SPARC, A Secreted Protein Associated with Cellular Proliferation, Inhibits Cell Spreading In Vitro and Exhibits Ca^{+2}-dependent Binding to the Extracellular Matrix

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Abstract. SPARC (Secreted Protein Acidic and Rich in Cysteine) is a Ca^{+2}-binding glycoprotein that is differentially associated with morphogenesis, remodeling, cellular migration, and proliferation. We show here that exogenous SPARC, added to cells in culture, was associated with profound changes in cell shape, caused rapid, partial detachment of a confluent monolayer, and inhibited spreading of newly plated cells.

Bovine aortic endothelial cells, exposed to 2–40 μg SPARC/ml per 2 × 10^6 cells, exhibited a rounded morphology in a dose-dependent manner but remained attached to plastic or collagen-coated surfaces. These round cells synthesized protein, uniformly excluded trypan blue, and grew in aggregates after replating in media without SPARC. SPARC caused rounding of bovine endothelial cells, fibroblasts, and smooth muscle cells; however, the cell lines F9, PYS-2, and 3T3 were not affected. The activity of native SPARC was inhibited by heat denaturation and prior incubation with anti–SPARC IgG. The effect of SPARC on endothelial cells appeared to be independent of the rounding phenomenon produced by the peptide GRGDSP. Immunofluorescence localization of SPARC on endothelial cells showed preferential distribution at the leading edges of membranous extensions.

SPARC bound Ca^{+2} in both amino- and carboxyl-terminal (EF-hand) domains and required this cation for maintenance of native structure. Solid-phase binding assays indicated a preferential affinity of native SPARC for several proteins comprising the extracellular matrix, including types III and V collagen, and thrombospondin. This binding was saturable, Ca^{+2} dependent, and inhibited by anti–SPARC IgG. Endothelial cells also failed to spread on a substrate of native type III collagen complexed with SPARC. We propose that SPARC is an extracellular modulator of Ca^{+2} and cation-sensitive proteins or proteinases, which facilitates changes in cellular shape and disengagement of cells from the extracellular matrix.

CELLULAR responses to certain types of injury and developmental signals often involve expression of the same genes. Vascular endothelial cells interact with components of the extracellular matrix during angiogenesis, intercellular adhesion, signal transduction, and maintenance of thromboresistance (Herman, 1987; Nicosia and Madri, 1987; Rodgers, 1988). A consequence of such interactions is the induction of gene products that facilitate or modulate cellular behavior. Studies from several laboratories have identified one of these gene products as SPARC (Secreted Protein Acidic and Rich in Cysteine), a secreted Ca^{+2}-binding protein that is associated with cellular differentiation, proliferation, stress, and certain developmental signals (Mason et al., 1986a,b; Sage et al., 1986; Holland et al., 1987). Molecular cloning and protein sequence analysis have shown that SPARC is identical to osteonectin, a protein originally described in bone but also found in platelets and fibroblasts (Termine et al., 1981; Otsuka et al., 1984; Wasi

et al., 1984; Mason et al., 1986b; Stenner et al., 1986; Young et al., 1986; Zung et al., 1986; Bolander et al., 1988). SPARC is also identical to BM-40, a product of a basement membrane–secreting tumor cell line (Dziadek et al., 1986; Mann et al., 1987), and to a 43-kD glycoprotein, synthesized in vitro by cells of the vessel wall, as well as other normal and transformed cells (Sage et al., 1981a, 1984). In endothelial cells this protein was identified as a "culture shock" protein that bound BSA and a 70-kD serum protein and was increased after endotoxin-mediated injury (Sage, 1986a,b; Sage et al., 1986).

The SPARC single-copy gene has been localized to mouse chromosome 11 in a region identified with several developmental anomalies (Mason et al., 1986a). SPARC is highly conserved with 92% sequence identity between the bovine and mouse protein (Bolander et al., 1988; Engel et al., 1987). The amino acid sequence revealed four unique domains: I, an acidic, Ca^{+2}-binding region; II, a Cys-enriched

domain containing a glycosylation site; III, a neutral, α-helical sequence containing a serine protease–sensitive site; and IV, a Ca^{+2}-binding EF-hand as found in other high affinity Ca^{+2}-binding proteins (Mason et al., 1986b; Engel et al., 1987). The affinity of SPARC for Ca^{+2} (3 × 10^{-7} M) (Romberg et al., 1985) is similar to that of calmodulin.

Since bone osteonectin/SPARC is now known to be a potent inhibitor of hydroxyapatite formation (Romberg et al., 1985), it has been suggested that the protein might inhibit (Engel et al., 1987) rather than promote (Termine et al., 1981) extracellular mineralization. Recent studies have shown tissue-specific and developmentally related expression of SPARC mRNA in fetal and adult mice (Holland et al., 1987). This work, as well as immunohistochemical data (Tung et al., 1985; Sage et al., 1989a,b), provided evidence for the association of SPARC with proliferating cells in several tissues. While the presence of SPARC in basement membranes and extracellular interstitia in vivo is controversial (Termine et al., 1981; Wasi et al., 1984; Tung et al., 1985; Dziadek et al., 1986; Sage et al., 1989a,b), it was found not to be incorporated into newly synthesized extracellular matrix by several different cell types which secrete the protein in vitro (Mason et al., 1986b; Sage et al., 1986).

In this study we have addressed the function of SPARC as a secreted but nonmatrix protein showing up-regulation during cellular migration and/or proliferation in vitro. We show that addition of purified SPARC to cells in culture elicited a rounded morphology that was correlated with an inhibition of cellular spreading. This effect of SPARC was specific, dose-dependent, Ca^{+2} sensitive, and nontoxic. The change in shape was accompanied by limited cellular aggregation and appeared distinct from an RGD-related mechanism (Hynes, 1987). Exogenous SPARC was localized to leading edges of extensions of the endothelial cell plasma membrane. We also demonstrate that SPARC bound specifically to several components of extracellular matrix: type III collagen, type V collagen, and thrombospondin. Since these proteins, as well as SPARC, are produced by endothelial cells, smooth muscle cells (SMCs),[1] and fibroblasts of the vessel wall (Sage et al., 1981a, 1984; Mumby et al., 1984b; Wight et al., 1985; Sage, 1986b), we propose that the rounding effect of SPARC on endothelial cells is mediated through an interaction of SPARC with specific matrix proteins. This interaction would be permissive for cellular migration and proliferation.

