of endothelial cell nuclei (compare localization of signal between Fig. 1, *E* and *F*), a measure that angiogenesis was ongoing at this time of development. Since the requirements for antigen preservation are different, it was not possible to detect SPARC protein and PCNA simultaneously in these sections. The data thus provide evidence that both SPARC mRNA and protein are expressed by endothelial cells undergoing developmental angiogenesis. In contrast, SPARC was not seen in the endothelium of large vessels, such as the aorta, at this time or in mature brain capillaries later in development, but it was present in some vascular smooth muscle cells, as reported by Wewer et al. (1988). Recent studies have shown that a second endothelial gene, von Willebrand factor, is also differentially expressed in capillaries (Coffin et al., 1991). It is, therefore, possible that expression of SPARC is temporally regulated during vessel maturation and subserves a function at specific stages early in this process.

The distribution of SPARC protein was indistinguishable from that of the corresponding mRNA. Fig. 1 *C* shows the pattern of reactivity of an anti-SPARC peptide IgG in 17-d-old mouse embryonic brain. The reactivity was eliminated by incubation of the antiserum with the immunogen, a synthetic peptide representing amino acids 16–35 of the mature protein (not shown). Expression was not observed in all vessel profiles; however, positive vessels were visible at all levels of the brain and were particularly evident near the meningial surfaces. We detected SPARC in a subset of vessels throughout the development of the embryonic brain (not shown). Vasculature of adult mice did not appear to express SPARC protein or mRNA by these methods. Cells immunoreactive for SPARC also appeared in dermal capillaries of an adult rat 3 d after the area received a full-thickness incisional wound (Fig. 1, *G* and *H*). Capillaries in unaffected regions of the dermis appeared negative.

SPARC and Peptides Derived from $SPARC_{113-130}$ Stimulate the Formation of Endothelial Cords In Vitro

Addition of SPARC to cultures of endothelial cells that formed cords and tubes in vitro (i.e., angiogenic cultures) was associated with an increase in the number of new cords over 1–4 d of treatment. Fig. 2 illustrates the culture system and the increased sprouting induced by incubation with SPARC (Fig. 2, *C* vs *D*). The number of cords was determined by a count of cord intersections (branchpoints) within a specific field (Fig. 3) according to a published method (Iruela-Arispe et al., 1991*c*). To control for the variation in cord number among individual culture wells, we normalized the counts to the number of cords present at day 1 for each well, and experiments were performed in triplicate. Treat-

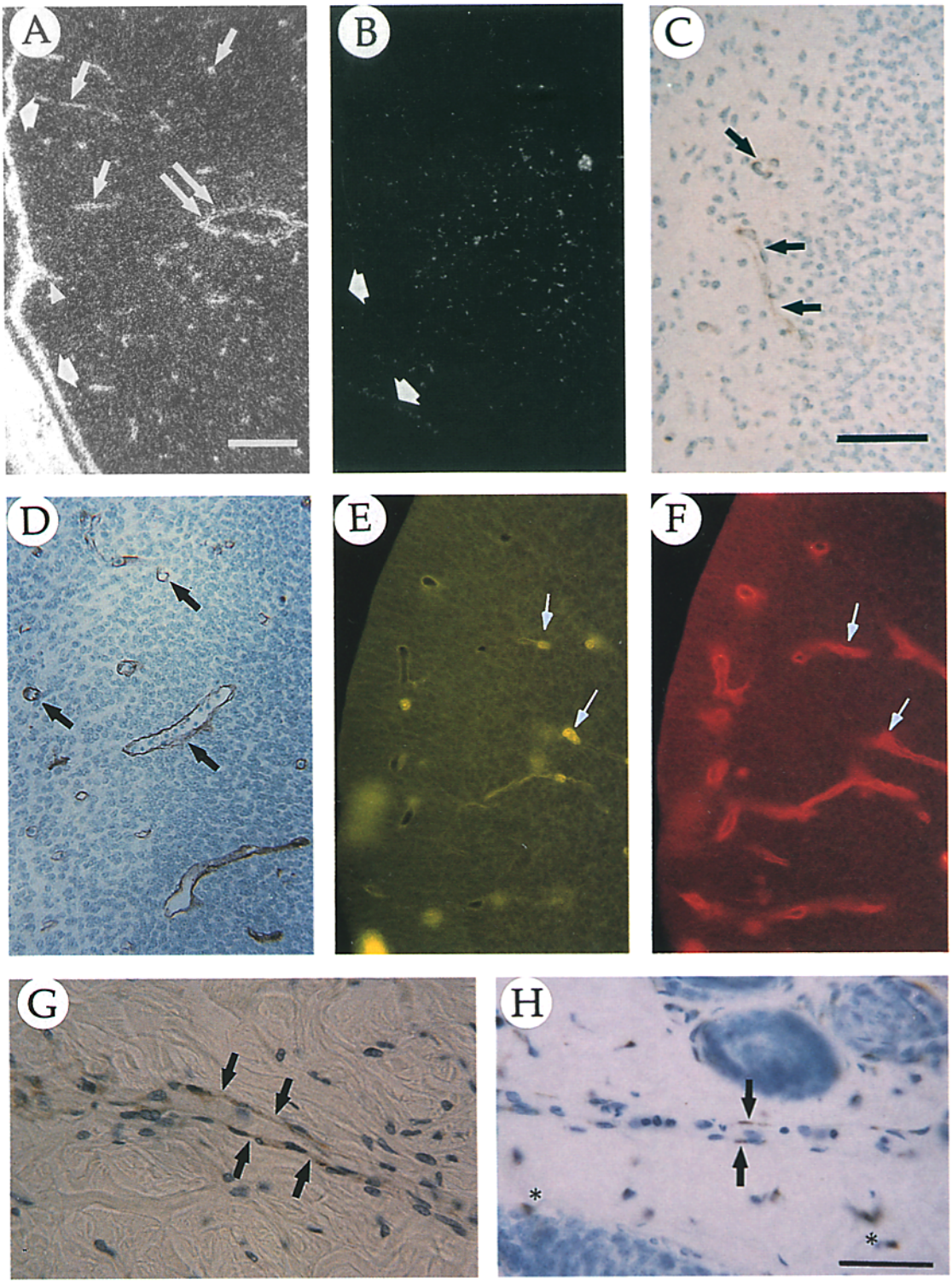

Figure 1. SPARC is expressed in capillaries of embryonic brain and in capillaries of dermal wounds. (*A–F*) 5-μm sections of brain tissue from 17-d-old mouse embryos. (*A*) In situ hybridization of SPARC mRNA (antisense probe). (*B*) Serial section to *A* (sense probe). (*C*) Immunohistochemical localization of SPARC protein. (*D*) Histochemical identification of endothelial cells with the B4 lectin of *B. simplicifolia*. *C* and *D* do not represent serial sections. (*E*) Immunohistochemical localization of proliferating cells with a monoclonal antibody against PCNA. (*F*) Same section shown in *E*, stained with the B4 lectin to identify capillaries. (*G* and *H*) Immunohistochemical localization of SPARC protein in full-thickness rat dermal wounds (3 d after injury). mRNA was detected by autoradiography of tissues after hybridization with [^{35}S]SPARC riboprobe RNA. SPARC protein was identified as a brown product after reaction with an affinity-purified anti-SPARC$_{16\text{-}35}$ antibody, used with an avidin-biotin-peroxidase detection system. PCNA was detected with an FITC-linked secondary antibody and a photomicroscope equipped with an epifluorescence imaging system. B4 lectin in D was labeled with biotin and is visualized as a brown reaction product in conjunction with an avidin-biotin-peroxidase detection system. B4 lectin in F was labeled with rhodamine. *Small arrows*, signal in capillaries; *double arrows*, signal in medium-sized vessels; *large arrows in A*, signal that corresponds to meningial fibroblasts; *arrows in E and F*, nuclei from proliferating endothelial cells. *Additional immunopositive cells in granulation tissue that possibly correspond to fibroblasts or macrophages. *Bars*, 50 μm in *A–F*, 100 μm in *G* and *H*.

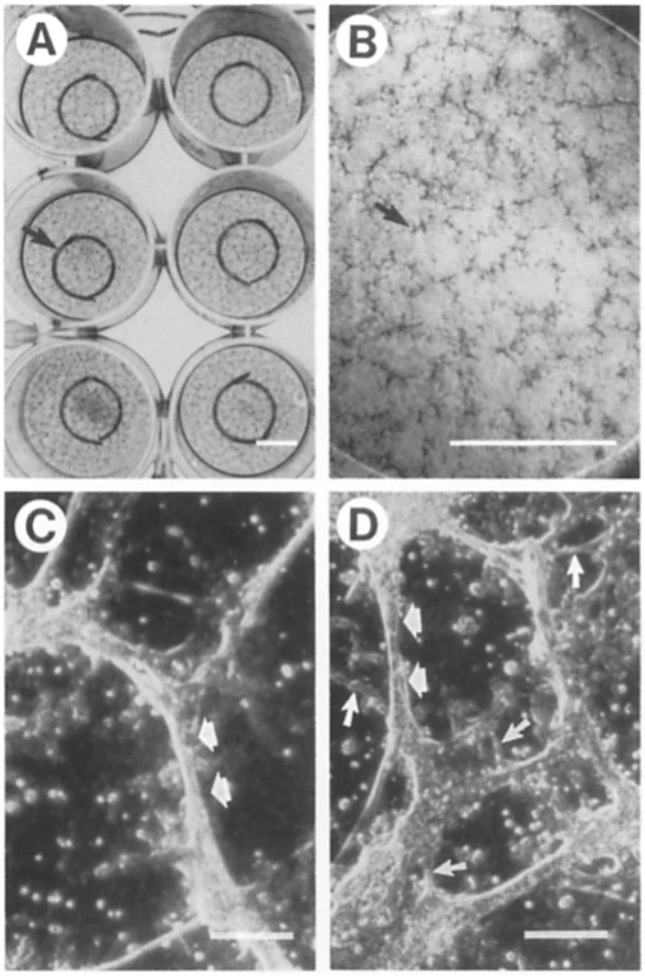

Figure 2. SPARC increases the number of cords produced by angiogenic endothelial cells in vitro. (*A*) Photograph of several culture wells with angiogenic clones; (*B*) photo of one well. Before photography, cells in *A* and *B* were fixed briefly in methyl-Carnoy's solution and stained with 1% toluidine blue to demonstrate the interconnecting network of endothelial cells. (*C* and *D*) High magnification photomicrographs illustrating the differences between unfixed cultures of angiogenic BAE cells treated for 48 h with control saline (PBS) (*C*) or 20 μg/ml SPARC (*D*). *Arrow in A*, identifies locator ring, drawn on the bottom of each well, that allowed photography of identical fields on successive days of culture; *arrows in B, C and D*, cords of endothelial cells; *large white arrows*, thick cords; *small white arrows*, small cords prevalent in *D*. *Bars*, 5 mm in *A* and *B*, 100 μm in *C* and *D*. Diameter of 1 culture well is 15.5 mm.

