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Inhibition of Endothelial Cell Proliferation by SPARC Is Mediated Through a Ca^{2+}-Binding EF-Hand Sequence

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Abstract SPARC (secreted protein, acidic and rich in cysteine, also known as osteonectin and BM-40) is a metal-binding glycoprotein secreted by a variety of cultured cells and characteristic of tissues undergoing morphogenesis, remodeling, and repair. Recently it has been shown that SPARC inhibits the progression of the endothelial cell cycle in mid-G_1, and that a synthetic peptide (amino acids 54–73 of secreted murine SPARC, peptide 2.1) from a cationic, disulfide-bonded region was in part responsible for the growth-suppressing activity [Funk and Sage (1991): Proc Natl Acad Sci USA 88:2648–2652]. Moreover, SPARC was shown to interact directly with bovine aortic endothelial (BAE) cells through a C-terminal EF-hand sequence comprising a high-affinity Ca^{2+}-binding site of SPARC and represented by a synthetic peptide (amino acids 254–273) termed 4.2 [Yost and Sage (1993): J Biol Chem 268:25790–25796]. In this study we show that peptide 4.2 is a more potent inhibitor of DNA synthesis that acts cooperatively with peptide 2.1 to diminish the incorporation of [^{3}H]-thymidine by both BAE and bovine capillary endothelial (BCE) cells. At concentrations of 0.019–0.26 mM peptide 4.2, thymidine incorporation by BAE cells was decreased incrementally, relative to control values, from approximately 100 to 10%. Although somewhat less responsive, BCE cells exhibited a dose-responsive decrement in thymidine incorporation, with a maximal inhibition of 55% at 0.39 mM. The inhibitory effect of peptide 4.2 was essentially independent of heparin and basic fibroblast growth factor and was blocked by anti-SPARC peptide 4.2 IgG, but not by antibodies specific for other domains of SPARC. To identify residues that were necessary for inhibition of DNA synthesis, we introduced single amino acid substitutions into synthetic peptide 4.2 and tested their activities and cell-surface binding characteristics on endothelial cells. Two peptides displayed null to diminished effects in the bioassays that were concentration-dependent: peptide 4.2 K, containing an $Asp_{258} \rightarrow$ Lys substitution, and peptide 4.2 AA, in which the two disulfide-bonded Cys (positions 255 and 271) were changed to Ala residues. Peptide 4.2 K, which failed to fulfill the EF-hand consensus formula, exhibited an anomalous fluorescence emission spectrum, in comparison with the wild-type 4.2 sequence, that was indicative of a compromised affinity for Ca^{2+}. Moreover, ablation of the disulfide bond in peptide 4.2 AA potentially destabilized the Ca^{2+}-binding loop structure, as assessed by fluorescence spectroscopy, such that the peptide competed poorly for the binding of [^{125}I]-peptide 4.2 to BAE cells. We conclude both that Ca^{2+}-coordinating Asp at position 258 and the conformation of peptide 4.2 are necessary for the inhibition of DNA synthesis by SPARC in cultured endothelial cells.

Key words: angiogenesis, EF-hand, endothelial cells, extracellular matrix, vascular biology

The Ca^{2+}-binding glycoprotein SPARC (secreted protein acidic and rich in cysteine) is expressed in many tissues and organs during embryogenesis and is typically found in epithelia and connective tissue during remodeling, wound healing, and atherogenesis [for a review, see Lane and Sage, 1994]. In *Xenopus laevis*, SPARC appears necessary for patterning and the development of a correct embryonic axis [Purcell et al., 1993], and transgenic experiments in the nematode *Caenorhabditis elegans* revealed altered morphology and viability coincident with the inappropriate expression of this protein [Schwarzbauer and Spencer, 1993].

Abbreviations used: BAE, bovine aortic endothelial; BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; wt, wild-type.

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SPARC also exhibits pleiotrophic effects on cultured cells. Together with the extracellular proteins tenascin and thrombospondin, it displays anti-adhesive properties that are generally manifested as a promotion of cell rounding and/or an inhibition of cell spreading [Sage and Bornstein, 1991; Chiquet-Ehrismann, 1991]. Consistent with these observations, Murphy-Ullrich and her colleagues have shown that SPARC diminishes the number of focal adhesions in cultured bovine aortic endothelial (BAE) cells [Murphy-Ullrich et al., 1991], and Goldblum et al. [1994] demonstrated changes in the cytoskeleton and barrier function in BAE cells exposed to SPARC. Studies with synthetic peptides representing the four distinct regions of SPARC (termed domains I–IV [Engel et al., 1987]) distinguished two Ca^{2+}-binding sequences that modulated endothelial cell shape [Lane and Sage, 1990]. The N-terminal acidic region also induced plasminogen activator inhibitor-1 and decreased both fibronectin and thrombospondin-1 in cultures of BAE cells that formed cords and tube-like structures in vitro [Lane et al., 1992]. It has been proposed that SPARC regulates the deposition or assembly of extracellular proteins through the induction of proteases or their inhibitors [Hasselaar et al., 1991; Tremble et al., 1993] and thus mediates the interaction of cells with their extracellular matrix [Lane and Sage, 1994].

In previous studies we established that SPARC inhibited the incorporation of [^{3}H]-thymidine by BAE cells and fibroblasts [Funk and Sage, 1991, 1993]. Moreover, we had shown that a synthetic peptide from SPARC domain II, peptide 2.1 (amino acids 54–73 of the secreted murine protein), was also inhibitory to the proliferation of endothelial cells at concentrations of 0.2–0.8 mM [Funk and Sage, 1991]. On fibroblasts, however, peptide 2.1 exhibited a biphasic effect: concentrations of 0.1–0.4 mM were stimulatory, whereas concentrations greater than 0.4 mM were inhibitory [Funk and Sage, 1993]. Peptide 2.1 is part of a highly disulfide-bonded, cationic sequence of SPARC that is similar to a region of the protein follistatin, which binds the growth factors activin and inhibin [Lane and Sage, 1994]. The inhibitory sequence appeared to reside in the first ten amino acids of the peptide (peptide 2.1a), which contains a high proportion of basic residues. The binding of SPARC to collagen is mediated by both peptide 2.1 and peptide 4.2, a C-terminal sequence (amino acids 254–273) from domain IV containing a Ca^{2+}-binding EF-hand (helix-loop-helix motif) [Lane and Sage, 1990]. Since there are no free sulfhydryl groups in SPARC, it has been proposed that a disulfide bond is formed which effectively juxtaposes domains II and IV [Engel et al., 1987]; this placement was confirmed by peptide mapping studies [Sage et al., 1984; Engel et al., 1987; Maurer et al., 1992].

We have recently shown that peptide 4.2 competed for the binding of SPARC to BAE cell monolayers and had noted that peptide 2.1 also competed for the binding of SPARC to these cells [Yost and Sage, 1993]. Therefore, it was possible that the inhibition of cell proliferation mediated by domain II of SPARC resulted from a direct binding of domain IV of the protein to receptors on endothelial cells [Yost and Sage, 1993]. The effect of the region comprising domain IV of SPARC on cell proliferation, however, had not been determined. We therefore tested the effect of SPARC peptide 4.2, as well as peptides with substitutions of amino acids within the 4.2 sequence, on the synthesis of DNA by bovine aortic and adrenal capillary endothelial cells in vitro. The requirement of basic fibroblast growth factor (bFGF) for the proliferation of microvascular endothelial cells allowed us to test the effect of peptide 4.2 on DNA synthesis stimulated directly by this cytokine. Our results indicate that SPARC peptide 4.2 is inhibitory to the proliferation of both aortic and capillary endothelial cells, although the effect was less pronounced in the bFGF-dependent cells. Substitution of amino acids within the partial EF-hand sequence of peptide 4.2 revealed two features of this region that were critical for the inhibitory activity: Asp_{258}, which is a coordinating residue for Ca^{2+}, and $cystine_{255-271}$, which stabilizes the Ca^{2+}-binding loop.