Materials and Methods

Cell Culture

Bovine aortic endothelial (BAE) cells and bovine aortic SMCs at low passage numbers were obtained from Dr. S. Schwartz (University of Washington, Seattle, WA) and were cultured as previously described (Sage et al., 1986). Bovine fetal calf ligament (FCL) fibroblasts and additional strains of BAE cells were prepared in our laboratory and grown in DME containing 10% FCS and antibiotics (Sage and Mecham, 1987). Swiss 3T3 cells (clone D1) were grown as described by Pentinnen et al. (1988). F9 cells were purchased from the American Type Culture Collection (Rockville, MD) and grown on gelatin-coated tissue culture plasticware in DME supplemented with 10% FCS. Mouse parietal yolk sac (PYS)-2 cells, gifts from Drs. B. Hogan (Vanderbilt University, Nashville, TN) and J. Lehman (Albany Medical College, Albany, NY), were cultured in DME containing 10% FCS and were passaged approximately twice per week. Experiments were performed

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1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; FCL, fetal calf ligament; Gla, γ-carboxyglutamic acid; PYS, parietal yolk sac; SMC, smooth muscle cell.

with several separate strains of BAE and FCL cells derived from primary culture.

Metabolic labeling of cells in vitro with [³H]proline and [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) was according to Sage et al. (1986). [³²P]Orthophosphoric acid (New England Nuclear, Boston, MA) was used as described by Engel et al. (1987). For determination of γ-carboxyglutamic acid (Gla), [¹⁴C]glutamic acid (Amersham Corp.) was added (5 μCi/ml) to subconfluent FCL cells in serum-free DME for 24 h.

Purification of SPARC Protein

Approximately 20 150-cm² plastic tissue culture plates (Falcon Labware, Oxnard, CA) of subconfluent PYS (mouse) or FCL (bovine) cells were incubated in serum-free DME for 18–24 h. The medium was subsequently decanted, clarified of cellular debris, and stirred overnight at 4°C in the presence of 50% (wt/vol) ammonium sulfate. Precipitated proteins were concentrated by centrifugation, dissolved in and dialyzed against a 4 M urea/50 mM Tris-HCl buffer (pH 8), and chromatographed on DEAE–cellulose at 4°C. Most of the BSA was eluted with 75 mM NaCl in the same buffer, while SPARC and laminin (PYS cells) or SPARC, fibronectin, and type I procollagen (FCL cells) were eluted with 175 mM NaCl (Sage et al., 1984, 1986). We have found that dialysis of this latter fraction against distilled water caused SPARC to precipitate selectively. This precipitation occurred in a relatively narrow pH range (3.5–5.5) and was inhibited by 10 mM EDTA. SPARC was purified further by chromatography on Sephadex G-200 in TBS at 4°C. Alternatively, SPARC was purified by carboxymethyl–cellulose chromatography at room temperature in a 2 M urea, 40 mM Na acetate buffer (pH 4.5), following an initial denaturation step at 40°C for 5–10 min. To monitor the purification, one dish of cells was radiolabeled with [³⁵S]methionine, but nonradioactive SPARC was used for Western or metal blotting (see below).

Bovine and mouse SPARC exhibited identical chromatographic and solubility properties and produced the same results when added to cells. SPARC from these two species was used interchangeably for the experiments described in this study.

SDS-PAGE, protein staining, fluorescence autoradiography, and scanning densitometry were performed as previously described (Sage et al., 1986). Molecular weight standards were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, MD) and Pharmacia Fine Chemicals (Piscataway, NJ).

Studies in Vitro with Exogenous SPARC

Immunohistological Studies. Immunofluorescence microscopy was performed on cells grown on glass coverslips in vitro as previously described (Sage et al., 1984, 1986). Antibody concentrations were determined by absorbance at 280 nm and extrapolation to a standard curve for normal rabbit IgG.

Addition of SPARC to Cells. Purified SPARC was solubilized in PBS at a concentration of 250 μg/ml as determined by optical density. Increasing amounts, from 0 to 10 μl, of SPARC solution were added to 30 μl of DME supplemented with 5% FCS in a 96-well plate. The total volume of each well was then brought up to 40 μl with PBS, and the plate was equilibrated for 10 min in a 5% CO_2 incubator at 37°C. Freshly trypsinized BAE cells (fourth passage) were washed by centrifugation in DME/10% FCS, counted with a hemocytometer, and diluted to 2 × 10⁶ cells/ml in DME/5% FCS. 20 μl of cell suspension was added to each well and the plate was returned to the incubator for 18 h before photography. Cultures were photographed using an inverted phase-contrast microscope (Carl Zeiss, Inc., Thornwood, NY), and the percentage of rounded vs. spread cells was quantitated by analysis of two photographed fields (3 × 5 in) per well. In this manner, a dose-response curve was obtained for BAE cells exposed to SPARC.

For experiments with peptides containing the sequences RGD and RGE (control), 10 μl of a 1 mg/ml stock solution of HPLC-purified GRGDSP or GRGESP (gifts from Dr. R. Heimark, University of Washington) in PBS were added to wells, and cells were plated as described above with and without 7 μl/well SPARC solution.

The biosynthetic products of SPARC-treated and control cells were determined as follows: young (fourth passage) primary cultures of FCL cells were trypsinized, washed in DME/10% FCS, and resuspended at a concentration of 1 × 10⁶ cells/ml in DME/1% FCS. 50 μl of cell suspension was added to wells containing 200 μl of DME/1% FCS and 25 μCi/ml (L-2, -3, -4, and -5)(³H)proline (Amersham Corp.), in the presence or absence of 6 μl (3 μg) SPARC solution.