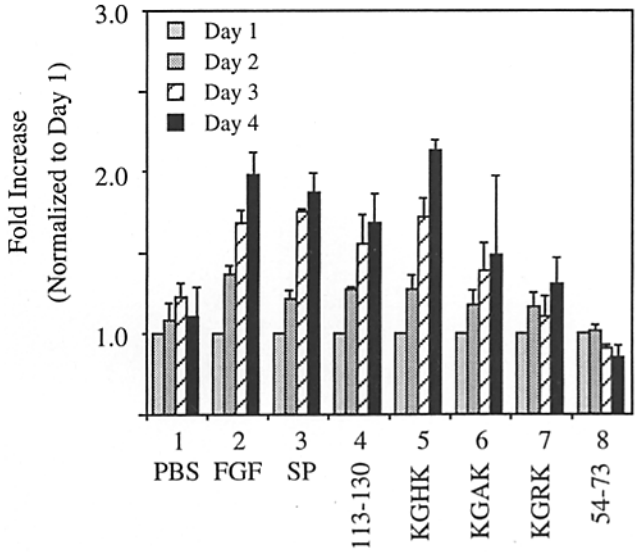

Figure 3. Effect of SPARC and $SPARC_{113-130}$ on the formation of endothelial cords. Clones of endothelial cells were grown until a network of cords was established (10–14 d). The cells were cultured for an additional 24 h in serum-free medium. Peptides were added in fresh serum-free medium (day 1); photomicrographs were taken at 24-h intervals. Reagents were replaced every 24 h. Counts of cord intersections were made at identical positions for each dish, and values were normalized to the value obtained at day 1 for each well. Averages of triplicate determinations, ± SE, were plotted. *1*, PBS control; *2*, 10 ng/ml bFGF; *3*, 750 nM SPARC (20 μg/ml); *4*, 125 μM $SPARC_{113-130}$ (250.0 μg/ml); *5*, 125 μM KGHK (58.6 μg/ml); *6*, 125 μM KGAK (50.4 μg/ml); *7*, 125 μM KGRK (61.0 μg/ml); and *8*, 125 μM $SPARC_{54-73}$ (250.0 μg/ml).

ment with SPARC resulted in a twofold increase in the number of cords within 2–4 d (Fig. 3, column *3*). Treatment with bFGF, a known stimulator of angiogenesis in vitro and in vivo (Montesano et al., 1986, and references therein), was associated with a similar increase (Fig. 3, column *2*). Exposure of angiogenic cultures to a variety of SPARC-derived peptides (Table I) revealed similar angiogenic stimulation by a synthetic peptide representing cationic region 2 (amino acids 113–130) of the Cys-rich portion of SPARC (Fig. 3, column *4*). Incubation of cultures with peptides representing sequences from cationic region 1 (amino acids 54–73) or with bovine serum albumin had no stimulatory effect and occasionally resulted in a slight decrease in the number of cords (Fig. 3, column *8*, and Table I). Further analysis demonstrated that a sequence of four amino acids, KGHK (amino acids 119–122), contained significant activity (column *5*). Substitution of the central His residue with Ala (column *6*) or Arg (column *7*) reduced the apparent stimulation. Further truncation of the active sequence resulted in a loss of stimulatory activity in this assay; GHK had little effect on the number of endothelial cords after 4 d; however, pretreatment of GHK with $CuSO_4$ resulted in significant angiogenic stimulation (not shown). Since treatment with $CuSO_4$ alone had a minor stimulatory effect, we confined our analysis to KGHK and longer peptides that did not require pretreatment to acquire activity.

$SPARC_{113-130}$ and KGHK Specifically Stimulate Angiogenesis In Vivo

Peptides identified as stimulators of angiogenesis in vitro were tested for their ability to induce angiogenesis in vivo. $SPARC_{113-130}$ (Fig. 4 *C*) and KGHK (Fig. 4 *D*) induced extensive angiogenic responses within 24 h in the absence of exogenously supplied $CuSO_4$. The response resulted in a characteristic radial pattern of both large and small vessels that culminated in a vascular knot at the center of the treated area. In contrast, $SPARC_{54-73}$ did not generate a significant response (Fig. 4 *A*), and disks that contained the substituted sequence KGRK elicited a very weak response (not shown). The angiogenic peptide bFGF stimulated vessel formation to

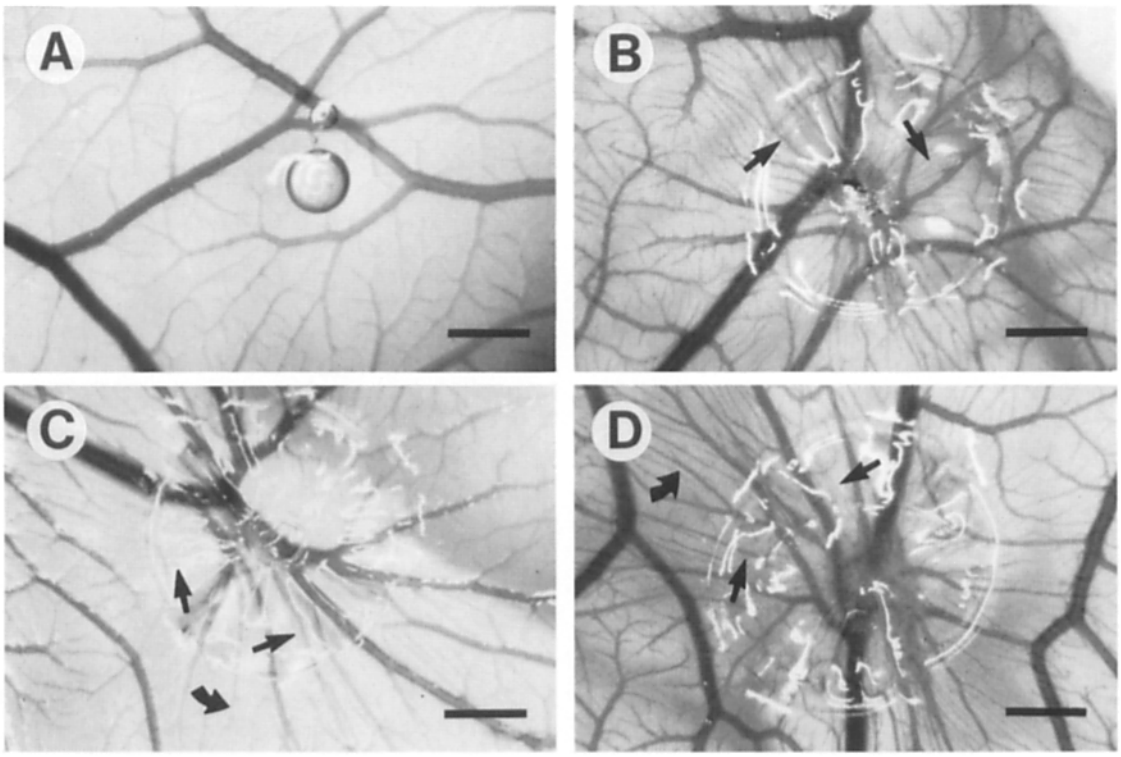

Figure 4. $SPARC_{113-130}$ and KGHK stimulate angiogenesis in the chick CAM. (*A–D*) Photographs of regions of the CAM after treatment with (*A*) $SPARC_{54-73}$ (266 μg), (*B*) bFGF (200 ng), (*C*) $SPARC_{113-130}$ (222 μg), and (*D*) KGHK (30 μg). Peptides were diluted in PBS and polymerized with methylcellulose to form slow-release disks. Disks were applied to the ectodermal surface of the chick CAM cultured ex ovo. CAMs were photographed after 24 h. *Small and large arrows*, radial vasculature that has developed within and outside the methylcellulose disks, respectively. *Bars*, 2 mm in *A*, 1 mm in *B–D*.

an extent that resembled the response to KGHK, both in magnitude and in the radial arrangement of the vessels (Fig. 4 *B*). Exposure to protamine, an inhibitor of vessel growth (Taylor and Folkman, 1982), resulted in the formation of an avascular zone (not shown). KGHK elicited angiogenic activity with applications as low as 50 ng/disk. Additional experiments have shown that the angiogenic effects of SPARC peptides were dose dependent and developed in the absence of immune cells or macrophages (Iruela-Arispe, M. L., T. F. Lane, D. Redmond, M. Reilly, R. Bolender, T. Kavanagh, and E. H. Sage, manuscript submitted for publication).