METHODS

Purification of SPARC and Synthesis of SPARC Peptides

Native SPARC was purified from the culture media of murine parietal yolk sac cells by chromatography on diethylaminoethyl-cellulose, as previously described [Sage et al., 1989a]. Peptides representing various regions of SPARC were synthesized and purified by high performance liquid chromatography as described by Lane and Sage [1990] and Lane et al. [1992]. Sequences of peptides from SPARC domain IV that were used in this study are shown in Figure 1. Antibodies specific for SPARC and SPARC

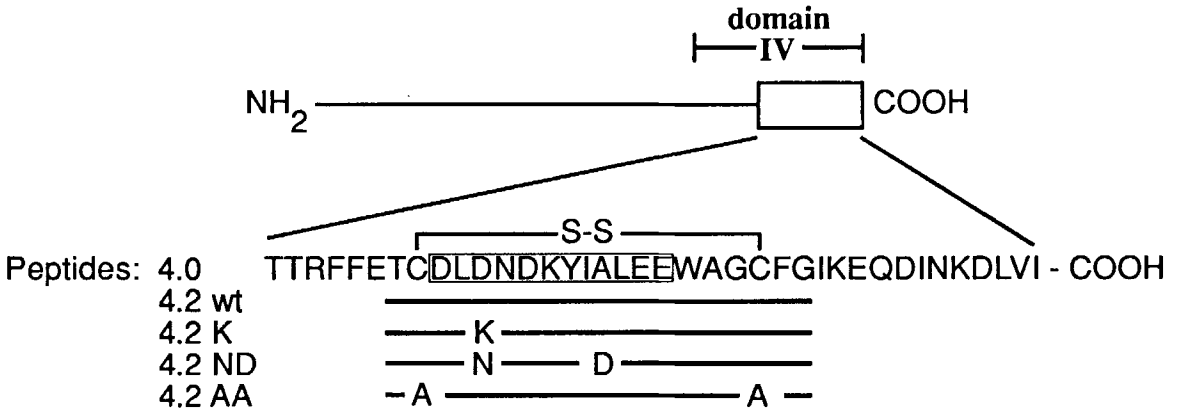

Fig. 1. Schematic representation of murine SPARC and peptide sequences from domain IV. The secreted form of SPARC (285 amino acids) is indicated by the horizontal line; the EF-hand sequence within domain IV is represented by the open box. The peptides used in this study are shown by the thick black lines, positioned under the wild-type sequence of SPARC indicated as peptide 4.0 (amino acids 248–285 of SPARC). The box denotes the Ca^{2+}-binding loop. Peptide 4.2 wt (amino acids 254–273) is a 20-mer derived from peptide 4.0. Synthetic peptides with one or two amino acid substitutions are shown as peptides 4.2 K, 4.2 ND, and 4.2 AA. The substituted residue is indicated relative to peptide 4.2 wt (e.g., peptide 4.2 K is identical to peptide 4.2 wt, with the exception of the replacement of residue 258 (a "D" in 4.2 wt) with a "K").

peptides 1.1, 2.1, and 4.2 were used as ammonium sulfate fractions or as affinity-purified reagents [Lane and Sage, 1990]. Anti-human osteonectin antiserum was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Reagents were tested for endotoxin by a *Limulus* amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA) [Hasselaar et al., 1991].

Typically, SPARC was solubilized in phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO) (PBS) at 0.1–0.4 mg/ml and was used immediately, as losses of activity have been noted upon storage at 4 or −20°C. Stock solutions of peptides (4 mM) were prepared by initial dissolution in 50 mM NaOH (60% of final volume), followed by addition of a 10X phosphate buffer (0.1 M Na_3 PO_4, 0.15 M NaCl, pH 6.5) (10% of final volume), titration of the solution to pH 7.7 with 1 N HCl, and adjustment to the final volume with H_2O. All buffers were sterile, and the concentrations of SPARC and peptide 4.2 (and its substituted derivatives) were determined by absorbance at 280 nm, according to the calculated extinction coefficients ($\epsilon_{280,\,1cm}$ = 0.82 for SPARC and 7.21 for peptides 4.2, 4.2 AA, 4.2 ND, and 4.2 K) [Lane and Sage, 1990].

Cell Culture

Bovine aortic endothelial (BAE) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing antibiotics and 10% fetal calf serum by volume (Hyclone, Logan, UT), as previously described [Funk and Sage, 1991]. Several different strains of cloned cells were used at passages between 6 and 12. Strains of BAE cells that formed cords and tubes in culture and/or exhibited a sprouting phenotype have been described [Iruela-Arispe et al., 1991]. Bovine capillary endothelial (BCE) cells derived from adrenal cortex were maintained on gelatin-coated dishes in DMEM supplemented with antibiotics, 10% calf serum (JRH Biosciences, Lenexa, KS), and 2–5 ng/ml bFGF (a gift from Takeda Inc., Osaka, Japan). A stock solution of bFGF (10–20 μg/ml in 2 M NaCl, 50 mM Tris-HCl, pH 7.4) was stored at −70°C, and fresh aliquots were thawed on a weekly basis for use in cell culture. Heparin (Sigma Chemical Co.) was prepared as a stock solution of 10 mg/ml in sterile H_2O and was used on cells at a final concentration of 33 μg/ml.

Assay for Incorporation of [^{3}H]-Thymidine

Incorporation of [methyl-^{3}H]-thymidine (81.3 Ci/mmol, 1 mCi/ml, New England Nuclear, Boston, MA) was measured in growth-arrested cultures of BAE and BCE cells that were replated at subconfluent density, as previously described [Shing et al., 1984; Shing, 1990; Funk and Sage, 1991]. Contact-inhibited cultures of BAE cells were rendered quiescent (>90% of the cells

were in G_0) by incubation in serum-free DMEM typically for 2–5 days (d). BCE cells were incubated from 1–2 d in DMEM containing 1–2% calf serum in the absence of bFGF; incorporation of thymidine under these conditions was low (<10% of the cpm incorporated by cells stimulated with 2–5 ng/ml bFGF, unpublished experiments). Cells were dissociated by brief exposure to a solution of trypsin/ethylenediaminetetraacetic acid (EDTA) (GIBCO) and were dispensed into 48-well tissue culture plates (Costar, Cambridge, MA) at a density of approximately 10,000 cells/well. Plating (growth) medium for BAE cells was DMEM containing 1 or 2% fetal calf serum, and for BCE cells, DMEM containing 1 or 2% calf serum and 1 or 2 ng/ml bFGF. A total of 67 experiments was performed that included variations in the concentrations of serum and bFGF for both types of endothelial cells. In some instances, cells were plated into wells coated with 1% gelatin in PBS; results were generally similar between cells plated on plastic vs. gelatin, although we noted smaller standard deviations from the mean in BCE cell replicates cultured on gelatin-treated plastic.

Immediately after subculture, SPARC peptides were added to respective replicate cultures at concentrations ranging from 6.6×10^{-6} to 5.2×10^{-1} mM. Controls included cells that received either no addition or volumes of PBS equivalent to those in which the peptides were delivered. For some experiments, SPARC was used at a final concentration of 20 μg/ml (0.6 μM). Cells were incubated with reagents for 18–24 h and were pulsed during the last 1–4 h with [^{3}H]-thymidine at 2.6 or 3.4 μCi/ml (stock solution of [^{3}H]-thymidine was 100 μCi/ml in PBS). Experiments were terminated by removal of the culture medium and fixation of the cell layers, according to Shing [1990]. Briefly, cells were washed once with PBS or DMEM, incubated 2 × 5 min in cold absolute methanol, treated with cold 5% trichloroacetic acid (2 × 20 min), washed once with deionized water, and solubilized in 250 μl 0.3 M NaOH. [^{3}H]-Thymidine cpm incorporated into cellular DNA were quantified by liquid scintillation counting in 2 ml Ecolume (ICN, Irvine, CA). Data were expressed as means ± S.D. of determinations performed in triplicate or sextuplicate.