Conditioned medium was collected after 1, 4, 20, and 48 h. Cells were washed with 200 μl of PBS, and the pooled medium and wash were clarified

by centrifugation and precipitated on ice with 10% trichloroacetic acid (final concentration by volume) in the presence of 0.1 mg/ml pepstatin A (Peninsula Laboratories, Burlingame, CA). Pellets were washed with 5% trichloroacetic acid followed by two washes in cold absolute ether and stored overnight at $-20°C$, before solubilization in Laemmli buffer (Laemmli, 1970) and analysis by PAGE. The relative amounts of radiolabeled SPARC and other proteins were then determined from an autoradiograph by scanning densitometry.

Other experiments were conducted in 12- and 24-well Costar (Cambridge, MA) dishes, as well as in 30-mm and 60-mm plates. Stock solutions of SPARC, ranging from 50 to 500 $\mu g/ml$, were added to appropriate volumes of media to produce final concentrations of 0.1–5 $\mu g/ml$. Samples were dissolved in PBS or DME and were dialyzed against the same buffer under sterile conditions. TBS as a solvent was found to have deleterious effects on BAE cells. Control additions included BSA, type I procollagen, and laminin; these proteins originated from the same chromatographic run (DEAE-cellulose or G-200) as the SPARC sample. Other controls on separate dishes of cells were addition of the sample buffer alone, heat or chromatographically denatured SPARC, SPARC in combination with affinity-purified anti-SPARC IgG or with normal rabbit IgG, and antibodies alone. A minimum of 15 separate preparations of SPARC were used, from both bovine and murine cells.

Preparation and Characterization of Antibodies. Native SPARC purified on G-200 was used to generate polyclonal antibodies in adult rabbits (Sage et al., 1989a,b). IgG, precipitated from whole antisera by addition of ammonium sulfate to a final concentration of 20% (wt/vol), was further purified by affinity chromatography on Sepharose CL-4B (Pharmacia Fine Chemicals) to which had been coupled purified SPARC, according to the manufacturer's instructions. Affinity-purified anti-SPARC IgG (typical concentrations ranged from 0.1–0.225 mg/ml) were reactive by ELISA at dilutions in excess of 1/2,048 and exhibited no reactivity toward BSA or FCS. Antibodies were characterized by ELISA, Western blotting, and radioimmune precipitation as previously described (Sage et al., 1984, 1986).

Time-lapse Video Microscopy. BAE or FCL cells which had been freshly dissociated with trypsin were plated at 2×10^6 cells/ml into T-25 flasks containing DME/10% FCS. Control medium was supplemented with PBS, while test medium was supplemented with SPARC. Some of the flasks were coated with Vitrogen (Collagen Corp., Palo Alto, CA), before plating of FCL cells. Immediately after plating, the cultures were briefly incubated at 37°C in a water-saturated 5% CO_2 atmosphere. The caps on the flasks were then tightened and the cells were videotaped continuously from 2 h to several days. The cells were magnified through a 20× objective attached to a Nikon Diaphot inverted microscope equipped with an extra-long working phase condenser (Nikon, Inc., Garden City, NY). The cells were imaged through a video camera (series 65; Dage-MTI, Inc., Wabash, MI) equipped with a high resolution vidicon tube, and were videotaped with a time-lapse video recorder (model TLC2051R; Gyyr Products, Anaheim, CA). During the videotaping, the cells were maintained within an incubator (Nikon, Inc.) attached directly to the microscope, and the temperature was regulated at 37°C by an automatic temperature control unit (Nikon, Inc.). The videotape was subsequently viewed on a monochrome monitor (model 925; Lenco, Jackson, MI), and selected frames were photographed directly from the screen using a 35-mm camera (Nikkormat) equipped with a 50-mm lens.

Structural Studies

Circular Dichroism. Far ultraviolet circular dichroism spectra were obtained at 20°C from a Cary 61 spectrometer, calibrated with D(−)-pantolactone, with a quartz cell of 0.1-mm path length. Protein concentrations ranged from 0.15 to 0.45 mg/ml (5.1–15.0 μM) in TBS containing 2.5–5.0 mM $CaCl_2$ or 12 mM EDTA. The molar ellipticity, θ, at 220 nm (degree $cm^2/dmol$) was calculated from a mean residue molecular weight of 110, determined from the amino acid composition of mouse (PYS cell) SPARC (Mason et al., 1986b). Protein concentration was determined by amino acid analysis (performed by Dr. D. Eyre, University of Washington) or in some cases from an extinction coefficient, $E_{1\ cm}^{0.1\%/280} = 1.076$ calculated from the compositional data and the molecular weight of 33,062 predicted from the cDNA sequence (Mason et al., 1986b).

Gla Analysis. [^{14}C]Glutamic acid-labeled SPARC isolated from FCL cells was hydrolyzed in vacuo in 1 N NaOH at 105°C for 5 h, neutralized with 2 M HCl, and lyophilized. Separation of acidic amino acids was performed essentially as described by McMullen et al. (1983) on a Dowex 50W-X8 column in 0.1 M ammonium formate, pH 2.2. Gradient elution was achieved with equal volumes (25 ml) of 0.1 ammonium formate, pH 2.9,

and 0.2 M ammonium formate, pH 3.65. Standards included prothrombin (a gift from Dr. K. Fujikawa, University of Washington), [^3H]aspartic acid, and [^{14}C]glutamic acid. Detection of radioactive peaks was by liquid scintillation counting; nonradioactive amino acids were detected by addition of 1.5 ml 0.2 M $NaBO_4$ (pH 9) to lyophilized fractions, followed by 500 μl fluorescamine (Fluoram; Roche Diagnostics, Nutley, NJ) (300 mg/100 ml acetone), and fluorimetry at an excitation wavelength of 390 nm and an emission wavelength of 480 nm. With this latter method, the limit of sensitivity was 1 nmol/ml and therefore required a minimum of 0.25 μM ($\sim$10 $\mu g/ml$) prothrombin (8 Gla/molecule) and $\sim$50 μg unlabeled SPARC (assuming 1–2 Gla/molecule). The occurrence of Gla in SPARC was also checked by HPLC of base-hydrolyzed protein isolated from FCL cells and was performed by Dr. D. Liska (University of Washington).