The effect of native SPARC protein on CAMs was variable. In some experiments, there was a modest increase in the number of vessels and in the appearance of their radial growth toward the disk. However, the effect was not as evident in other experiments. Because the disks are acellular, we tentatively concluded that direct cell contact, as provided by the assays in vitro, might be required for a more vigorous response. Alternatively, SPARC binds to a number of extracellular matrix components and might not diffuse into the tissue effectively. Another possibility is that SPARC must be degraded before cell contact to elicit an angiogenic response. Since mapping identified a potential Cu^{2+}-binding domain in SPARC as a source of angiogenic activity, we wanted to determine whether this sequence bound Cu^{2+}, and whether Cu^{2+}-binding and angiogenic stimulation were independent activities.

Two Cationic Regions of SPARC Represent Copper-binding Sites

A previous study has shown that SPARC binds copper ions (Vernon and Sage, 1989). Copper-binding proteins are known to accumulate at sites of tissue repair and, under some circumstances, to be angiogenic in vivo (Raju et al., 1982). Therefore, it was important to determine whether the angiogenic activity of SPARC was related to Cu^{2+} binding. Immobilized metal affinity chromatography (IMAC) has been used on several occasions to identify interactions between proteins and divalent cations (Porath et al., 1975). Initial experiments with SPARC demonstrated that the native protein bound to Cu^{2+}-charged IMAC columns with high affinity. Retention of SPARC on these matrices required the presence of immobilized metal, but the protein was not released under several typical elution regimes (low pH, low ionic strength, and gradients of imidazole). Instead, efficient elution of SPARC required large, stepwise additions of imidazole (Fig. 5, *A* and *B*) or EDTA. These results indicated that SPARC bound Cu^{2+} with high affinity, and/or that multiple sites on the protein combined to form a highly stable interaction with immobilized Cu^{2+}.

We decided to use this procedure to scan for SPARC peptides with Cu^{2+}-binding capacity. Fig. 5 *C* shows the results of multiple analyses in which purified preparations of synthetic peptides were subjected to analysis by Cu-IMAC. Peptides from both cationic regions of the follistatin-like domain

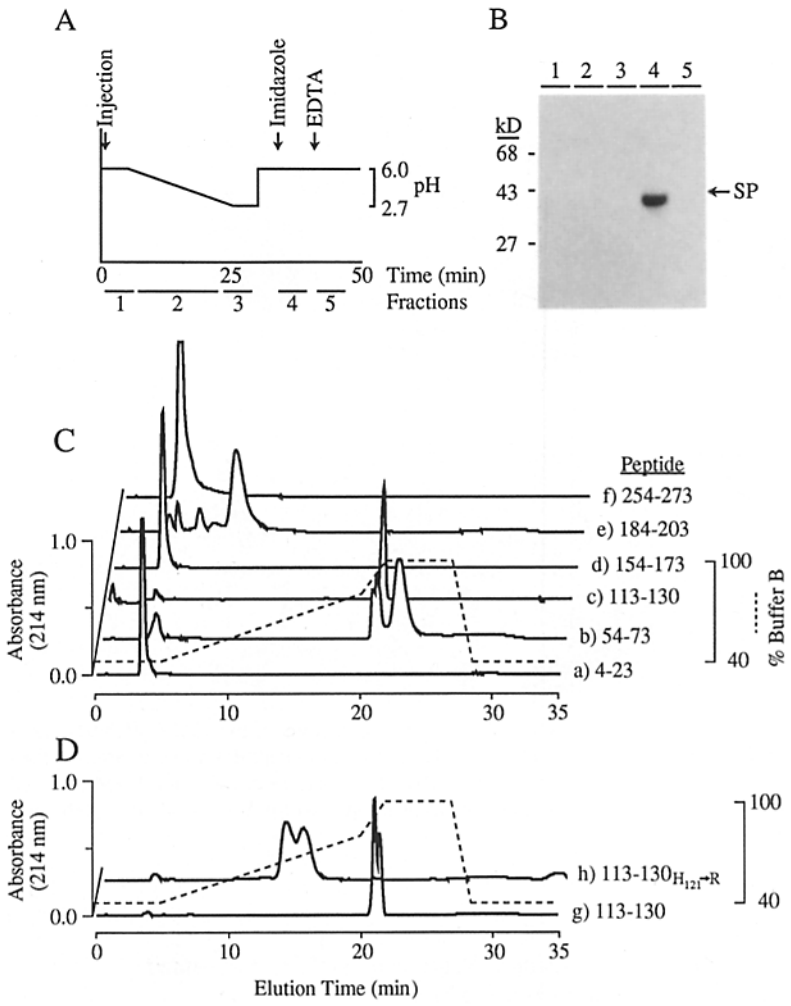

Figure 5. $SPARC_{54-73}$ and $SPARC_{113-130}$ represent potential copper-binding domains in SPARC. Purified SPARC protein (100 μg) was applied to an immobilized Cu^{2+}-affinity column (Cu^{2+}-IMAC). Pooled fractions were collected as indicated, dialyzed, lyophilized, and assayed for the presence of SPARC protein. (*A*) Schematic diagram of a typical gradient profile from IMAC. (*B*) Results of an immunoblot of proteins from each pooled fraction after resolution by SDS-PAGE. SPARC bound to the column (fraction 1) and was not eluted by decreasing pH (fractions 2 and 3). Release of bound SPARC required the addition of imidazole (50 mM) (a specific competitor for bound Cu^{2+}) (fraction 4). EDTA (a chelator of divalent cations) was used to strip remaining proteins from the column (fraction 5). (*C*) 1-mM solutions of synthetic peptides that represent several regions of the SPARC protein were applied to Cu^{2+}-IMAC and were eluted with a gradient of decreasing pH. Peptides shown in chromatograms a, d, and f did not bind under the conditions used (pH 6.0). The release of peptide $SPARC_{184-203}$ was retarded, and the peptide was subsequently eluted under isocratic conditions (*e*). Peptides $SPARC_{54-73}$ (*b*) and $SPARC_{113-130}$ (*c*) were retained and eluted at a pH of 4.85–4.95. SPARC and SPARC peptides did not bind to these columns in the absence of immobilized copper (not shown). (*D*) Peptides with mutations within the proposed Cu^{2+}-binding regions were analyzed by Cu^{2+}-IMAC, and their elution patterns were compared with those of wild-type sequences: (*g*) $SPARC_{113-130}$ and (*h*) a mutated version of $SPARC_{113-130}$ in which His_{121} has been replaced by Arg. Chromatography of samples in *C* and *D* used a gradient of increasing buffer B that resulted in decreasing pH: buffer A, 20 mM $NaPO_4$/0.5 M NaCl (pH 7.0); buffer B, 100 mM $NaPO_4$/0.5 M NaCl (pH 2.7). Elution was monitored at 214 nm.

($SPARC_{54-73}$ and $SPARC_{113-130}$) (Lane and Sage, 1994) bound and were eluted with gradients of decreasing pH at similar positions (pH 4.85–4.95) (Fig. 5 *C*, profiles *b* and *c*). Peptides from other regions of the protein did not bind or interacted weakly with the resin, and peptides from the α-helical domain III failed to bind with high affinity (Fig. 5 *C*, profiles *d* and *e*). The delayed elution of peptide $SPARC_{184-203}$ resulted from a weak interaction with the column. Since this peptide also eluted at the same position when chromatography was performed under isocratic conditions (pH 6.0), the interaction of $SPARC_{184-203}$ was most likely nonspecific. Peptides that coincided with the two previously characterized Ca^{2+}-binding domains ($SPARC_{4-23}$ and $SPARC_{254-273}$) failed to bind immobilized Cu^{2+} (Fig. 5 *C*, profiles *a* and *f*). The formation of multiple peaks in the IMAC profile of $SPARC_{54-73}$ appeared to result from alternate complexes between the resin and the Cys residues in this peptide. When we tested a 10–amino acid peptide that included the first cationic sequence ($SPARC_{55-64C59-S}$, corresponding to amino acids 55–64 with a Ser residue in place of the Cys normally found at position 59), the peptide eluted as a single peak in a position similar to that of $SPARC_{113-130}$ (not shown).

To characterize further the amino acids involved in the binding of $SPARC_{113-130}$ to immobilized Cu^{2+}, we analyzed peptides with mutations in the cationic region by IMAC. In Fig. 5 D (profile *g*), $SPARC_{113-130}$ appears as an elution control. In profile *h*, the elution of $SPARC_{113-130H121-R}$ is shown This peptide represents a mutated sequence in which His_{121} is converted to an Arg. $SPARC_{113-130H121-R}$ bound to the Cu^{2+}-charged resin but eluted considerably earlier in the gradient (pH 5.5) than did the wild-type sequence. These data indicate that His_{121} is important for Cu^{2+}-binding activity within this sequence of SPARC, but that other residues also appear to contribute to the affinity.

It has recently been recognized that ionspray (or electrospray ionization) can be used for the study of peptide-metal interactions (Hutchens et al., 1992). Since peptides less than five amino acids in length did not bind Cu^{2+}-IMAC, we used ionspray mass spectrometry (Covey et al., 1988) to determine whether the fragments of $SPARC_{113-130}$ used in the previous growth assays continued to associate with copper ions. As shown in Fig. 6 (*A*), the predicted mass of KGHK (468.3 D) is in good agreement with the observed value of the protonated molecule (MH^+ = 469.3 D). After the addition of 50 μM $CuSO_4$ (KGHK:Cu, 1:2.5), two peaks of mass/charge ratio 530.3 and 532.3 were observed.