Measurements of Intrinsic Fluorescence

SPARC peptides were dissolved into 0.01 M Tris-HCl/0.15 M NaCl/0.05 M $CaCl_2$ (pH 9.0), at a concentration of 0.8–1.4 mM. Samples were dialyzed extensively against Chelex (Bio-Rad) treated with 0.01 M Tris-HCl/0.15 M NaCl at pH 9.0 to remove unbound Ca^{2+}. Peptide solutions were diluted to 8–14 μM in the same buffer that contained varying concentrations of EDTA.

Fluorescence measurements were performed with a Perkin-Elmer LS-50B luminescence spectrometer that was controlled by FLDM software operating on a DEC 386SX computer. The emission spectra (305–500 nm, slit width = 4.5 nm) of peptide or blank solutions were collected at 800 nm/min after excitation at 290 nm (slit width = 4.5 nm). All measurements were performed at ambient temperature. The spectra of solutions that contained no peptide were subtracted from the spectra of those containing peptide. The peak height at 354 nm of each spectrum was determined and was used to calculate the percent change in fluorescence intensity (FI) from a control sample of peptide that contained no EDTA, by the following formula:

$$[(FI_{sample+EDTA}) - (FI_{sample-EDTA})/(FI_{sample-EDTA})] \times 100.$$

To ascertain which aromatic amino acids were contributing to the observed emission spectra, we determined the fluorescence emission profiles of Trp, Tyr, and Phe at the same conditions used for the synthetic peptides of SPARC. Trp was identified as the residue that emitted fluorescencee light typical of the spectra that we observed for the peptides.

Binding of [^{125}I]-SPARC Peptides to Bovine Aortic Endothelial Cells

SPARC peptide 4.2 was iodinated and added to confluent BAE cells as previously described [Yost and Sage, 1993]. Competition for binding was performed with unlabeled peptides (4.2, 4.2 ND, 4.2 AA, and 4.2 K), and data were analyzed as percent of [^{125}I]-peptide cpm bound in the absence of competitor [Yost and Sage, 1993].

RESULTS

In Figure 1 are shown the peptides that were used in this study. Peptide 4.0 is a 38-mer that contains additional amino acid residues flanking both the N- and C-terminal residues of peptide 4.2. Within the EF-hand loop sequence, D_{258} was changed to a K (peptide 4.2 K); for production of peptide 4.2 ND, D_{258} was changed to an N, and A_{264} in this sequence was changed to a D (Fig. 1).

In peptide 4.2 AA, the disulfide bond was removed by the substitution of the two Cys residues with Ala residues at positions 255 and 271 (Fig. 1). The peptides were purified by high performance liquid chromatography, and we observed no cytotoxic effects coincident with their use on cells.

A consistent property of SPARC has been its ability to effect changes in the shape of cultured endothelial cells and fibroblasts [Sage et al., 1989b]. In contrast, changes in BAE and BCE cell shape were not seen with peptides 4.2, 4.2 AA, 4.2 K, or 4.2 ND, at concentrations from 0.013–0.4 mM (data not shown). Since biphasic effects on cell proliferation had been reported for SPARC and SPARC peptide 2.1 [Funk and Sage, 1993], it was important to examine the peptides over a wide range of concentrations. Peptide 3.2 was used routinely as a control for the preparation of the synthetic peptides.

Initially, we compared the effect of SPARC and peptides from domains II, III, and IV of SPARC on the incorporation of [^{3}H]-thymidine by BAE and BCE cells. Since these capillary endothelial cells require bFGF for growth [Folkman and Shing, 1992], we were obliged to treat both types of cells with peptides in the presence of 1% fetal calf serum and 5 ng/ml bFGF. The concentration of peptides used in Figure 2 (0.39 mM) represents a mid-range value that has been effective for several SPARC peptides that we have previously studied (0.1–0.8 mM) [Lane and Sage, 1990; Hasselaar and Sage, 1992; Lane et al., 1992]; SPARC was used at its predetermined ED_{50} of 0.6 μM [Funk and Sage, 1991]. As shown in Figure 2, both SPARC and peptide 4.2 significantly diminished the incorporation of [^{3}H]-thymidine by aortic and capillary endothelial cells (to approximately 8 and 45%, respectively, of control values). In contrast, peptide 2.1 b, representing the inactive C-terminal half of peptide 2.1 [Funk and Sage, 1993], appeared to be slightly stimulatory (15–20% over control), and peptide 3.2 inhibited incorporation by approximately 10–20% in both cell types (Fig. 2). Therefore, in the presence of bFGF, peptide 4.2 mimicked the activity of SPARC, since it inhibited the incorporation of [^{3}H]-thymidine by aortic and capillary endothelial cells. However, the effect was less pronounced on capillary endothelium. The diminution in DNA synthesis that was seen with peptide 4.2 was reversed by ~90% by the addition of anti-peptide 4.2 IgG to the culture medium. The activity of peptide 4.2 was not affected by anti-osteonectin monoclonal antibody, which is specific for a domain I sequence, or by other anti-peptide antibodies (not shown).

We had reported that SPARC and two Ca^{2+}-binding peptides of SPARC inhibited the incorporation of thymidine in BAE cells stimulated by bFGF [Hasselaar and Sage, 1992]. To confirm this observation, we cultured cells in the

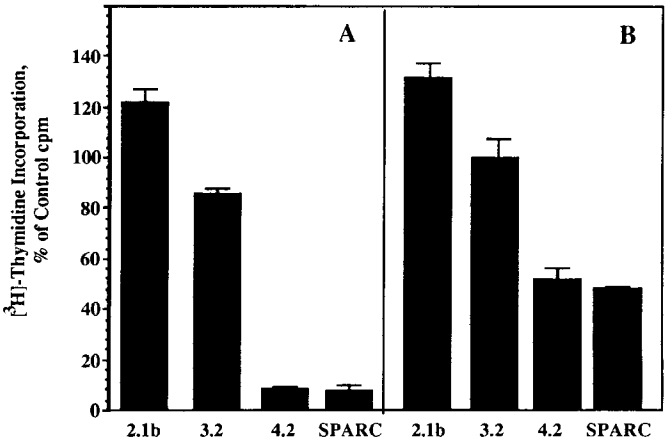

Fig. 2. Comparative effects of SPARC and SPARC peptides on the incorporation of [^{3}H]-thymidine by bovine aortic and capillary endothelial cells. Aortic (**A**) and capillary cells (**B**) were rendered quiescent by culture for 5 days in DMEM and in DMEM containing 1% calf serum, respectively. Both cultures were dissociated in trypsin/EDTA and were replated at equal densities in DMEM containing 1% serum and 5 ng/ml bFGF. Respective cultures were subsequently incubated with SPARC (0.6 μM) or SPARC peptides 2.1 b, 3.2, or 4.2 (0.39 mM, final concentrations) for an interval of 19 h that included a 2 h pulse with [^{3}H]-thymidine at 3.4 μCi/ml. Bars represent the means ± S.D. derived from experiments performed in triplicate; values were normalized to cpm incorporated by control cultures that were treated with PBS alone (A: 21,902 ± 384; B: 44,816 ± 300 cpm).