Metal blotting. Standardized solutions of Ca^{+2}-binding proteins (calmodulin, troponin C) and control proteins including (a) a Cu^{+2}-binding protein (apoazurin; a gift from Dr. E. Adman, University of Washington), (b) an Fe^{+2}-binding protein (transferrin; Collaborative Research, Inc., Waltham, MA), and (c) nonmetal-binding proteins (BSA, insulin, and M_r standards) were resolved, both reduced and unreduced, on 10 or 15% SDS gels and were subsequently transferred electrophoretically to nitrocellulose at 0.5 mA for 2 h. Incubation with ^{45}Ca and washing were done following closely to the protocol of Davis et al. (1986). ^{45}Ca (0.2, 2, and 20 μM) (New England Nuclear) was added to blots previously washed three times for 10 min in a 60 mM KCl/5 mM $MgCl_2$/10 mM imidazole buffer (pH 6.8), followed by a 5-min wash in 50% ethanol, air drying, and exposure to x-ray film. To assess the Ca^{+2}-binding of different domains of SPARC, the protein was subjected to limited cleavage with trypsin (Sage et al., 1984).

Enzymatic Assays. Preparations of SPARC were tested for tissue plasminogen activator activity with a standard assay kit (Spectrolyse) purchased from American Diagnostica Inc. (New York).

Solid-phase Binding Assays. Direct binding assays were conducted in polystyrene 96-well plates or in Removawells (Immunlon; Dynatech Laboratories, Inc., Alexandria, VA). Native collagen types I (lathyritic rat skin), II (bovine cartilage), III (bovine skin), IV (human and bovine placenta), V (human fetal membrane), and VIII (bovine Descemet's membrane) were prepared in our laboratory by limited pepsin digestion (except for type I) and differential salt fractionation (Sage et al., 1981b). Collagens were denatured at room temperature in 1 M urea/50 mM Tris-HCl (pH 7.4); in some cases, individual collagen chains or CNBr peptides separated on carboxymethyl-cellulose were used. Collagens were solubilized in TBS (50 $\mu g/ml$) and adsorbed to polystyrene wells as previously described (Galvin et al., 1987). Coating efficiency among the different collagen types, quantitated by amino acid analysis, was not appreciably different ($\sim$10%) (Parsons et al., 1983; Galvin et al., 1987), and adsorption of fibrillar and nonfibrillar collagens to plastic surfaces was judged uniform by immunofluorescence (Parsons et al., 1983). Human platelet thrombospondin was a gift from Dr. G. Raugi (Veterans Administration Medical Center, Seattle, WA); human plasma fibronectin was a gift from Dr. P. Bornstein (University of Washington) or was purchased from Imcera Bioproducts, Inc. (Terre Haute, IN); bovine endothelial heparan sulfate proteoglycan was a gift from Dr. M. Kinsella (University of Washington); mouse laminin was isolated from PYS cells in our laboratory; bovine type VI collagen was a gift from Dr. J. Wu (University of Washington); bovine plasma fibrinogen and plasminogen were from Dr. E. Davie (University of Washington); and BSA (Pentex, fraction V) and normal rabbit IgG (Miles Laboratories, Inc., Elkhart, IN) were purchased from commercial suppliers. Human recombinant tissue plasminogen activator was a gift from Dr. S. Busby (Zymogenetics Inc., Seattle, WA). Native SPARC was iodinated with Na-^{125}I and Iodo-Gen or with Bolton-Hunter reagent (Pierce Chemical Co., Rockford, IL) and was purified either by extensive dialysis against PBS or by passage over Sephadex G-25 (Pharmacia Fine Chemicals). The iodinated protein migrated as a single M_r 43,000 band on SDS-PAGE and had a specific activity of 1.3–42 $\times 10^6$ cpm/μg. All proteins used in these assays were examined for purity by SDS-PAGE and were quantitated by amino acid analysis, absorbance at 230 or 280 nm, or Bradford assay.

From 0.4 to 12 μg protein was added to each well in a total volume of 100 μl TBS. Quantitation of adsorbed protein was by amino acid analysis (AAA Laboratory, Mercer Island, WA) of material solubilized from wells in constant boiling HCl. After incubation overnight at 4°C, supernates were removed and wells were blocked with 100 μl ovalbumin (1–10 mg/ml in TBS) for 1–2 h at room temperature. After one to two washes in TBS containing either Ca^{+2} or EDTA (see below), ^{125}I-SPARC was added (10^5–10^6 cpm in a total volume of 90–100 μl TBS) and incubated at 4°C overnight. Conditions were varied with respect to inclusion of Ca^{+2} or EDTA in TBS (Galvin et al., 1987), concentration of coated protein, and amounts of radio-

active SPARC. For some experiments, normal rabbit IgG or anti–SPARC IgG (39–78 μg of an ammonium sulfate precipitate) were incubated with SPARC before addition to the blocked wells. Wells were subsequently washed two to three times with TBS containing 5–100 mM $CaCl_2$ or 1–10 mM EDTA, snapped off, and assayed in a γ-scintillation counter.

Results

Structural Characteristics of the SPARC Protein

For functional studies of SPARC, it was necessary to purify larger amounts of the protein in an intact, native form than had previously been described in the literature. In this regard, we found that SPARC could be separated from its major contaminant, BSA, by dialysis against water at pH 5.5. The acidic pI of SPARC (pI 4.3) is most likely responsible for its Ca^{+2}-dependent precipitation (Mason et al., 1986b; Engel et al., 1987). Isolation of SPARC from tissue culture medium in the presence of Ca^{+2} and protease inhibitors resulted in minimal degradation, and subsequent chromatography on Sephadex G-200 in TBS produced a homogeneous preparation of SPARC with retention of native structure. In Fig. 1 is shown a typical purification on G-200 (Fig. 1 A), and the corresponding analysis by SDS-PAGE of peak area II (Fig. 1 B, lanes 1 and 2). Radioimmune precipitation of [35S]methionine-labeled PYS cell culture medium proteins (Fig. 1 B, lane 3) with affinity-purified anti–SPARC IgG produced a species of M_r 43,000 (Fig. 1 B, lane 4, arrow) which comigrated with purified SPARC (Fig. 1 B, lane 2). Further conformation of the specificity of the antibody is shown by the immunoblot of a PYS cell layer lysate (Fig. 1 B, lane 5). Several preparations of bovine or murine SPARC, as shown in Fig. 1 B, lane 1), as well as the monospecific anti–SPARC IgG, were used for the subsequent experiments described in this study.