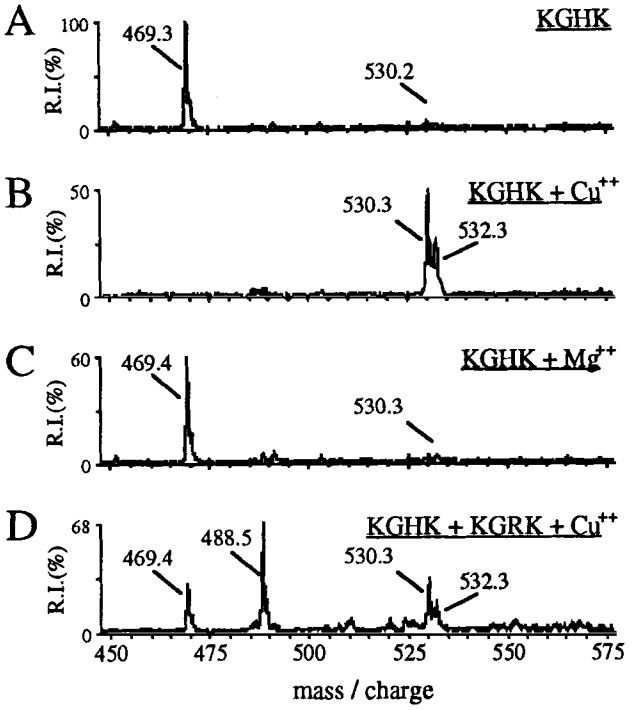

Figure 6. Demonstration of Cu^{2+} binding by isolation of the Cu^{2+}-KGHK complex. 20 μM solutions of KGHK were prepared in 20 mM NH_4HCO_3 (pH 7.5) and were analyzed by mass spectrometry. The masses of relevant ions are indicated. (*A*) KGHK alone (MH^+ = 469.3). (*B*) KGHK + 50 μM $CuSO_4$: the 469-D ion seen in *A* is absent and a doublet of 530.3/532.3 D has appeared ([KGHK + Cu − H]$^+$; ^{63}Cu and ^{65}Cu appear in the expected isotopic ratio). Since the KGHK/Cu spectra show no evidence of free KGHK, a conversion of 100% to KGHK:Cu complexes is indicated. KGHK was also treated with 50 μM $MgSO_4$ (*C*). The presence of a 530-D ion (KGHK:Cu; 1:1) in *C* indicates that residual copper ions were bound in the presence of excess Mg. (*D*) Results of a direct competition between KGHK (MH^+ = 469.3) and KGRK (MH^+ = 488.5) for copper ions present in limiting amounts. 10-μM peptides were mixed with 8 μM Cu^{2+}. The resulting spectra demonstrate that a KGRK:Cu complex was not formed ([KGRK + Cu − H]$^+$ = 550.5), and that all copper ions were sequestered in a KGHK:Cu complex ([KGHK + Cu − H]$^+$ = 530.3/532.3).

These ions are consistent with KGHK (468.3) and with the complex (peptide+Cu-H)$^+$ that contains both ^{63}Cu and ^{65}Cu in the expected isotopic ratio. Thus, KGHK formed a 1:1 complex with copper ion (Fig. 6, *A* vs *B*). Similar 1:1 complexes did not form with equivalent amounts of another divalent cation, magnesium (Fig. 6 *C*). Interestingly, a trace amount of KGHK:Cu^{2+} complex could be identified in buffers that lacked exogenously supplied salts (Fig. 6 *A*). This complex was not displaced by 50 μM Mg^{2+} (a result confirmed by comparison of peaks at mass/charge ratio 530.2 in *A* and *C* at higher resolution; not shown). Substitution of the His residue with either Arg or Ala eliminated high affinity Cu^{2+}-binding. In *D*, KGHK and KGRK were allowed to compete for limiting amounts of copper ions. The figure demonstrates that the available Cu^{2+} was bound by KGHK. Additional experiments conducted with KGRK alone demonstrated that binding of Cu^{2+} did not occur at equimolar ratios of Cu^{2+} and peptide, and that variable results were obtained at higher ratios of Cu^{2+} (not shown).

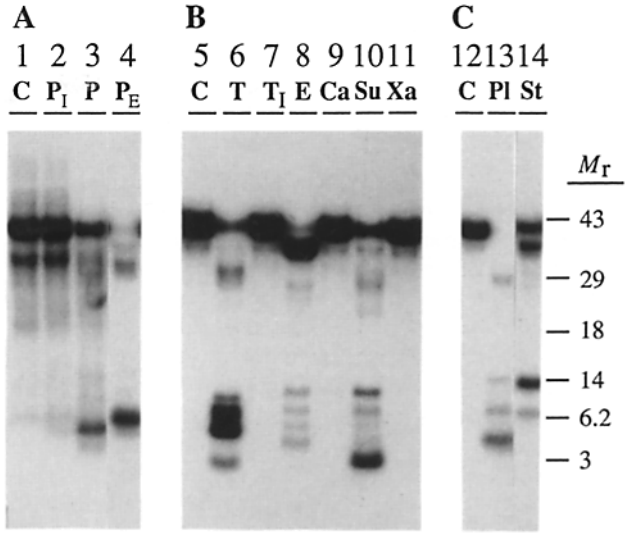

Figure 7. SPARC is degraded by platelet releasate and extracellular serine proteases. (*A*) ^{125}I murine SPARC was incubated at 37°C with buffer (lane *1*, *C*), a releasate from thrombin-activated human platelets (lane *3*, *P*), or platelet releasate that contained PMSF, a broad-spectrum serine protease inhibitor (lane *2*, P_I). Addition of EDTA increased the degradation of SPARC in platelet releasate (lane *4*, P_E). (*B*) [^{125}I]SPARC isolated from human platelets was incubated with buffer (lane *5*, *C*), trypsin (lane *6*, *T*), trypsin with PMSF (lane *7*, T_I), elastase (lane *8*, *E*), bacterial collagenase (lane *9*, *Ca*), subtilisin (lane *10*, *Su*), or Factor Xa (lane *11*, *Xa*). (*C*) [^{125}I]SPARC isolated from human platelets was incubated with buffer (lane *12*, *C*), plasmin (lane *13*, *Pl*), or stromelysin (lane *14*, *St*). Enzyme-to-substrate molar ratios were <1:100 and all reactions contained excess BSA as an internal control (not shown). Digests were resolved by SDS-PAGE on a 10–20% gradient gel. The positions of protein ($M_r \times 10^{-3}$) standards are indicated on the right.

Fragmentation of the Cu:KGHK complex resulted in the release of daughter ions consistent with a Cu:KGHK complex. Cu^{2+} remained associated with peptide fragments throughout the analysis. The smallest complex detected included Cu:GH in a 1:1 molar ratio.

To confirm these results, we carried out a spectroscopic analysis of SPARC, $SPARC_{113-130}$, and KGHK. Addition of copper ions to the intact protein or to the peptides resulted in an increase in absorbance at 580 nm. This binding was saturable and was not displaced with 100-fold excesses of Na, K, Ca, or Mg ions (not shown). In conjunction with the Cu^{2+}-IMAC analysis, these results support the conclusion that Ca^{2+}- and Cu^{2+}-binding sites in SPARC are functionally independent.

SPARC is Degraded by Extracellular Proteases

To address the potential for proteolysis of SPARC in extracellular fluids associated with wound repair, we analyzed the effects of activated human platelet releasate on the stability of native SPARC. Fig. 7 *A* (lane *3*) shows that [^{125}I]SPARC was degraded by human platelet releasate, and that the activity was inhibited (lane *2*) by PMSF, a broad-spectrum inhibitor of serine protease activity. SPARC was relatively stable to thrombin (Sage et al., 1984), which is routinely used for the activation of platelets, but was degraded to low molecular weight components after exposure to freshly isolated platelet

releasate. Since platelet releasate contains SPARC, we analyzed immunoblots of proteins from similar releasates for evidence of proteolytic activity directed against the endogenous protein. These experiments showed that endogenous platelet SPARC was rapidly degraded into fragments similar in size to those produced from the iodinated exogenous protein (not shown). We chose iodinated SPARC for routine assays because more fragments could be analyzed by radiography, in comparison to immunoblotting that would have required peptide-specific antisera. Staining of the gels with Coomassie blue showed that many of the proteins present in the releasates were undegraded. Treatment of the platelet releasate with EDTA, a chelator of divalent cations, increased the extent of SPARC degradation (lane *4*), a result which indicated that metalloproteinases were not required for the activity.

We exposed iodinated SPARC to a variety of purified proteolytic enzymes and analyzed the pattern of degradation. As shown in Fig. 7 *B* (lane *6*), trypsin degraded SPARC to components of low molecular weight; the activity was inhibited by PMSF (lane *7*). Elastase (lane *8*) released a stable fragment of ~40 kD, whereas bacterial collagenase (lane *9*) and clotting Factor Xa (lane *11*) had minimal or no effect. Subtilisin (lane *10*), a broad-spectrum serine protease related to mammalian dibasic endoproteases (Barr, 1991), and stromelysin, a matrix-degrading metalloprotease (lane *14*), also degraded SPARC. The specificity of these reactions was monitored by addition of a 100-fold molar excess of BSA to the reactions. SPARC was preferentially degraded over BSA by all enzymes tested (not shown).

Plasmin, a serine protease related to trypsin and implicated in a variety of processes associated with angiogenesis, degraded SPARC to low molecular weight components (Fig. 7, lane *13*; Sage et al., 1984). Inclusion of EDTA augmented the susceptibility of SPARC to plasmin (not shown). The enhanced degradation of SPARC in the presence of EDTA indicated that local changes in the concentration of metal ions (presumably Ca^{2+} or Cu^{2+}) could alter the stability of SPARC to proteolysis. Similar effects of divalent cations on the susceptibility of SPARC to degradation have been described for several other proteinases (Maurer et al., 1992).