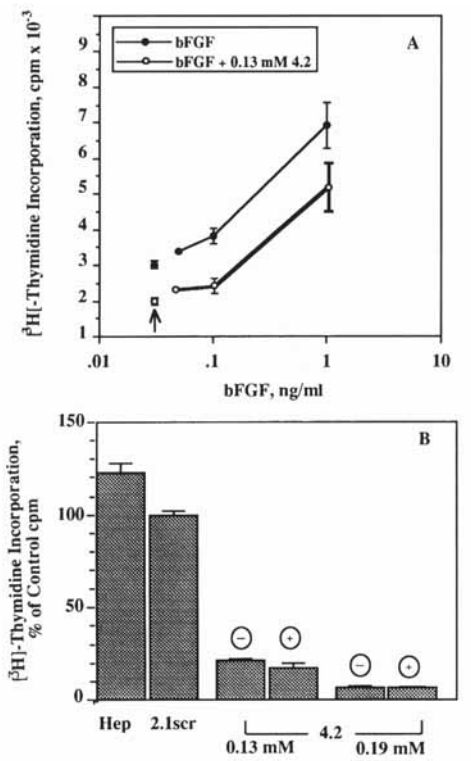

Fig. 3. Effect of bFGF and heparin on the incorporation of [^{3}H]-thymidine by bovine aortic endothelial cells exposed to SPARC peptide 4.2. **A:** Peptide 4.2 diminishes the incorporation of thymidine by cells cultured in the presence of bFGF. Cells growth-arrested for 5 days in the absence of fetal calf serum were dissociated in trypsin/EDTA and were subsequently plated in DMEM containing 1% serum and different amounts of bFGF (0.05–1.0 ng/ml) at one-third of the original density. Peptide 4.2 was added at a final concentration of 0.13 mM, and cells were incubated for a total of 20 h that included a 40 min pulse with 2.6 μCi/ml [^{3}H]-thymidine. Solid circles represent thymidine incorporation by cells cultured in the absence of peptide 4.2, and open circles designate incorporation by cells cultured in the same amounts of bFGF but with added peptide 4.2. Arrow indicates incorporation in the absence of bFGF. Total cpm ± S.D. derived from triplicate samples are shown. **B:** Heparin in concert with bFGF does not affect the response of cells to peptide 4.2. Growth-arrested cells were released with trypsin/EDTA and were replated at subconfluent density in DMEM supplemented with 1% fetal calf serum and 5 ng/ml bFGF, ± 33 μg/ml heparin. Immediately thereafter, SPARC peptide 2.1scr (0.33 mM, final concentration) or peptide 4.2 (0.13 and 0.196 mM, final concentrations) was added to the cultures. Data shown were combined from 2 separate experiments performed in triplicate; bars represent means ± S.D. normalized to the respective cpm incorporated into control cellular DNA: Bars labeled Hep (heparin), 2.1scr, and 4.2− (without heparin) represent values expressed as percent of control cpm derived from cells that received only PBS (54,352 ± 1,863); bars labeled 4.2+ (peptide in the presence of heparin) represent values expressed as percent of control cpm derived from cells treated with heparin alone. Incubations ranged from 17.5–22.5 h that included a pulse with 3.4 μCi/ml [^{3}H]-thymidine for 1–2 h.

TABLE I. Effect of SPARC Peptide 4.2 on the Incorporation of [^{3}H]-Thymidine by Bovine Capillary Endothelial Cells Cultured in Different Concentrations of bFGF[a]

	bFGF (ng/ml)			
	0.2	0.5	1.0	2.0
0.066	88.8 ± 3.8	84.1 ± 6.7	86.9 ± 6.3	90.4 ± 7.0
0.13	78.4 ± 5.4	77.9 ± 4.7	77.1 ± 3.3	84.6 ± 8.2
0.26	73.5 ± 1.9	79.0 ± 1.7	71.9 ± 1.9	83.4 ± 8.7

[a]Equal aliquots of cells were plated in DMEM containing 2% calf serum and different concentrations of bFGF. Immediately thereafter, SPARC peptide 4.2 (0.066, 0.13, and 0.26 mM, final concentrations) was added to the respective wells, and incubation proceeded for a total of 22 h that included a 1.5 h pulse with [^{3}H]-thymidine at 3.4 μCi/ml. Data are expressed as percent of cpm in control wells receiving no peptide for each concentration of bFGF. Control cpm were arbitrarily defined as 100%. Values represent means ± S.D. from experimental determinations performed in triplicate. Absolute cpm ± S.D. in the absence of peptide 4.2 were 28,783 ± 2,831 at a concentration of 0.2 ng/ml bFGF.

presence of different amounts of bFGF, with or without heparin, a glycosaminoglycan known to modulate the response of cells to this cytokine. As shown in Figure 3A, peptide 4.2, at a concentration of 0.13 mM, diminished the incorporation of thymidine by BAE cells by approximately 30%, regardless of the concentration of bFGF that was present in these cultures. This result was not altered by heparin (33 μg/ml) (Fig. 3B). Heparin itself, or a scrambled version of SPARC peptide 2.1 (2.1 scr), had a slightly stimulatory effect or no effect on thymidine incorporation, respectively (Fig. 3B). Although we were unable to perform this experiment on BCE cells (the cells failed to proliferate in the absence of bFGF), we did culture these cells in different concentrations of bFGF and peptide 4.2. As shown in Table I, at any given concentration of peptide 4.2, the level of inhibition was nearly equivalent in cells grown in 0.2–2.0 ng/ml bFGF. Heparin also did not modulate the effect of SPARC on these cells in the presence of bFGF (data not shown).

A dose-response effect on the inhibition of [^{3}H]-thymidine incorporation by BAE cells cultured in the presence of peptide 4.2 is shown in Figure 4. At concentrations of 0.019–0.26 mM peptide 4.2, thymidine incorporation, expressed as percent of control cpm, decreased from 107 to 10% (Fig. 4). In the absence of fetal calf serum, the cells were more sensitive to peptide 4.2 (Fig.

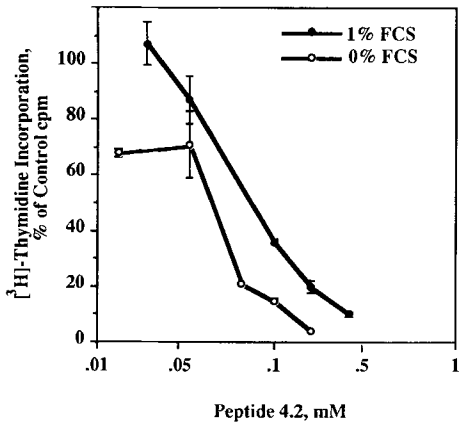

Fig. 4. Incorporation of [^{3}H]-thymidine by bovine aortic endothelial cells is diminished by SPARC peptide 4.2 in a dose-dependent manner. Confluent, growth-arrested cultures were dissociated in trypsin/EDTA and were subsequently replated in DMEM alone (open circles) or in DMEM containing 1% fetal calf serum (solid circles). Cells were incubated for 19.5 h (no serum) or 17.25 h (1% serum) that included a 2 or 1 h pulse with 3.4 μCi/ml [^{3}H]-thymidine, respectively. Incorporated cpm are expressed as percent of cpm derived from control cultures that received either PBS alone (1% serum, 4,183 ± 337 cpm) or no additions (no serum, 2,717 ± 293 cpm). Points represent means ± S.D. from triplicate determinations.

TABLE II. Effect of SPARC Peptides 4.2 and 4.2 AA on the Incorporation of [^{3}H]-Thymidine by Bovine Capillary Endothelial Cells[a]

Peptide	Concentration (mM)	% of control cpm[2]
4.2	6.6×10^{-6}	104 ± 4.1
	1.3×10^{-4}	101 ± 4.8
	6.6×10^{-4}	101 ± 1.5
	1.3×10^{-3}	98.0 ± 3.2
	6.6×10^{-3}	96.2 ± 4.0
	1.3×10^{-2}	82.3 ± 5.0
	3.3×10^{-2}	72.0 ± 2.5
	6.6×10^{-2}	70.6 ± 0.4
	1.3×10^{-1}	74.7 ± 5.6
	2.6×10^{-1}	64.3 ± 0
4.2 AA	1.3×10^{-2}	105 ± 9.8
	1.3×10^{-1}	102 ± 1.3
	1.9×10^{-1}	91.9 ± 4.1

[a]Equal aliquots of quiescent cells were plated into gelatin-coated plastic wells containing 2% calf serum and 2 ng/ml bFGF. Immediately thereafter, different concentrations of peptide 4.2 were added to respective wells. Cells were incubated with peptides for a total of 24 h that included a 3.5 h pulse with [^{3}H]-thymidine (3.4 μCi/ml). Each concentration was delivered in an aliquot of PBS; cpm were expressed as percent of control cpm obtained with addition of PBS alone and represent the mean of triplicate or sextuplicate determinations ± S.D. from 2 separate experiments.