The amino acid sequence predicted from mouse SPARC cDNA revealed two putative Ca^{+2}-binding domains: the glutamic acid–rich NH_2-terminal domain I and the EF-hand–containing domain IV (Engel et al., 1987). We examined this binding directly by a blotting procedure in conjunction with radioactive metal ligands. Cleavage of native SPARC with trypsin produces two major fragments, one of ~M_r 28,000 (containing domain I) and the other of ~M_r 6,000 (containing domain IV) (Sage et al., 1984; Engel et al., 1987). In Fig. 2 we show that both fragments in the reduced form (labeled I and II) bound $^{45}Ca^{+2}$. Control experiments showed that the EF-hand–containing proteins calmodulin and troponin C also bound the metal, whereas transferrin, insulin, and apoazurin were negative (data not shown). Both transferrin ($^{55}Fe^{+2}$) and apoazurin ($^{64}Cu^{+2}$) bound their respective metal ligands, while BSA and protein markers were negative (except for β-lactoglobulin which bound $^{45}Ca^{+2}$) (not shown). The relationship between the micrograms of SPARC blotted and the intensity of the band after exposure to the metal isotope was nearly linear by scanning densitometry.

It has been estimated that from five to seven Ca^{+2} are bound by SPARC domain I (Engel et al., 1987; Sage et al., 1989b), which we speculate has a lower affinity for Ca^{+2} than that of the EF-hand. The M_r 28,000 fragment retains most of the Ca^{+2}-dependent binding activity towards type III collagen that has been shown with the intact protein (Sage, H., unpublished experiments; and see experiments below).

Since the mechanism of Ca^{+2} binding by domain I is not known, we examined SPARC for the presence of Gla. With a lower limit of one residue Gla per molecule of SPARC, we were unable to detect this amino acid in SPARC.

The role of phosphorylation in the function of SPARC is unclear. Although this posttranslational modification has been reported in bone osteonectin (Uchiyama et al., 1986) and in SPARC from PYS-2 cells (Engel et al., 1987), it was not found in porcine calvarial osteonectin (Domenicucci et al., 1988) or in SPARC produced by endothelial cells (Sage et al., 1984; and data not shown). Examination of FCL cells after an extended labeling showed a minor phosphorylated species of M_r 43,000 (data not presented). We concluded that the addition of phosphate group(s) to SPARC is cell or tissue-specific and reflects a specialized functional adaptation. Furthermore, this posttranslational modification was not required for the ability of SPARC to inhibit cell spreading in vitro (see below).

SPARC Inhibits Cell Spreading In Vitro

In earlier experiments we had shown that SPARC was not mitogenic for BAE cells in vitro (Sage et al., 1986). The association of SPARC with proliferating cells also prompted us to test its potential activity as a "scatter factor" on Madin-Darby canine kidney (MDCK) cells (Stoker et al., 1987). Unlike the fibroblast protein that causes dispersion of epithelial cells, SPARC in this assay was negative (data not shown). We then directed our efforts toward examining the effects of exogenous native SPARC on cell behavior in vitro, as well as its potential interaction with components of the extracellular matrix. When purified SPARC was added to freshly trypsinized FCL fibroblasts, the cells failed to spread. This effect was seen on collagen-coated as well as plastic substrata in the presence of 1–10% FCS. As shown in Fig. 3 A, most of the cells plated in medium lacking SPARC were attached and spread within 2 h. In contrast, cells plated in the presence of SPARC at a concentration of 1.0 μg/ml attached but retained a rounded morphology (Fig. 3 B). These cells could not be dislodged by shaking the dish but could be removed by treatment with trypsin/EDTA. SPARC in a native conformation was required for the rounding effect. After heat denaturation, or purification under denaturing conditions, SPARC produced the flattened circular dichroism spectrum as shown in Fig. 3 C and was unable to induce rounding. For comparison, the spectrum of an active sample is shown in Fig. 3 D, with a shoulder at 220 nm signifying the presence of α-helix. Engel et al. (1987) reported an overall α-helical content for a tissue form of SPARC (BM-40) of 25–30%, and a θ_{220} of −5,600 degrees $cm^2/dmol^{-1}$, which is similar to the value we obtained (−4,500 degrees $cm^2/dmol$). The cells shown in Fig. 3 B excluded trypan blue (>90%), incorporated [3H]proline into protein, and remained viable for several days.