Proteolysis of SPARC Leads to the Release of KGHK and GHK

Analysis of peptides released from SPARC demonstrated that the dibasic amino acid sequence -KK- at position 117–118 was susceptible to cleavage by both trypsin and plasmin. Fig. 8 shows the elution profiles from hydrophilic interaction chromatography of SPARC (*C*) and the peptide $SPARC_{113-130}$ (*B*) after digestion with trypsin. Mass spectral analysis of the fractions eluting at 23 min revealed that the

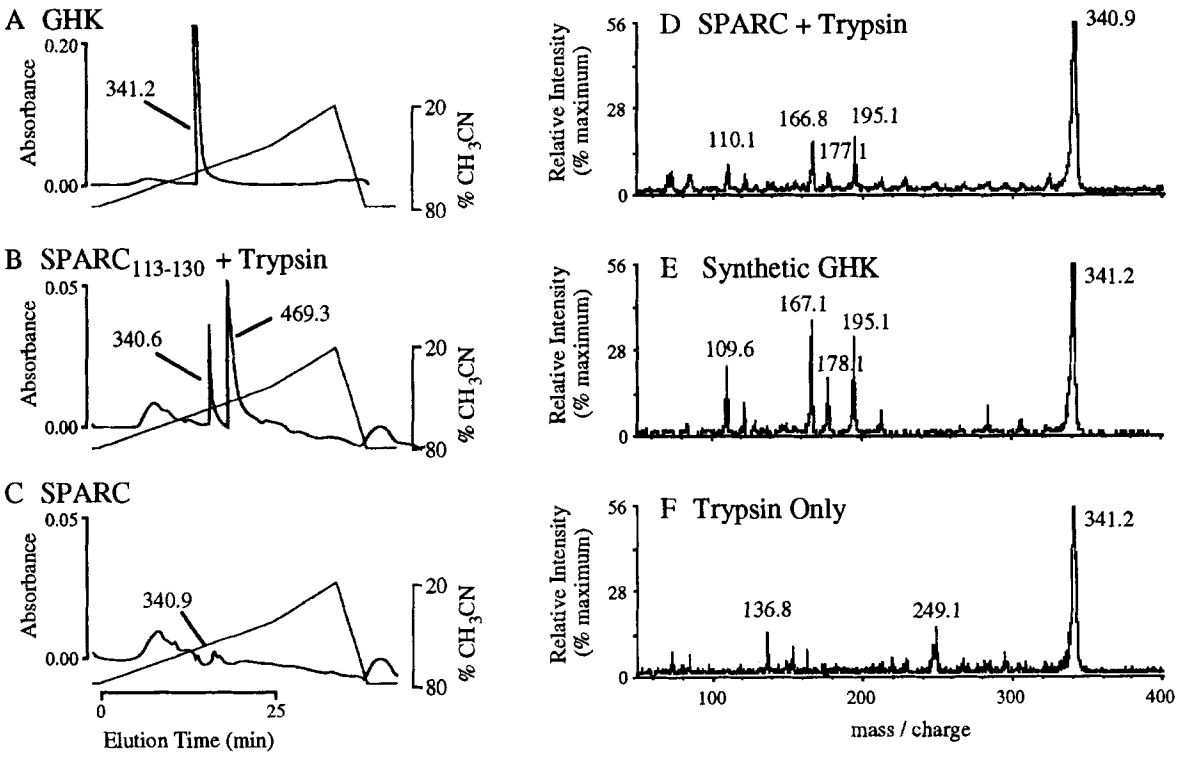

Figure 8. Digestion of SPARC and $SPARC_{113-130}$ with trypsin releases peptides containing GHK. SPARC (6 nmol), or a synthetic peptide that spans amino acids 113–130 (200 nmol), was treated with trypsin (1:100, enzyme/substrate molar ratio) for 2 h at 37°C. The solutions were lyophilized and chromatographed on reverse-phase HPLC; unbound fractions were collected (not shown). (*A*, *B*, and *C*) Hydrophilic interaction chromatography of peptide fractions; peptides were eluted with gradients of acetonitrile. In *A*, synthetic GHK (88 nmol) was chromatographed as a standard. In *B* and *C*, the unbound fractions of digested SPARC and $SPARC_{113-130}$ were analyzed by hydrophilic interaction chromatography. Fractions eluting at 23 min contained GHK by tandem mass spectrometry (not shown). The second peak in the digest of peptide 113–130 (*B*), at 26 min, was identified as KGHK (not shown). (*D*, *E*, and *F*) Tandem mass spectrometry profiles of 3 μmol SPARC after treatment with trypsin (*D*), 3 μmol GHK (*E*), and a hydrolysis control with trypsin alone (*F*), respectively. The masses (in atomic mass units [D]) of product ions released by ionization of the 341 ± 0.5 D parent ion are shown.

peptide GHK ($SPARC_{120-122}$; calculated atomic mass = 340.2 D) was present. KGHK ($SPARC_{119-122}$; calculated atomic mass = 468.6 D) was found in fractions of the peptide digest that eluted at 26 min (*B*). KGHK was seen in variable amounts; extended digestion times resulted in a decrease in the amount of KGHK and a subsequent increase in GHK.

For samples that contained low amounts of SPARC, the use of hydrophilic interaction chromatography was required to remove salts from the peptides before mass spectrometry. However, GHK could be identified directly by mass spectral analysis of the entire digest when larger amounts of protein were studied. Fig. 8, *D*, *E*, and *F*, show tandem mass spectra of unfractionated digests. Tryptic cleavage of SPARC (Fig. 8 *D*) released a peptide (MH^+), with a mass/charge ratio of 341, which fragmented in a manner identical to that of the GHK standard (*E*). The pattern of product ions was completely consistent with the identification of GHK in these samples. Control digests in which the substrate was omitted (Fig. 8 *F*) or of SPARC in the absence of enzymes (not shown) did not produce ions of this type. These controls demonstrate that GHK was not present as a contaminant in the initial preparations.

Mapping of Potential Proteolytic Sites for Plasmin and Trypsin

The activity of purified proteases on the two cationic regions of SPARC encompassed by domain II was analyzed by incubation of enzymes with peptides that represented these sequences. As an initial screen for protease-sensitive sites within SPARC, peptides that represented both cationic sites were exposed to proteolytic enzymes for 1–2 h and were analyzed by high resolution gel electrophoresis. The results of these experiments are shown in Table II. The peptide $SPARC_{113-130}$ was digested completely by exposure to either trypsin-TPCK or plasmin (Molar ratio of enzyme to substrate was <1:100). The same peptide was resistant to similar concentrations of activated thrombin, Factor Xa, or bacterial collagenase. A substituted peptide sequence in which Lys_{119} was replaced by Ala ($SPARC_{113-130K119-A}$ Table I) was more resistant to trypsin than was the wild-type sequence and was poorly cleaved by plasmin. These results indicate that the -KK- sequence is required for efficient recognition and cleavage by plasmin. Comparable digestion of $SPARC_{54-73}$, which contains an inverted KGHK site (KHGK; $SPARC_{60-63}$), required higher doses of trypsin and appeared resistant to plasmin.

Mapping of enzymatic cleavage sites was accomplished by hydrophilic interaction chromatography. Tandem mass spectrometry was used to confirm the sequences of the proteolytic fragments identified by hydrophilic interaction chromatography. In Fig. 9 *A*, the elution profile of $SPARC_{113-127}$ is shown after digestion with plasmin or trypsin. Undigested controls are presented in Fig. 9 *B* for reference. A summary of observed cleavage sites within both cationic regions ($SPARC_{54-73}$ and $SPARC_{113-127}$) is presented as a schematic diagram in Fig. 9 *C*. Cleavage of $SPARC_{113-127}$ resulted in fragments consistent with cleavage after the KK dibasic sequence (after K_{119}). Trypsin also cut efficiently between the K residues (after K_{118}) and after GHK (K_{122}). The fragments recovered from the chromatographic analysis of the tryptic digest were consistent with release of KGHK and GHK. Plasmin cleaved peptide bonds COOH-terminal to K_{118} and K_{122}, but the appearance of these products in low yield indicated that the reaction was considerably slower than that observed with trypsin. The fragments recovered from hydrophilic interaction chromatography of the plasmin digest were consistent with release of an NH_2-terminal sequence and of KGHK and GHK in lower yields.

*Table II. Enzymatic Susceptibility of Peptides from Basic Regions of SPARC**

Enzyme	$SPARC_{113-130}$	$SPARC_{113-130K119-A}$	$SPARC_{54-73}$
Trypsin	High‡ (5)	Moderate (2)	Low (3)
Plasmin	High (4)	Low (2)	Stable (3)
Thrombin	Stable (4)	ND	ND
Factor Xa	Stable (2)	ND	ND
Bacterial collagenase	Stable (2)	ND	ND
Elastase	Moderate (3)	ND	ND

* 20 μg of peptide (see Table I for complete sequences) was digested with 1 μg of enzyme (enzyme to substrate molar ratios were <1:100), and aliquots were removed for analysis on SDS-PAGE as described in Materials and Methods. Digestion was for 2 h in Hepes-buffered saline, pH 7.5, 10 mM $CaCl_2$, at 37°C.

‡ Degree of activity was assessed by the loss of Coomassie blue-stained protein: high, 90–100% reduction in stained protein; moderate, 40–60% reduction; low, 10–20% reduction; stable, no evidence of reduction; ND, not determined. The number of experiments is indicated in parentheses.

Analysis of the digestion products of $SPARC_{54-73}$ indicated that this region was not cleaved by plasmin and served as a very inefficient substrate for trypsin (summarized in Fig. 9 *C*).