4). A dose-responsive effect of peptide 4.2 was also seen in BCE cells (Table II).

We next compared the effects of the wt SPARC peptide, 4.0 (a 38-mer containing the peptide 4.2 sequence), with the mutant peptides 4.2 AA and 4.2 ND. A dose-response curve for BAE cells is shown in Figure 5A. Peptide 4.2 AA was less effective than peptides 4.0 and 4.2 ND in the inhibition of thymidine incorporation by these cells. Wild-type peptides 4.0 and 4.2 produced equivalent levels of inhibition in aortic as well as in capillary endothelial cells (not shown). The combined effect of peptides from domains II and IV of SPARC on the incorporation of thymidine by BAE cells is shown in Figure 5B. An inhibition of ~25% occurred in the presence of 0.26 mM peptide 2.1, as previously reported [Funk and Sage, 1991], whereas peptide 4.2 at 0.13 mM diminished incorporation by ~70%. Peptide 4.2, in combination with peptide 2.1scr or 2.1, reduced the levels of [^{3}H]-cpm that were incorporated by BAE cells incubated with 2.1scr or 2.1 alone (Fig. 5B). Therefore, the inhibitory effect of peptide 2.1 was further enhanced by peptide 4.2. Higher concentrations of both peptides resulted in an increased inhibition of incorporated [^{3}H]-thymidine (not shown).

The effects of peptides from domains II and IV of SPARC on the incorporation of thymidine by BCE cells are shown in Table II and Figure 6. In comparison with peptide 4.2, peptide 4.2 AA exhibited minimal activity in [^{3}H]-thymidine incorporation assays (Table II). In contrast, at a concentration of 0.26 mM, peptide 2.1 and 4.2 ND inhibited the incorporation of thymidine by approximately 45%, relative to control. Peptide 4.2 had a lesser effect (approximately 75% of control), and peptide 4.2 K was nearly 90% of control. Peptide 2.1scr, used at twice the concentration (0.52 mM), was nearly equivalent to the control cpm. An interesting effect was observed when equimolar concentrations of peptide 2.1 and either 4.2 or mutant 4.2 peptides were used together. In combination with peptide 2.1, the inhibitory effect of peptide 4.2 was >90% (Fig. 6). An enhancement of inhibition was also observed with peptide 4.2 ND in combination with peptide 2.1; in contrast, an augmentation of thymidine incorporation was seen with peptide 4.2 K in the presence of peptide 2.1 (Fig. 6). These data indicate that wt domains II and IV acted synergistically to inhibit the synthesis of DNA by BCE cells grown in the presence of

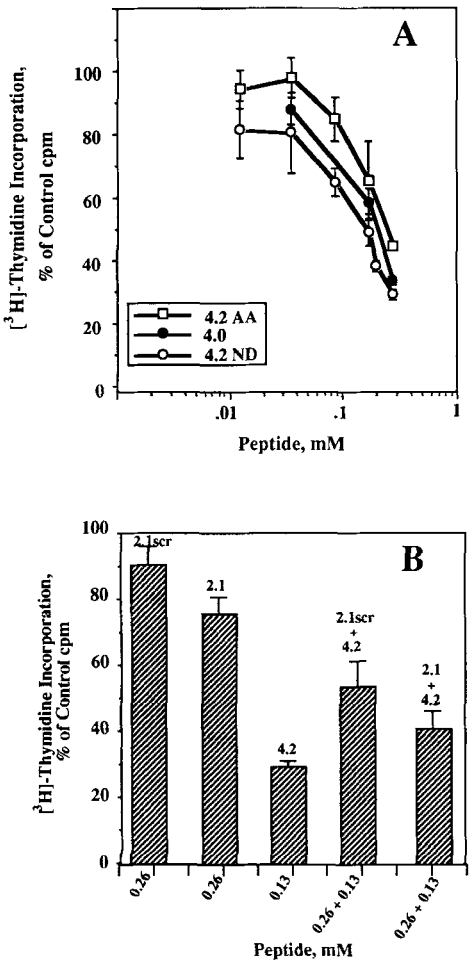

Fig. 5. Inhibition of thymidine incorporation by bovine aortic endothelial cells cultured in the presence of SPARC peptides from domains II and IV. Cells were maintained in a quiescent state without serum for 7 d. After dissociation in trypsin/EDTA, cells were plated in DMEM containing 1% fetal calf serum (**A**) or 2% fetal calf serum (**B**) and were exposed to peptides at the concentrations shown for a 24 h period that included a 1 h pulse with 2.6 μCi/ml [^{3}H]-thymidine (A) or for 20 h including a 3 h pulse with 3.4 μCi/ml [^{3}H]-thymidine (B). Incorporation of thymidine (cpm) in the presence of peptides was assessed relative to control cultures that had received equal volumes (20–40 μl) of PBS alone. These control values ranged from 8,160 ± 370 to 7,500 ± 688 cpm (A) and from 12,553 ± 1,102 to 9,977 ± 286 cpm (B). Points represent mean ± S.D. from triplicate determinations.

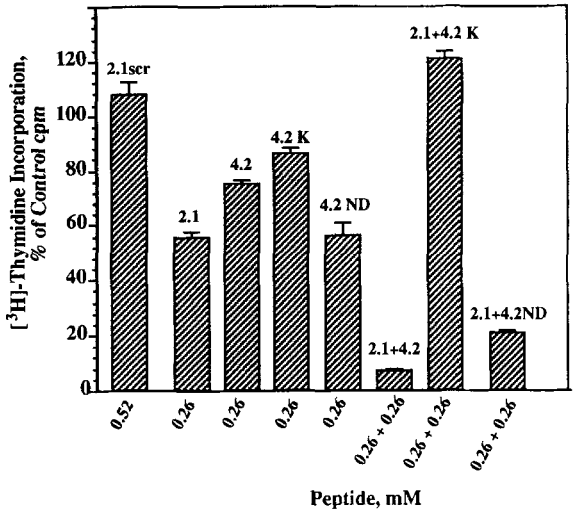

Fig. 6. Effects of peptides from domains II and IV of SPARC on the incorporation of thymidine by capillary endothelial cells. Equal numbers of quiescent BCE cells were plated at subconfluent density in DMEM containing 1% calf serum and 2 ng/ml bFGF. Immediately thereafter, peptides were added to cells at the concentrations shown, and incubation was maintained for 22.5 h, inclusive of 1.5 h pulse of [^{3}H]-thymidine at 3.4 μCi/ml. Bars represent means of incorporated cpm ± S.D. from triplicate determinations; values have been normalized to cpm incorporated by control cells that received equal volumes of PBS alone. [^{3}H]-cpm corresponding to 20, 40, and 60 μl PBS were 67,790 ± 4,813, 60,794 ± 5,530, and 59,895 ± 839, respectively.

bFGF. However, BAE cells, which bind native SPARC through domain IV [Yost and Sage, 1993], appear to respond directly to peptide 4.2, and the inhibitory effect on DNA synthesis was not enhanced by peptide 2.1 (Fig. 5B).