Video microscopy was also performed to monitor the response of a given field of FCL and endothelial (BAE) cells to SPARC as a function of time. In one experiment, FCL cell controls were spread 39 min after plating, while SPARC-treated cells were still round at 31 min (data not shown). BAE cells, which require somewhat longer times for spreading, showed very active membrane activity and began to spread at 1 h, 22 min. SPARC-treated BAE cells were rounded after
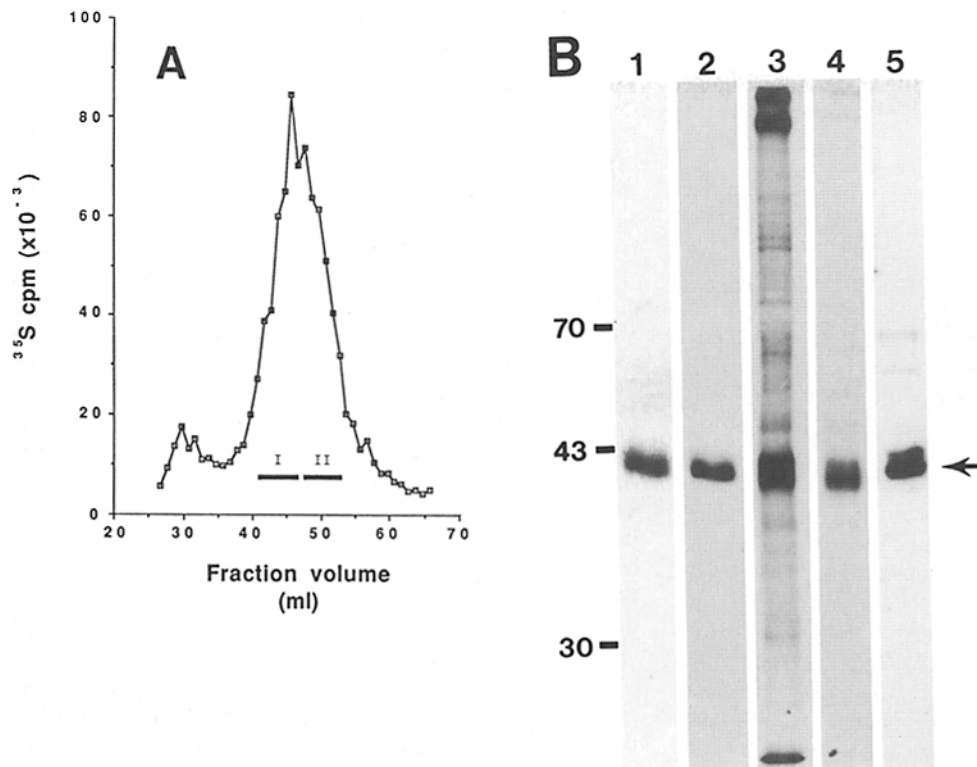
Figure 1. Purification of SPARC protein. [35S]Methionine–SPARC was isolated from PYS cell culture medium by ammonium sulfate precipitation and DEAE–cellulose chromatography. A fraction enriched in SPARC was purified further by chromatography on Sephadex G-200 in TBS at 4°C. (*A*) Elution profile showing two subfractions of the major peak. Pool II contained the most homogeneous preparations of SPARC. (*B*) Analysis of SPARC pool II as shown in *A* is depicted in lanes *1* and *2* of a 10% SDS–polyacrylamide gel. PAGE was performed under reducing conditions, and proteins were stained with Coomassie blue (lane *1*) or were detected by fluorescence autoradiography (lane *2*). Molecular mass standards in kilodaltons are shown on the left. Lanes *3* and *4* show an autoradiograph of [35S]methionine-radiolabeled proteins secreted by PYS cells before (lane *3*) and after radioimmune precipitation with anti–SPARC IgG and staphylococcal protein A (lane *4*). Lane *5* represents a PYS cell layer lysate blotted onto nitrocellulose and subsequently probed with anti–SPARC IgG and Protein A. The arrow (on the right) indicates SPARC (M_r 43,000) as the specific component of the radioimmune complex.

1 h, 25 min, although a few cells in the process of spreading were apparent (not shown).

The ability of SPARC to inhibit cell spreading was concentration dependent. In Table I are listed concentrations of SPARC, from 0–42 μg/ml, with the corresponding percentage of rounded cells scored 24 h after the addition of SPARC. The degree of activity varied somewhat with different preparations; we now know that this variability is due to the proportion of native molecules in a given sample. It was a consistent finding that not all of the cells became round in the presence of SPARC. This result could be due to cellular heterogeneity, position in the cell cycle at the time of addition, or internalization of SPARC at 37°C over 24 h. The rounding effect was also blunted, although never eliminated, in higher concentrations of FCS (10–20%).

The biosynthetic profiles of control and SPARC-treated cells, examined at several time intervals, were found to be identical. Moreover, the levels of SPARC production were nearly equivalent and were insignificant compared to the amounts of exogenous SPARC added to the cultures (data not shown). Although it is possible that SPARC was acting as a chelating agent in cell culture, we are inclined to believe that SPARC was not present in sufficient quantities in these experiments to depress Ca^{+2} levels below those required for BAE cell spreading. The concentrations of SPARC used in these experiments ranged from 16.7 nM to 1.7 μM, in DME containing 1.6 mM Ca^{+2}. In related experiments, we have found that (*a*) cells spread normally in DME formulated without cations, (*b*) cell rounding occurred between 0.05 and 0.1 mM EGTA in DME without cations in the absence of SPARC, and (*c*) DME with cations containing 0.2 mM EGTA or 0.2 mM EGTA plus SPARC spread normally. However, cell behavior in the absence of Ca^{+2} is difficult to interpret, and EGTA is known to have deleterious effects on the structure of SPARC.

As shown in Fig. 4, SPARC had a marked effect on the morphology of BAE cells. Attached, spread cells exposed to 0.5 μg/ml SPARC in 5% FCS assumed a rounded morphology but remained attached to the plastic or collagen-coated surface (Fig. 4, compare *A* with *B*). This morphology, associated with a lack of spreading, occurred with freshly trypsinized, attached but subconfluent, and superconfluent cells, and was seen within 1 h after addition of SPARC (somewhat longer intervals were required in the case of sprouting [Cotta-Pereira et al., 1980] or superconfluent cultures). More SPARC was required to produce this effect when higher concentrations of serum were used. The rounded cells, although still attached, could be dislodged by vigorous shaking or pipetting; when replated without SPARC, they appeared as clusters or aggregates (Fig. 4 *C*) which eventually proliferated with the apparent absence of cell spreading (Fig. 4 *D*). The rounded cells synthesized protein and excluded trypan blue to the same extent as untreated BAE cells. Type I procollagen and BSA, used in equimolar concentrations to those of SPARC, had no effect (data not shown).