Discussion

In this study, we have demonstrated the coincidence of SPARC with endothelial reorganization in vivo and provided evidence that SPARC protein functions to support the growth of new vessels. SPARC was shown to be expressed transiently and specifically in nascent capillaries during a period of rapid angiogenesis in the embryonic brain and in healing dermal wounds. Moreover, addition of SPARC to cultures of endothelial cells undergoing angiogenesis in vitro resulted in an increased number of cords, an effect mimicked by synthetic peptides from cationic region 2 of SPARC ($SPARC_{113-130}$), a sequence that we show binds copper ion. $SPARC_{113-130}$ and smaller peptides derived from this sequence were also shown to be potent stimulators of angiogenesis in vivo, but a second Cu^{2+}-binding peptide (from cationic region 1) had no effect. Substitution of the amino acid His_{121} with either Arg or Ala decreased Cu^{2+} binding and diminished the stimulation. Proteolysis of SPARC, or of synthetic peptides that overlapped amino acids 113–130, resulted in the release of the peptides KGHK and GHK.

Detection of gene transcription by endothelial cells in vivo can be difficult, since these cells are characterized by low amounts of cytoplasm and correspondingly low levels of mRNA. Therefore, we decided to look for SPARC in tissues in which both endothelial migration and proliferation were enhanced, and in which the levels of SPARC expression in adjacent cell types was thought to be low. This strategy allowed us to detect expression in capillary endothelial cells from embryonic brain and from adult skin in response to wound repair. Our results indicate that SPARC is synthesized by endothelial cells in vivo and that principally growing or differentiating cells are implicated. Differential synthesis of

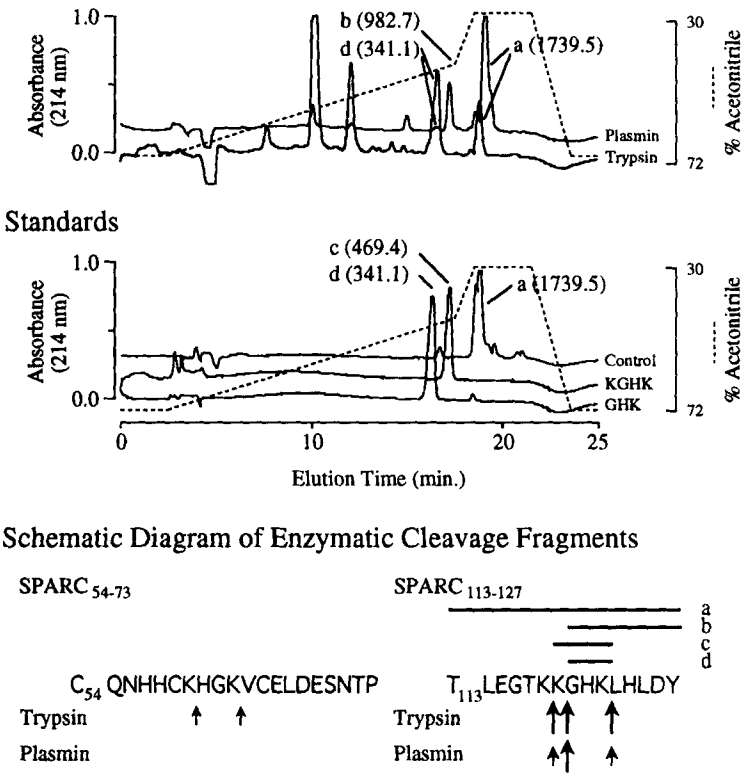

Figure 9. Identification of proteolytic cleavage sites in synthetic peptides. (*A*) Peptide $SPARC_{113-127}$ was treated with either trypsin (1:300, enzyme/substrate molar ratio) or plasmin (1:100, enzyme/substrate molar ratio) at 37°C for 2 h. Digests were resolved on an hydrophilic interaction chromatography column developed with a gradient of acetonitrile. (*B*) Chromatograms of undigested peptide (*control*) and synthetic peptide standards (KGHK and GHK). Peaks of absorbance (214 nm) were collected for sequence analysis by mass spectrometry, and the mass of each peptide is indicated in parentheses next to each relevant peak. *Lower case letters*, elution positions of the fragments presented in *C*. (*C*) Schematic of the SPARC sequence shows the location of observed cleavage sites in $SPARC_{54-73}$ and $SPARC_{113-127}$; *lower case letters*, peptide identified in the chromatograms shown in *A*; *large and small arrows*, major and minor cleavage sites, respectively. The sequence representing peptide $SPARC_{54-73}$ was unaffected by plasmin, and treatment with trypsin resulted in very low rates of cleavage (not shown).

SPARC might reflect one means by which the availability of the protein is regulated during the response of endothelial cells to angiogenic signals.

We have shown that an assay of endothelial cord formation in vitro produced results with SPARC peptides that were consistent with those obtained from a CAM assay in vivo. Although SPARC inhibits the proliferation of cultured, subconfluent endothelial cells (Funk and Sage, 1991, 1993), the present study is consistent with the hypothesis that SPARC and/or certain SPARC peptides can enhance the angiogenic response in vitro. Angiogenesis can be viewed as a series of interactions between endothelial cells and their immediate extracellular matrix; e.g., the production of matrix-degrading proteases, acquisition of an invasive phenotype, extension of endothelial cords, formation of patent capillary networks, reestablishment of a basement membrane, and the return to a quiescent state (Folkman and Klagsbrun, 1987; Ingber and Folkman, 1989*b*). The ability of growth factors to modulate this process presumably relies on their intervention at specific steps in the pathway, since these proteins can interact with several cell types, bind to various ligands in the extracellular milieu, or recruit inflammatory cells that release secondary growth stimulators. Proliferation assays can, however, be misleading (Sholley, 1984; Williams et al., 1989) because not all steps in the process of capillary formation are dependent on endothelial cell mitosis. Although cell proliferation in vitro remains the simplest and most frequently used assay for the screening of angiogenic substances, it is also instructive to study culture systems that present more complex interactions between endothelial cells and their extracellular matrix.

SPARC has been described as a temporally regulated component of extracellular matrices. However, with the possible exception of bone and certain tumors, SPARC protein is not generally observed as a stable extracellular component. SPARC has been shown to associate with extracellular matrices and to regulate matrix deposition (Termine et al., 1981; Romberg et al., 1986; Mayer et al., 1991; Lane et al., 1992). The effects of SPARC on cells are consistent with a role in the regulation of cell-matrix interactions: the protein is a potent inhibitor of cellular spreading (Sage et al., 1989*b*; Lane and Sage, 1990) and has recently been shown to inhibit endothelial proliferation in vitro (Funk and Sage, 1991 and 1993). Although SPARC has been characterized as a regulator of cell-substrate interactions (Sage and Bornstein, 1991), our observation that proteolysis of SPARC can lead to the release of diffusable, bioactive peptides indicates that additional functions should be considered.

We have demonstrated that SPARC can be cleaved by plasmin and propose that other enzymes might also contribute to the release of KGHK and GHK from cationic region 2. SPARC was cleaved by proteases that are present in tissues and mediate matrix turnover, such as elastase and stromelysin, and by the broad-spectrum serine proteases trypsin and subtilisin. Interestingly, SPARC was cleaved by thrombin, a potent activator of endothelial cells and platelets, to yield a stable intermediate that might subsequently be susceptible to other enzymes. Previous studies have also shown that SPARC is digested by α-chymotrypsin and pepsin (Sage et al., 1984), human leukocyte elastase (Maurer et al., 1992), and an uncharacterized metalloprotease secreted by osteoblasts (Tyree, 1989). The susceptibility of SPARC to protease cleavage by leukocyte elastase and α-chymotrypsin is dependent on the concentration of calcium ion (Maurer et

al., 1992). Since a major chymotrypsin-sensitive site is located at amino acid 109 (Maurer et al., 1992), amino acids near the GHK site appear to be exposed and susceptible to proteolysis. The sequence GHK occurs in a variety of proteins but is underrepresented in proteins that are secreted from mammalian cells. The pair of basic residues (KK) that flanks the GHK site makes the context of the GHK sequence, as a conserved motif, unique to vertebrate SPARC proteins and provides a target for a variety of enzymes. Such sites are frequently cleaved by serine proteases and by members of the subtilisin family of endoproteinases. Our analysis of GHK sequences in other proteins indicates that they could have arisen by chance, since these sites are not generally conserved across species.

The involvement of copper and copper complexes in angiogenesis has received considerable interest. Copper has been shown to accumulate in tissues before vascularization (Ziche et al., 1982; Apelgot et al., 1986) and systemically during an inflammatory response (reviewed in Linder, 1991). Copper-deficient animals have a diminished response to angiogenic stimulation (Ziche et al., 1982) and fail to vascularize implanted tumors effectively (Brem et al., 1990). The concentration of available copper complexes directly affects the synthesis of fibronectin and collagen by endothelial cells (Hannan and McAuslan, 1982), as well as their migration in vitro (Alessandri et al., 1983). These observations have led to the hypothesis that copper ion is required for one or several steps in blood vessel formation. Previous studies with several copper-containing complexes, including Cu:GHK, have shown an extensive stimulation of new vessel growth in vivo, whereas apoGHK had little effect (Raju et al., 1982). Studies by the same group showed that Cu:GHK affected endothelial migration minimally in vitro (Alessandri et al., 1983). In the present studies, both KGHK and GHK stimulated cord formation in vitro; however, GHK required previous treatment with copper salts to acquire activity. We have shown that KGHK is a potent stimulator of angiogenesis, both in the CAM in vivo and in an assay of endothelial cord stimulation in vitro. Although KGHK was shown to bind Cu^{2+}, the peptide did not appear to require previous loading with Cu^{2+} to manifest angiogenic activity. At the present time, we are unable to distinguish the effect of KGHK from that of the Cu:KGHK complex. Amino acid substitutions that eliminated Cu^{2+} binding also diminished angiogenesis. Thus, there is a formal possibility that copper ion contributed by the cells is required for KGHK activity, a hypothesis that we could not test because our cells have an absolute growth requirement for trace levels of copper.