We had noted that, on both aortic and capillary endothelial cells, peptide 4.2 K had minimal activity compared to that of peptide 4.2; in fact, of all the domain IV mutant peptides that we had synthesized, peptide 4.2 K was the least active in the inhibition assays. As a control for the amount of total peptide added to the cultures, we titrated the inhibitory effect of peptide 4.2 on thymidine incorporation by BAE cells with increasing additions of peptide 4.2 K, as shown in Figure 7. An equimolar mixture of peptides 4.2 and 4.2 K (0.13 mM each) produced an inhibition of thymidine incorporation of 70%, relative to control. As the concentration of peptide 4.2 increased, and that of peptide 4.2 K correspondingly decreased, the incorporation of [^{3}H]-thymidine was diminished, as previously shown. Similar results were obtained in other experiments with different relative concentrations of peptides 4.2 and 4.2 K (Table III). These experiments also served as controls for the different molarities of peptide added to cells, e.g., in dose-response experiments.

At this point, our finding with respect to the SPARC peptide 4.2 sequence can be summarized as follows: (1) peptide 4.2 inhibited the incorporation of [^{3}H]-thymidine by both BAE and BCE cells, (2) peptide 4.2 ND behaved similarly to the wt peptide (4.2) in this assay, (3)

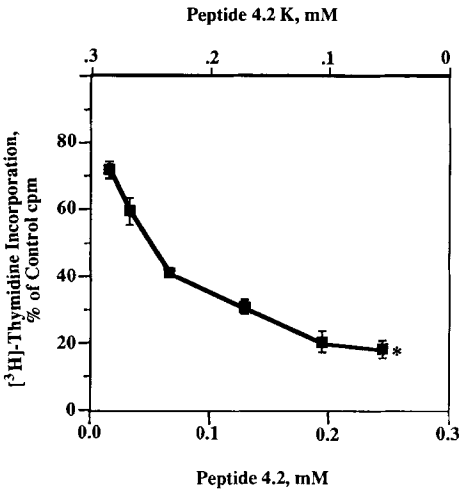

Fig. 7. Comparative effect of SPARC peptides 4.2 and 4.2 K on the incorporation of thymidine by bovine aortic endothelial cells. Cells were growth-arrested for 2 days in DMEM and were subsequently replated in DMEM containing 1% fetal calf serum. Immediately thereafter, peptides 4.2 and 4.2 K were added simultaneously to cultures at the concentrations shown, for a period of 26 h that included a pulse of 2.5 h with 2.6 μCi/ml [^{3}H]-thymidine. Points represent means ± S.D. of triplicate determinations, and data are expressed as percent of cpm incorporated by control cultures in the presence of 0.0006 mM peptide 4.2 (131,176 ± 10,274 cpm). The peptide mixtures were delivered in an equal volume of 20 μl and represent a total of 0.26 mM. Points denote decreasing concentrations of peptide 4.2 K in combination with increasing concentrations of peptide 4.2. An equimolar mixture of 4.2 and 4.2 K (0.13 mM each) produced an inhibition of 70%. Asterisk denotes percent inhibition achieved with peptide 4.2 alone at a concentration of 0.26 mM.

peptide 4.2 AA was less inhibitory than peptides 4.2 wt and 4.2 ND, and (4) peptide 4.2 K displayed substantially less inhibitory activity relative to peptide 4.2 wt, especially at concentrations ≤ 0.2 mM. We therefore questioned whether these peptides exhibited different activities in competitive binding assays on BAE cells. As shown in Figure 8, peptides 4.2 wt and 4.2 ND competed effectively for the binding of [^{125}I]-SPARC peptide 4.2 to these cells, whereas peptides 4.2 AA and 4.2 K did not compete at concentrations that produced >50% displacement by 4.2 wt and 4.2 ND. At the highest concentrations used (60 μmol, approximately a 2,000-fold molar excess over [^{125}I]-peptide 4.2), peptides 4.2 AA and 4.2 K displaced 25 and 50%, respectively, of bound [^{125}I]-cpm; in contrast, peptides 4.2 wt and 4.2 ND displaced >90% of bound [^{125}I]-cpm (Fig. 8).

SPARC contains an EF-hand sequence in domain IV and binds, in this region, one Ca^{2+} with a K_d of 0.2–0.3 × 10^{-6} M [Engel et al., 1987].

TABLE III. Effect of SPARC Peptide 4.2 on the Incorporation of [^{3}H]-Thymidine by Bovine Aortic Endothelial Cells: Titration With Mutant Peptide 4.2 K[a]

Molar ratio (4.2:4.2 K)	Total concentration (mM) 4.2	4.2 K	[^{3}H]cpm (% of control)[b]
—	0.13		35.5 ± 2.4
—	0.196		15.3 ± 1.6
—	0.26		7.5 ± 1.5
—		0.196	72.0 ± 3.0
—		0.26	64.2 ± 2.9
1:3	0.033 +	0.099	67.7 ± 3.7
1:1	0.066 +	0.066	50.9 ± 2.3
3:1	0.099 +	0.033	41.8 ± 2.8
1:1	0.13 +	0.13	25.1 ± 1.2

[a]Cells were incubated for 3 days in DMEM without serum prior to the inception of the experiment. Subsequently, quiescent cells were released with trypsin/EDTA and were replated in DMEM containing 1% fetal calf serum. Peptides 4.2 and 4.2 K were added immediately thereafter to respective wells at the concentrations shown. Incubation with peptides continued for a total of 26 h that included a 4 h pulse with 2.6 μCi/ml [^{3}H]-thymidine.

[b][^{3}H]-thymidine cpm relative to untreated (control) cells, the incorporation of which was defined as 100%, shown as means of triplicate determinations ± S.D. Absolute number of cpm obtained without addition of PBS or peptides was 49,723 ± 466.3.

Since the wt peptides 4.0 and 4.2 are predicted to interact with Ca^{2+} [Bairoch, 1992] (unpublished experiments), we measured by intrinsic fluorescence spectroscopy the interaction of both wt and mutant SPARC peptides with this cation. Figure 9 shows the intrinsic fluorescence emission spectra of peptide 4.2. The intensity of the emission at 354 nm displayed a concentration-dependent increase, in which peak height is proportional to the mass of the peptide in solution (see inset). Similar dose-response spectra were obtained with peptides 4.0, 4.2 ND, 4.2 AA, and 4.2 K (not shown). From emission spectra of the aromatic amino acids taken after excitation at 290 nm (where primarily Trp absorbs), we concluded that the contribution of Tyr and Phe was negligible, since their emission maxima occur at 310 and 328 nm, respectively, and that of Trp is typically seen at 354 nm (data not shown).

We next tested each of the domain IV peptides with respect to their interaction with Ca^{2+} by measurement of the intrinsic fluorescence of the Trp residue that is placed identically in all the peptides (Fig. 1). Figure 10 shows the changes in the fluorescence intensity of the five peptides

(previously saturated with Ca^{2+}) that occurred upon the addition of EDTA. A dose-dependent increase in peak height at 354 nm (represented in Fig. 10 as a percent change in intensity) was observed for all peptides except 4.2 K. This result was interesting, as only the sequence of peptide 4.2 K failed to fit the consensus loop sequence of the helix-loop-helix EF-hand motif [Bairoch, 1992]. Approximately 100–300 μM EDTA was required to produce 50% of the maximal effect of the shift in λ max that was induced by EDTA in all peptides except 4.2 K.