Addition of SPARC to sparsely plated cultures initially caused a retraction of the elongate shape, followed by the rounded morphology seen with confluent BAE cells (Fig. 4
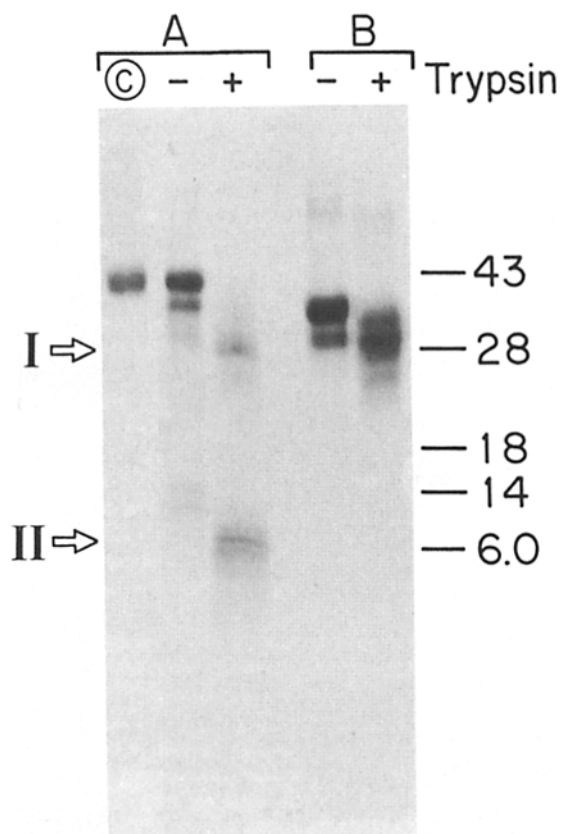
Figure 2. Binding of Ca^{+2} to domains I and IV of SPARC. Equal amounts (1 μg) of native SPARC protein were incubated at 37°C with or without trypsin (0.01 μg), and the digest was resolved by SDS-PAGE (10% gel) in the presence (*A*) and absence (*B*) of DTT. After electrophoretic transfer onto nitrocellulose, the filter was incubated with $^{45}Ca^{+2}$, washed extensively, and exposed to x-ray film. *C* denotes unincubated enzyme control. The presence of a second band (M_r 40,000) in the lanes (−) that were incubated at 37°C without enzyme, is due to an endogenous proteolytic activity in the SPARC sample. Trypsin cleavage produced two major fragments: I, the amino-terminal, glycosylated polypeptide (amino acids 1–197) of apparent M_r 28,000; and II, a sequence of ∼M_r 6,000 at the carboxyl end of the molecule. Both fragments I and II displayed an affinity for Ca^{+2}. Molecular mass markers in kilodaltons are indicated on right.

E). When these round cells (Fig. 4 *E*) were replated, foci of cells were apparent among areas of confluent cells in selected areas of the dishes (Fig. 4 *F*). Although the round cells attached and grew (Fig. 4, *D* and *F*), the ability of the cells to spread depended on the concentration of added SPARC and on the duration of treatment. Hence, the SPARC-treated BAE cells in Fig. 4 *D* (0.5 μg/ml for 24 h) proliferated but did not spread even after 10 d in culture with several changes of DME/10% FCS. In contrast, cells receiving 0.25 μg/ml SPARC for 6 h demonstrated a cobblestone-like monolayer interspersed with foci of round cells within 3 d after replating in fresh DME/10% FCS in the absence of SPARC (Fig. 4 *F*).

A series of control experiments performed with BAE cells is shown in Fig. 5. A laminin-containing fraction from Sephadex G-200 (peak I in Fig. 1 *A*) was ineffective (Fig. 5 *A*). BAE cells receiving equivalent amounts of native (Fig. 5 *B*) and denatured SPARC (Fig. 5 *C*) are also shown in Fig.

5. In the presence of the latter, the cells proliferated and appeared, 24 h later, as a normal endothelial monolayer (Fig. 5 *C*). That the effect of SPARC was specific was demonstrated by incubation of 0.75 μg/ml SPARC with affinity-purified anti-SPARC IgG. As shown in Fig. 5 *D*, there was no observable effect on cell morphology in the presence of the immune complexes. Antibody alone or normal rabbit IgG was also without effect on the monolayer (data not shown).

Although SPARC inhibited spreading in cultures of bovine endothelial cells and fibroblasts, not all the cells that we examined were affected in a similar manner. The transformed murine cell lines PYS-2 and F9 (undifferentiated) did not respond to SPARC, and the 3T3 cell line also was refractory (data not shown). Bovine SMC exhibited a lesser response, scored as percent of rounded cells, than was seen with equivalent concentrations of SPARC added to BAE and FCL cells. These results, as well as the biochemical controls already described, show that the effect of SPARC was not one of overall toxicity or inhibition of a general cellular metabolic pathway. Primary cells (e.g., murine skin fibroblasts), or cells at low passage, isolated from nonmalignant tissue, also were sensitive to added SPARC.

The effect of SPARC on BAE cells also appeared to be independent of the rounding phenomenon produced by a synthetic peptide containing the sequence RGD. In Fig. 6 are shown cultures that received RGD (Fig. 6, *A*, *D*, and *G*) and RGD plus SPARC (Fig. 6, *B*, *E*, and *H*), photographed at 1 (Fig. 6, *A–C*), 4 (Fig. 6, *D–F*), and 10 h (Fig. 6, *G–I*) after the addition. While both reagents caused a similar rounded morphology, after 10 h the RGD effect was diminished (possibly because the cells synthesized additional receptors and/or attachment proteins). Cells in the presence of RGD + SPARC, however, remained largely unspread. The control peptide RGE had no effect on the cells (Fig. 6 *F*), and it did not alter the ability of SPARC to inhibit cell spreading (Fig. 6, *C* and *I*). We have also recently observed that the anti-endothelial cell vitronectin receptor monoclonal antibody LM609 (Cheresh, 1987) did not interfere with the rounding of BAE cells plated onto SPARC-coated dishes (Cheresh, D., and H. Sage, unpublished experiments). These experiments suggest that SPARC affects cells independently from an RGD-related mechanism, in that the cells were unable to use alternate means for spreading in the presence of SPARC.

Immunofluorescence localization of exogenous SPARC on sparsely plated BAE cells is shown in Fig. 7. The protein was concentrated at the leading edges of membranous extensions (Fig. 7 *B*), which were barely perceptible by phase microscopy (Fig. 7 *A*, *arrows*). SPARC was not bound in detectable amounts onto the plastic surface; there was minimal extracellular matrix produced by these <24-h cultures. This finding is consistent with the binding of SPARC to BAE cell surfaces, especially in the areas of membranous ruffling. We have also recently demonstrated that ^{125}I-SPARC binds to BAE cells with high affinity (Sage, H., and S. Funk, unpublished experiments).