The production of SPARC by endothelial cells of developing capillaries, as well as by platelets and other connective tissue cells, provides a source of the protein that correlates temporally and spatially with the formation of neovascular sprouts during development and tissue repair. We propose that SPARC contributes to the formation of new vessel growth initially through its effects on endothelial cell shape and protein synthesis (Sage et al., 1989*b*; Lane et al., 1992). Subsequently, the protein is cleaved into bioactive peptides, one or more of which stimulate the morphogenesis of cords. We propose the following sequence for the activities of SPARC: (*a*) tissues undergoing remodeling and vascular growth produce high levels of SPARC; (*b*) SPARC is bound by the extracellular matrix or is released into a soluble compartment; (*c*) SPARC interacts with cells to modulate adhesion, proliferation, and matrix deposition; (*d*) stimulation of endothelial cells, platelet degranulation, or recruitment of inflammatory cells results in the release of specific proteases that degrade SPARC; and (*e*) cleavage products of SPARC manifest new functions and access new compartments not available to the intact protein. Cleavage of SPARC by plasmin provides an intriguing pathway, since this proteinase is closely regulated during angiogenesis (Rifkin et al., 1982; Pepper and Montesano, 1990; Bacharach et al., 1992). The activation of plasmin itself has been shown to be controlled by various angiogenic growth factors (Montesano and Orci, 1985; Montesano et al., 1986), and SPARC is a feedback regulator of this process through its increase of PAI-1 expression in angiogenic endothelial cells (Hasselaar et al., 1991; Lane et al., 1992).

From our data, it appears that SPARC could be cleaved by several proteases present in extracellular fluids. Although SPARC has traditionally been viewed as a regulator of cell-matrix interactions and matrix synthesis (Sage and Bornstein, 1991; Sage et al., 1992), the intact protein could also be viewed as a source of bioactive peptides associated with a larger domain. Long regulatory sequences have been identified as masking domains (latency structures) or as targeting signals in peptide hormones, growth factors, and enzymes (Neurath, 1989; Roberts and Sporn, 1992). Studies that have demonstrated the association of SPARC with developing or remodeling extracellular matrices could be reinterpreted in the context of a precursor protein that releases active peptides through specific proteolysis. Thus, SPARC might be a regulator of the distribution and activity of KGHK or GHK.

In view of the existing evidence for the selective expression of SPARC during angiogenesis, and the literature describing the expression of this gene during development and tissue remodeling, the fate of SPARC in extracellular fluids is of considerable importance. We provide evidence that SPARC might regulate vascular growth, a function that is enhanced by the action of specific proteases. The sequence GHK in a highly conserved, protease-sensitive domain of SPARC provides a potential substrate for the action of these enzymes. Since the source of GHK in plasma (or serum) is not known, we propose that at least a portion of this circulating peptide is derived from SPARC.

The authors thank Drs. May Reed and Pauli Puolakkainen for the rat wound tissue, and Drs. J. Meijers, W. Kisiel, and G. Goldberg for enzymes. We also thank Dr. J. Bassuk for assistance with the computer searches, and Drs. R. Bolender, J. Yost, E. Adman, and E. Raines for valuable discussions throughout these experiments.

These experiments were funded in part by National Institutes of Health grants GM-40711 and HL-03174. T. F. Lane was supported by a National Institutes of Health Predoctoral training grant (NRSA) HL-07312. M. L. Iruela-Arispe and R. S. Johnson were supported by grants from the American Heart Association, National and Washington Affiliates, respectively.

Received for publication 7 May 1993 and in revised form 31 December 1993.

References

Alessandri, G., K. S. Raju, and P. N. Gullino. 1983. Mobilization of capillary endothelium in vitro induced by effectors of angiogenesis in vivo. *Cancer Res.* 43:1790–1797.

Apelgot, S., J. Coppey, A. Fromentin, E. Guille, M.-F. Poupon, and A. Rousselet. 1986. Altered distribution of copper (64-Cu) in tumor-bearing mice and rats. *Anticancer Res.* 6:159–164.

Auerbach, R., L. Kubai, D. Knighton, and J. Folkman. 1974. A simple procedure for the long-term cultivation of chicken embryos. *Dev. Biol.* 41: 391–394.

Bacharach, E., A. Itin, and E. Keshet. 1992. In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. *Proc. Natl. Acad. Sci. USA.* 89:10686–10690.

Barr, P. J. 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. *Cell.* 66:1–3.

Brem, S. S., D. Zagzag, A. M. Tsanaclis, S. Gately, M. P. Elkouby, and S. E. Brien. 1990. Inhibition of angiogenesis and tumor growth in the brain: suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. *Am. J. Pathol.* 137:1121–1142.

Cawston, T. E., and G. Murphy. 1981. Mammalian collagenases. *Methods Enzymol.* 80:711–734.

Coffin, J. D., J. Harrison, S. Schwartz, and R. Heimark. 1991. Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. *Dev. Biol.* 148:51–62.

Covey, T. R., R. F. Bonner, B. I. Shushan, and J. Henion. 1988. The determination of protein, oligonucleotide and peptide molecular weights by ionspray mass spectrometry. *Rapid Commun. Mass Spectrom.* 2:249–256.

D'Amore, P. A. 1992. Mechanisms of endothelial growth control. *Am. J. Respir. Cell Mol. Biol.* 6:1–8.

Damjanovski, S., F. Liu, and M. Ringuette. 1992. Molecular analysis of *Xenopus laevis* SPARC (secreted protein, acidic, rich in cysteine). *Biochem. J.* 281:513–517.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387–395.

Dziadek, M., M. Paulsson, M. Aumailley, and R. Timpl. 1986. Purification and tissue distribution of a small protein (BM-40) extracted from a basement membrane tumor. *Eur. J. Biochem.* 161:455–464.

Engel, J. 1989. EGF-like domains in extracellular matrix proteins: localized signals for growth and differentiation? *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 251:1–7.

Fenn, J. B., M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science (Wash. DC).* 246:64–71.

Flaumenhaft, R., and D. B. Rifkin. 1992. The extracellular regulation of growth factor action. *Mol. Biol. Cell.* 3:1057–1065.

Folkman, J., and A. Moscona. 1978. Role of cell shape in growth control. *Nature (Lond.).* 273:345–349.

Folkman, J., and M. Klagsbrun. 1987. Angiogenic factors. *Science (Wash. DC).* 235:442–447.

Form, D. M., B. M. Pratt, and J. A. Madri. 1986. Endothelial cells proliferation during angiogenesis. *Lab. Invest.* 55:521–530.

Fujisawa, R., and Y. Kuboki. 1989. Changes in levels of osteonectin in bovine dentine during tooth development. *Arch. Oral Biol.* 34:89–92.

Funk, S. E., and E. H. Sage. 1991. The Ca^{2+}-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA.* 88:2648–2652.

Funk, S. E., and E. H. Sage. 1993. Differential effects of SPARC and cationic SPARC peptides on DNA synthesis by endothelial cells and fibroblasts. *J. Cell. Physiol.* 154:53–63.

Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.

Hannan, G. N., and B. R. McAuslan. 1982. Modulation of synthesis of specific proteins in endothelial cells by copper, cadmium, and disulfiram: an early response to an angiogenic inducer of cell migration. *J. Cell. Physiol.* 111:207–212.

Hasselaar, P., D. J. Loskutoff, M. Sawdey, and E. H. Sage. 1991. SPARC induces the expression of type 1 plasminogen activator inhibitor in cultured bovine aortic endothelial cells. *J. Biol. Chem.* 266:13178–13184.

Holland, P. W. H., S. J. Harper, J. H. McVey, and B. L. M. Hogan. 1987. In vivo expression of mRNA for the Ca^{++}-binding protein SPARC (osteonectin) revealed by in situ hybridization. *J. Cell Biol.* 105:473–482.

Hunt, D. F., J. R. I. Yates, J. Shabanowitz, S. Winston, and C. R. Hauer. 1986. Protein sequencing by tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA.* 83:6233–6237.

Hunter, W. M., and F. C. Greenwood. 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.).* 194: 495–496.

Hutchens, T. W., R. W. Nelson, M. H. Allen, C. M. Li, and T. T. Yip. 1992. Peptide-metal ion interactions in solution: detection by laser desorption time-of-flight mass spectrometry and electrospray ionization mass spectrometry. *Biol. Mass Spectrom.* 21:151–159.

Ingber, D. E. 1990. Fibronectin controls capillary endothelial cell growth by modulating cell shape. *Proc. Natl. Acad. Sci. USA.* 87:3579–3583.

Ingber, D. E., and J. Folkman. 1989*a*. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. *J. Cell Biol.* 109:317–330.

Ingber, D. E., and J. Folkman. 1989*b*. How does extracellular matrix control capillary morphogenesis? *Cell.* 58:803–805.

Iruela-Arispe, M. L., P. Hasselaar, and H. Sage. 1991*a*. Differential expression of extracellular proteins is correlated with angiogenesis in vitro. *Lab. Invest.* 64:174–186.

Iruela-Arispe, M. L., C. A. Diglio, and E. H. Sage. 1991*b*. Modulation of extracellular matrix proteins by endothelial cells undergoing angiogenesis in vitro. *Arterioscler. Thromb.* 11:805–815.