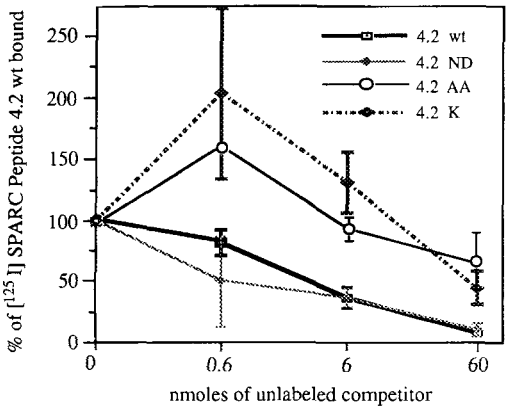

Fig. 8. Binding of [^{125}I]-SPARC peptide 4.2 to bovine aortic endothelial cells: Competition by mutant 4.2 peptides. Confluent BAE cells were incubated at 4°C with [^{125}I]-SPARC peptide 4.2. To separate wells were added molar excesses of unlabeled competitor peptides: 4.2, 4.2 ND, 4.2 AA, or 4.2 K. Cells were solubilized in 1N NaOH, and radioactivity was measured in a γ counter. Data represent means ± S.E. of 2 experiments performed in triplicate and are expressed as the percent of [^{125}I]-*peptide 4.2 wt bound in* the absence of competitor.

Peptide 4.2 AA has the same amino acid sequence as peptide 4.2, except that the two Cys, which are outside the Ca^{2+}-binding loop, were changed to Ala. As shown in Figure 10, both 4.2 and 4.2 AA produced biphasic curves, indicative of a similar response to the removal of Ca^{2+} by EDTA. The near-absence of an inflection in the curve corresponding to peptide 4.2 AA indicated that the optimal structure for interaction with Ca^{2+} is likely to require a disulfide bridge between Cys_{255} and Cys_{271}. It therefore appears that the Asp at position 258 and the conformation of peptide 4.2 are both critical to the effect of this sequence on DNA synthesis by endothelial cells.

DISCUSSION

Several conclusions can be drawn from the experiments described in this communication: (1) SPARC domain IV contains a Ca^{2+}-binding EF-hand sequence that inhibits the incorporation of [^{3}H]-thymidine by macro- and microvas-

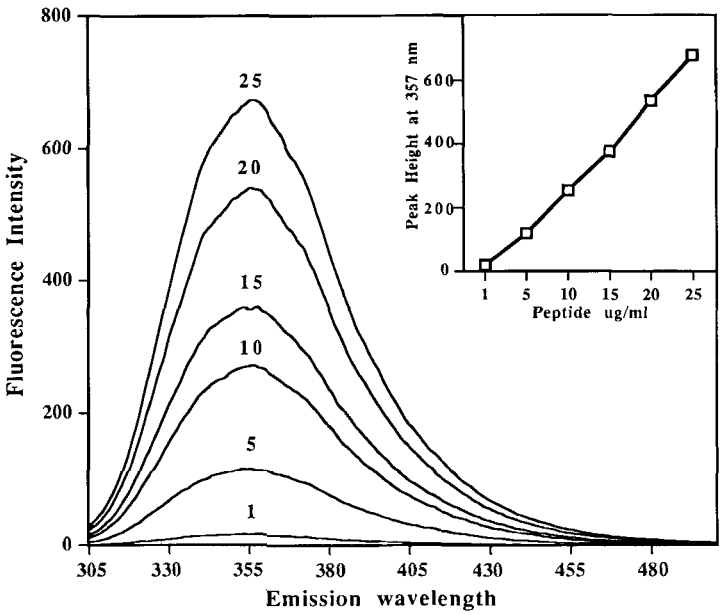

Fig. 9. Fluorescence intensity of SPARC peptide 4.2. SPARC peptide 4.2 was dissolved in 0.01 M Tris-HCl/0.15 M NaCl/0.05 M $CaCl_2$ (pH 9.0). After removal of free Ca^{2+}, the intrinsic fluorescence intensity of solutions containing 1–25 μg/ml peptide 4.2 was measured in a luminescence spectrometer at an excition wavelength of 290 nm and an emission wavelength range of 305–500 nm. The inset displays peak height at 357 nm as a function of peptide concentration.

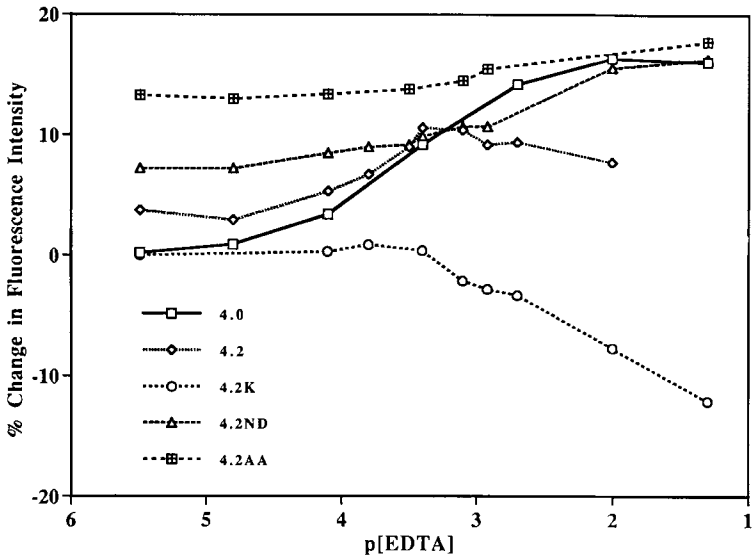

Fig. 10. Removal of bound Ca^{2+} alters the intrinsic fluorescence of SPARC peptides from the EF-hand region. SPARC peptides were dissolved in 0.01 M Tris-HCl/0.15 M NaCl/0.05 mM $CaCl_2$ (pH 9.0), at a concentration of 0.8–1.4 mM. Samples were dialyzed extensively against Chelex-treated 0.01 M Tris-HCl/0.15 M NaCl (pH 9.0) and were diluted to 20–30 μM in the same buffer that contained varying concentrations of EDTA. Plots show the percent change in intrinsic fluorescence at 354 nm as a function of pEDTA.

cular endothelial cells; (2) peptides comprising part of the helix-loop-helix motif (4.0, a 38-mer, and 4.2, a 20-mer) mimic the inhibitory activity of SPARC and exhibit a more pronounced effect than that previously described for another inhibitory sequence, peptide 2.1 [Funk and Sage, 1991]; (3) peptides 2.1 and 4.2 appear to act cooperatively in the inhibition of thymidine incorporation; (4) a decrease in thymidine incorporation of 50% below control levels was achieved with approximately 0.08 mM peptide 4.2 in BAE cells and 0.39 mM peptide 4.2 in BCE cells; (5) the inhibitory effect was not altered by the presence of heparin and/or bFGF in cultures of BAE cells; (6) an amino acid substitution of Asp_{258} with a Lys at this position significantly decreased the activity of the resulting peptide, 4.2 K, in proliferation assays on both cell types; (7) a 200-fold molar excess of peptide 4.2 K displayed no competition for the binding of [^{125}I]-peptide 4.2 wt to BAE cells, a concentration at which 50% of bound cpm were displaced by 4.2 wt or 4.2 ND (containing neutral mutations); (8) substitution of Cys_{255} and Cys_{271}, which form a disulfide bond at the base of the Ca^{2+}-binding loop, with Ala at each position produced a peptide, 4.2 AA, that in proliferation assays exhibited reduced (BAE cells) or minimal inhibitory activity (BCE cells) at concentrations < 0.2 mM; (9) peptide 4.2 AA, at a 200-fold molar excess over [^{125}I]-peptide 4.2 wt, was as ineffective as peptide 4.2 K in competition binding assays; (10) both peptides 4.2 K and 4.2 AA exhibited anomalous fluorescence emission spectra in the presence of EDTA, in comparison with the changes in fluorescence intensity observed with peptides 4.0, 4.2, and 4.2 ND. It therefore appears that Ca^{2+}-binding, resulting in part from Asp_{258} and an intact disulfide loop, are necessary for the inhibitory activity of the EF-hand region on endothelial cell proliferation.

It has long been appreciated that Ca^{2+} is necessary for maintenance of the α-helical conformation of SPARC and at least some of its biological properties. Engel et al. [1987] proposed that cooperative binding of Ca^{2+} at the 6–8 low-affinity sites residing at the N-terminus was critical for α-helix formation in this region of the protein, possibly by neutralization of the high density of negative charge imparted by the Glu residues. Other investigators have shown that Ca^{2+} is necessary for the binding of SPARC (osteonectin, BM-40) to several types of collagen and other extracellular proteins [Sage et al., 1989b; Mayer et al., 1991; reviewed in Lane and Sage, 1994]. The interaction of SPARC with collagen types I, III, and IV occurs through domain IV [Lane and Sage, 1990; Mayer et al.,

1991], and was shown to be dependent on a triple-helical conformation, bound Ca^{2+}, and the presence of an intact disulfide bond [Mayer et al., 1991]. An extensive study of the low-affinity ($K_d \geq 10$ mM) and high-affinity Ca^{2+}-binding site ($K_d = 0.6$ μM) on SPARC (osteonectin) was performed by Maurer et al. [1992], who demonstrated an increase in α-helicity of 33%, and a change in intrinsic fluorescence of approximately 60%, upon the reversible binding of Ca^{2+} to the protein. Moreover, a significant increase in proteolytic susceptibility was noted near the EF-hand region. A major implication of the study by Maurer et al. [1992] is that the removal of Ca^{2+} from SPARC produces a more asymmetric molecular shape. In the context of our work, we predict that a SPARC containing mutations in domain IV which eliminate the binding of Ca^{2+} would fail to interact with cognate receptors on endothelial cells and would thus exhibit a null effect on cell proliferation. Our data with synthetic wt and mutant peptides from domain IV support this proposal, but it will be necessary to test the native, intact protein with the engineered substitutions for confirmation of the model. These studies are in progress with recombinant and mutated SPARC expressed in *E. coli* [Bassuk et al., unpublished experiments].

Several independent studies have evaluated Ca^{2+}-binding activity in mutated EF-hands in, for example, troponin C and calmodulins from different species [Dotson and Putkey, 1993; Maune et al., 1992; Starovasnik et al., 1993]. A conserved bidentate Glu at position 12 in loop IV of *Drosophila melanogaster* calmodulin was shown to be critical for the stabilization of the F-helix within the EF-hand structure [Maune et al., 1992]. Moreover, fragments of calmodulin [Starovasnik et al., 1993] and troponin C [Marsden et al., 1990] have been useful for the determination of binding affinities corresponding to different regions of the proteins, as well as their cooperative interactions. Measurement of differences in free energy showed that approximately 60% of the Ca^{2+}-binding affinity of intact troponin C resided in a 34-residue helix-loop-helix peptide, whereas the remainder was accounted for by cooperativity with a second Ca^{2+}-binding site [Marsden et al., 1990].

We chose to examine the interaction of SPARC peptides from domain IV with Ca^{2+} by fluorescence spectroscopy, as previous experiments with these peptides (e.g., binding of ^{45}Ca by an enzyme-linked immunosorbent assay, dot-blot assay, or equilibrium chromatography) had been largely unsuccessful, due to low sensitivity and/or irreproducibility of the method. Since this region contains a functional EF-hand defined by a helix-loop-helix sequence motif [Engel et al., 1987], the peptides were predicted to interact with Ca^{2+}, although they lacked complete α-helical stretches. Comparison of the sequence of each peptide with the revised consensus formula for EF-hands [Bairoch, 1992] showed that only peptide 4.2 K failed to fit the formula. Since peptide 4.2 K displayed a unique emission spectrum that decreased in intensity when EDTA was added to the peptide solution, we concluded that the interaction of Ca^{2+} with 4.2 K was clearly different from that between Ca^{2+} and peptides 4.0, 4.2, 4.2 ND, and 4.2 AA. The likely basis for this difference is the inability of peptide 4.2 K to form a Ca^{2+}-binding loop structure that resembles the EF-hand motif present in many Ca^{2+}-binding proteins. It was also interesting that peptide 4.2 AA, containing substitutions that eliminate formation of cystine, exhibited compromised activities in thymidine incorporation and cell-binding assays. We interpret these data to indicate the requirement of an intact disulfide bond for stabilization of the Ca^{2+}-binding loop in SPARC domain IV. In their studies on SPARC from *C. elegans*, Schwarzbauer and Spencer [1993] showed that a fusion protein containing the EF-hand motif bound 5-fold less Ca^{2+} after reduction of the disulfide bond. Supportive results were observed with mutant forms of troponin C, in which an engineered disulfide bond with a single domain was shown to alter the Ca^{2+}-binding properties of this protein [Grabarek et al., 1990]. EF-hands of intracellular proteins generally lack disulfide bonds. The covalent S-S bridges found in extracellular proteins might therefore stabilize Ca^{2+}-binding loops that are exposed to concentrations of Ca^{2+} far in excess of those that normally exist within cells [Engel et al., 1987].

The band patterns and relative intensities of peptides derived from domain IV were insensitive to changes in excitation wavelength in the region of 280–300 nm. We chose 290 nm as an excitation wavelength because it provided the greatest separation of the Trp emission spectrum from the endogenous Rayleigh scattering peak. Our combined results indicate that the primary fluorophore in SPARC domain IV peptides is Trp, rather than Phe or Tyr. The removal of Ca^{2+} from the peptide apparently re-

sulted in the perturbation of the single Trp residue that is immediately C-terminal to the EF-hand loop motif. Such conformational changes induced by the removal of Ca^{2+} indicate an enhanced exposure of the aromatic side chain to the aqueous solvent. It therefore seems likely that domain IV peptides exert their apparently sequence-specific effects on cells because they undergo conformational changes upon the reversible binding of Ca^{2+}.

Finally, we note that there appeared to be a cooperative interaction between peptides 2.1 and 4.2, from SPARC domains II and IV, respectively, with regard to the inhibition of DNA synthesis in both aortic and capillary endothelial cells. The effect of peptide 4.2 was dominant over that of peptide 2.1. Although a synergistic inhibition between peptides 2.1 and 4.2 was more apparent in capillary vs. aortic cells, it was interesting that peptide 2.1 did not enhance the activity of peptide 4.2 K but was seen to augment the inhibition conferred by peptide 4.2 ND (a mutant displaying activities and physical properties similar to those of peptide 4.2 wt). It is possible that the cooperative effect is the result of adventitious disulfide bonding or exchange between peptides 2.1 and 4.2. We had previously reported that peptide 2.1 competed for the binding of [^{125}I]-SPARC, but did not compete for the binding of [^{125}I]-peptide 4.2, to BAE cells [Yost and Sage, 1993]. It is therefore likely that the affinity of peptide 4.2 for a SPARC receptor on BAE cells is considerably greater than that of peptide 2.1 for the same receptor; however, sequences in domain II are clearly involved in the association of native SPARC with the endothelial cell surface. The signal for interruption of the cell cycle might be mediated indirectly by a sequence in domain II, as this region is highly similar to a Cys-rich region in follistatin, which binds the cytokines activin and inhibin [see Lane and Sage, 1994]. Although we do not know whether the corresponding sequence in SPARC interacts with any growth factors, it has been shown that SPARC binds to the B-chain of platelet-derived growth factor and prevents the association of this factor with its receptors on fibroblasts [Raines et al., 1992]. In contrast, the inhibition of endothelial cell proliferation by SPARC is more likely to result from an interaction with cell-surface receptors via domain IV. We also propose that the Ca^{2+}-dependent binding of SPARC to extracellular matrix proteins influences the availability and accessibility of growth factors to the cell surface. SPARC (or peptides resulting from proteolytic degradation of SPARC) [Tyree, 1989; Lane et al., 1994] could thus be regarded as an extracellular regulator of cytokine activity [Flaumenhaft and Rifkin, 1992].

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