Iruela-Arispe, L., P. Bornstein, and H. Sage. 1991*c*. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. *Proc. Natl. Acad. Sci. USA.* 88:5026–5030.

Iruela-Arispe, L., D. J. Liska, E. H. Sage, and P. Bornstein. 1993. Differential expression of thrombospondin 1, 2, and 3 during murine development. *Dev. Dynamics.* 197:40–56.

Lane, T. F., M. L. Iruela-Arispe, and E. H. Sage. 1992. Regulation of gene expression by SPARC during angiogenesis in vitro: changes in fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1. *J. Biol. Chem.* 267:16736–16745.

Lane, T. F., and E. H. Sage. 1990. Functional mapping of SPARC: Peptides from two distinct sites modulate cell adhesion. *J. Cell Biol.* 111:3065–3076.

Lane, T. F., and E. H. Sage. 1994. The biology of SPARC: a protein that modulates cell-matrix interactions. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 8:163–173.

Linder, M. C. 1991. Biochemistry of Copper. Plenum Press, New York.

Mason, I. J., A. Taylor, J.G. Williams, H. Sage, and B. L. M. Hogan. 1986*a*. Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell "culture shock" glycoprotein. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1465–1472.

Mason, I. J., D. Murphy, M. Munke, U. Francke, R. W. Elliott, and B. L. M. Hogan. 1986*b*. Developmental and transformation-sensitive expression of the SPARC gene on mouse chromosome 11. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1831–1837.

Maurer, P., U. Mayer, M. P. J. Bruch, K. Mann, R. Landwehr, J. Engel, and R. Timpl. 1992. High-affinity and low-affinity calcium binding and stability of the multidomain extracellular 40-kDa basement membrane glycoprotein (BM-40/SPARC/osteonectin). *Eur. J. Biochem.* 205:233–240.

Mayer, U., M. Aumailley, K. Mann, R. Timpl, and J. Engel. 1991. Calcium-dependent binding of basement membrane protein BM-40 (osteonectin, SPARC) to basement membrane collagen type IV. *Eur. J. Biochem.* 198: 141–150.

McAuslan, B. R., G. N. Hannan, W. Reilly, R. G. Whittaker, and M. Florence. 1980. Reappraisal of evidence for the role of copper in angiogenesis. The nature of an active copper complex. *In* CSIRO Symposium on the Importance of Copper in Biology and Medicine. B. R. McAuslan, editor. Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia. pp. 42–49.

Meijers, J. C. M., P. N. M. Tijburg, and B. N. Bouma. 1987. Inhibition of human blood coagulation factor Xa by α2-macroglobulin. *Biochemistry.* 26:5932–5937.

Montesano, R., and L. Orci. 1985. Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell.* 42:467–477.

Montesano, R., J. D. Vassalli, A. Baird, A. Guillemin, and L. Orci. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. *Proc. Natl. Acad. Sci. USA.* 83:7297–7301.

Neurath, H. 1989. Proteolytic processing and physiological regulation. *Trends Biochem. Sci.* 14:268–271.

Otsuka, K., K. L. Yao, S. Wasi, P. S. Tung, J. E. Aubin, J. Sodek, and J. D. Termine. 1984. Biosynthesis of osteonectin by fetal porcine calvarial cells in vitro. *J. Biol. Chem.* 259:9805–9812.

Panayotou, G., P. End, M. Aumailley, R. Timpl, and J. Engel. 1989. Domains of laminin with growth-factor activity. *Cell.* 56:93–101.

Pepper, M. S., and R. Montesano. 1990. Proteolytic balance and capillary morphogenesis. *Cell. Diff. Dev.* 32:319–327.

Pickart, L. 1983. The biological effects and mechanism of action of the plasma tripeptide glycyl-L-histidyl-L-lysine. *Lymphokines.* 8:425–446.

Pickart, L., and S. Lovejoy. 1987. Biological activity of human plasma copper-binding growth factor glycyl-L-histidyl-L-lysine. *Methods Enzymol.* 147: 314–328.

Porath, J., J. Carlsson, I. Olsson, and G. Belfrage. 1975. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature (Lond.).* 258:598–599.

Rafferty, K. A. 1970. Methods in experimental embryology of the mouse. Johns Hopkins University Press, Baltimore, MD.

Raines, E. W., T. F. Lane, M. L. Iruela-Arispe, R. Ross, and E. H. Sage. 1992. The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. *Proc. Natl. Acad. Sci. USA.* 89:1281–1285.

Raju, K. S., G. Alessandri, M. Ziche, and P. N. Gullino. 1982. Ceruloplasmin, copper ions and angiogenesis. *J. Natl. Cancer Inst.* 69:81–88.

Reed, M. J., P. Puolakkainen, T. F. Lane, D. Dickerson, P. Bornstein, and E. H. Sage. 1993. Differential expression of SPARC and thrombospondin 1 in wound repair: immunolocalization and in situ hybridization. *J. Histochem. Cytochem.* 41:1467–1477.

Rifkin, D. B., J. L. Gross, D. Moscatelli, and E. Jaffe. 1982. Proteases and angiogenesis: production of plasminogen activator and collagenase by endothelial cells. *In* Pathobiology of the Endothelial Cell. H. L. Nossel and H. J. Vogel, editors. Academic Press, New York. pp. 191–197.

Roberts, A. B., and M. B. Sporn. 1992. Differential expression of the TGF-beta

isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol. Reprod. Dev.* 32:91–98.

Romberg, R. W., P. G. Werness, B. L. Riggs, and K. G. Mann. 1986. Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. *Biochemistry.* 25:1176–1180.

Sage, E. H., and P. Bornstein. 1991. Extracellular proteins that modulate cell-matrix interactions: SPARC, tenascin, and thrombospondin. *J. Biol. Cell.* 266:14831–14834.

Sage, E. H., T. F. Lane, M. L. Iruela-Arispe, and S. E. Funk. 1992. SPARC: A protein that modulates cell cycle and cell-matrix interactions. *In* Chemistry and Biology of Mineralized Tissues. H. Slavkin and P. Price, editors. Elsevier. The Netherlands. pp. 235–242.

Sage, E. H., R. B. Vernon, J. Decker, S. Funk, and M. L. Iruela-Arispe. 1989*a*. Distribution of the calcium-binding protein SPARC in tissues of embryonic and adult mice. *J. Histochem. Cytochem.* 37:819–829.

Sage, H., R. B. Vernon, S. E. Funk, E. A. Everitt, and J. Angello. 1989*b*. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca^{2+}-dependent binding to the extracellular matrix. *J. Cell Biol.* 109:341–356.

Sage, H., C. Johnson, and P. Bornstein. 1984. Characterization of a novel serum albumin-binding glycoprotein secreted by endothelial cells in culture. *J. Biol. Chem.* 259:3993–4007.

Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

Schulz, A., G. Jundt, K.-H. Berghäuser, P. Gehron-Robey, and J. D. Termine. 1988. Immunohistochemical study of osteonectin in various types of osteosarcoma. *Am. J. Pathol.* 132:233–238.

Schwarzbauer, J., and C. S. Spencer. 1993. The *Caenorhabditis elegans* homolog of the extracellular calcium binding protein SPARC/osteonectin affects nematode body morphology and mobility. *Mol. Biol. Cell.* 4:941–952.

Sholley, M. M., G. P. Ferguson, H. R. Seibel, J. L. Montour, and J. D. Wilson. 1984. Mechanisms of neovascularization. Vascular sprouting can occur without proliferation of endothelial cells. *Lab. Invest.* 51:624–634.

Taylor, S., and J. Folkman. 1982. Protamine is an inhibitor of angiogenesis. *Nature (Lond.).* 297:307–312.

Termine, J. D., A. B. Belcourt, K. M. Conn, and H. K. Kleinman. 1981. Mineral and collagen-binding proteins of fetal calf bone. *J. Biol. Chem.* 256:10403–10408.

Tyree, B. 1989. The partial degradation of osteonectin by a bone-derived metalloprotease enhances binding to type I collagen. *J. Bone Min. Res.* 4: 877–883.

Vernon, R. B., and E. H. Sage. 1989. The calcium-binding protein SPARC is secreted by Leydig and Sertoli cells of the adult mouse testis. *Biol. Reprod.* 40:1329–1340.

Waisman, D. M., and H. Rasmussen. 1983. A reexamination of the chelex competitive calcium binding assay. *Cell Calcium.* 4:89–105.

Wasi, S., K. Otsuka, K. L. Yao, P. S. Tung, J. E. Aubin, J. Sodek, and J. D. Termine. 1984. An osteonectin-like protein in porcine periodontal ligament and its synthesis by periodontal ligament fibroblasts. *Can. J. Biochem. Cell Biol.* 62:470–478.

Wewer, U. M., R. Albrechtsen, L. W. Fisher, M. F. Young, and J. D. Termine. 1988. Osteonectin/SPARC/BM-40 in human decidua and carcinoma, tissues characterized by de novo formation of basement membrane. *Am. J. Pathol.* 132:345–355.

Whitson, S. W., W. Harrison, M. K. Dunlap, D. E. Bowers, Jr., L. W. Fisher, P. G. Robey, and J. D. Termine. 1984. Fetal bovine bone cells synthesize bone-specific matrix proteins. *J. Cell Biol.* 99:607–614.

Williams, L. R., W. Risau, H.G. Zerwes, H. Drexler, A. Aguzzi, and E. F. Wagner. 1989. Endothelioma cells expressing the polyoma middle T onogene induce hemangiomas by host cell recruitment. *Cell.* 57:1053–1063.

Ziche, M., J. Jones, and P. N. Gullino. 1982. Role of prostataglandin E_1 and copper in angiogenesis. *J. Natl. Cancer Inst.* 69:475–482.