SPARC Binds to Several Components of the Extracellular Matrix

A solid-phase binding assay was used to measure the affinity of native radiolabeled SPARC for certain components of the
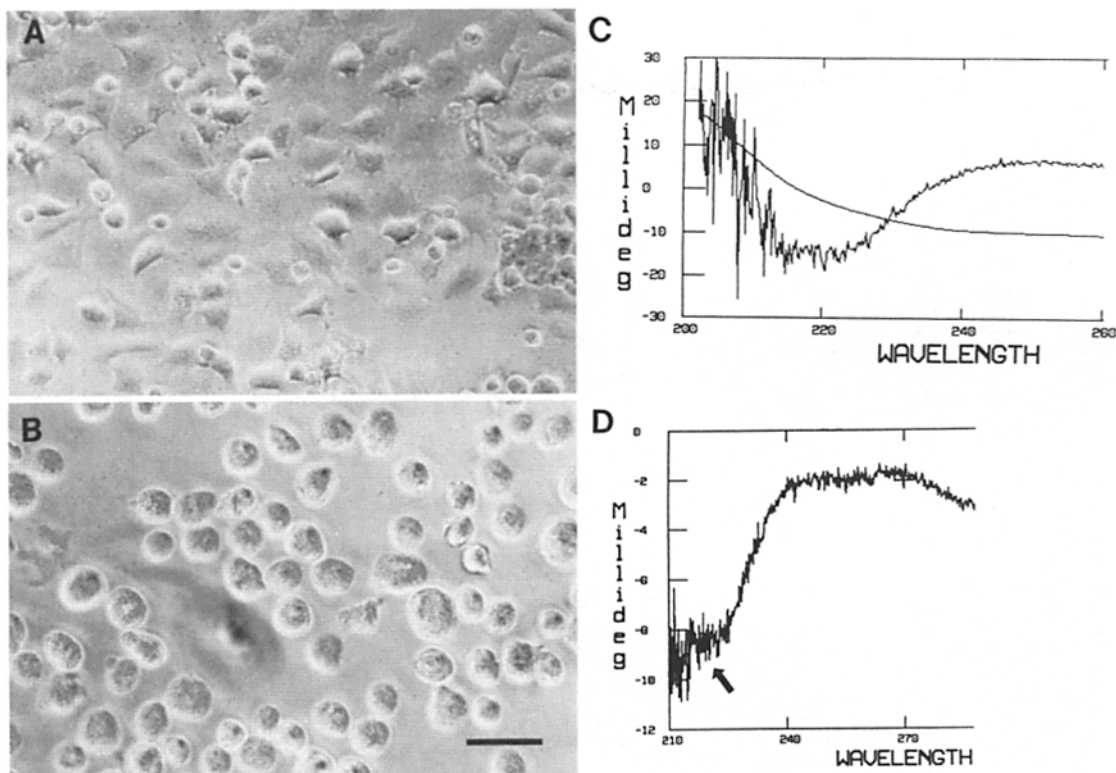
Figure 3. Effect of native SPARC on fibroblasts in vitro. (*A* and *B*) FCL fibroblasts were trypsinized and plated on collagen-coated plastic in DME/10% FCS in the absence (*A*) or presence (*B*) of native SPARC (1.0 μg/ml). In *A* (2 h after plating), the majority of cells are attached and spread. In *B*, cells in the presence of SPARC for 2 h have attached (during the first 15 min after plating) but have failed to spread. (*C* and *D*) Circular dichroism spectra of denatured (*C*) and native (*D*) SPARC. SPARC (0.15 mg/ml, 5.1 μM) in TBS containing 2.5 mM CaCl$_2$ was measured at 20°C. The shoulder at 220 nm (*arrow*) in D is indicative of α-helical content; θ_{220} was calculated as −4,500 degrees cm^2/dmol. This sample elicited the rounded morphology as shown in *B*. In contrast, the spectrum in *C* shows a sample of denatured SPARC that was ineffective on the cells, resulting in a morphology similar to that shown in *A*. Bar, 30 μm.

blood clotting cascade, as well as secreted proteins which contribute to the formation of basement membranes or extracellular matrices. 25 separate assays, performed with several preparations of radiolabeled SPARC and a variety of experimental conditions, produced reproducible trends. Individual experiments have been shown to illustrate internal

Table I. Relationship between SPARC Concentration and Inhibition of BAE Cell Spreading on Plastic Substrata*

SPARC addition	Rounded cells‡
μg/ml	%
0	9.0
2.0	14.5 ± 1.5
4.0	43.5 ± 0.5
12.5	46.0 ± 0
20.0	52.5 ± 2.5
29.0	61.0 ± 5.0
42.0	68

* Trypsinized BAE cells were resuspended in DME/5% FCS at 2 × 10⁶ cells/ml. Equal aliquots (20 μl each) were added to microtiter wells in the presence of increasing concentrations of native murine SPARC. Scoring was performed 24 h later on two photographed fields.
‡ Expressed as mean ± SEM.

consistency; measurements varied from 2–12% (SEM). Since SPARC can form a complex with BSA under certain conditions (Sage et al., 1984; Domenicucci et al., 1988), we routinely subtracted counts per minute of SPARC bound to control (BSA-coated) wells and used ovalbumin as a blocking agent.

The most significant interaction between ^{125}I-SPARC and these proteins was observed with thrombospondin and native collagen types III and V (Table II and Fig. 8). Incubation of ^{125}I-SPARC with anti–SPARC IgG, before addition to protein-coated wells, resulted in an 84–94% inhibition of binding (data not shown). Between 0.26 and 1.62 pmol SPARC were bound per picomole of collagen types I–V (Table II). Conversion of picomoles adsorbed collagens to molar concentrations (0.17–2.8 × 10⁻⁸ M) showed that the amounts of SPARC bound to collagen were from 20 to 300 times higher than those reported by Galvin et al. (1987) for thrombospondin bound to collagen in the presence of Ca⁺² (∼9 fmol thrombospondin bound to type V collagen at a concentration of 10⁻⁸ M). Fig. 8 *A* shows a comparison of the affinity of SPARC for several of these proteins. Wells coated with type III collagen clearly demonstrated the highest level of binding, which was linear with respect to the amounts of collagen adsorbed to the wells (not shown). Native type V collagen exhibited greater affinity for SPARC than types I, II, and IV
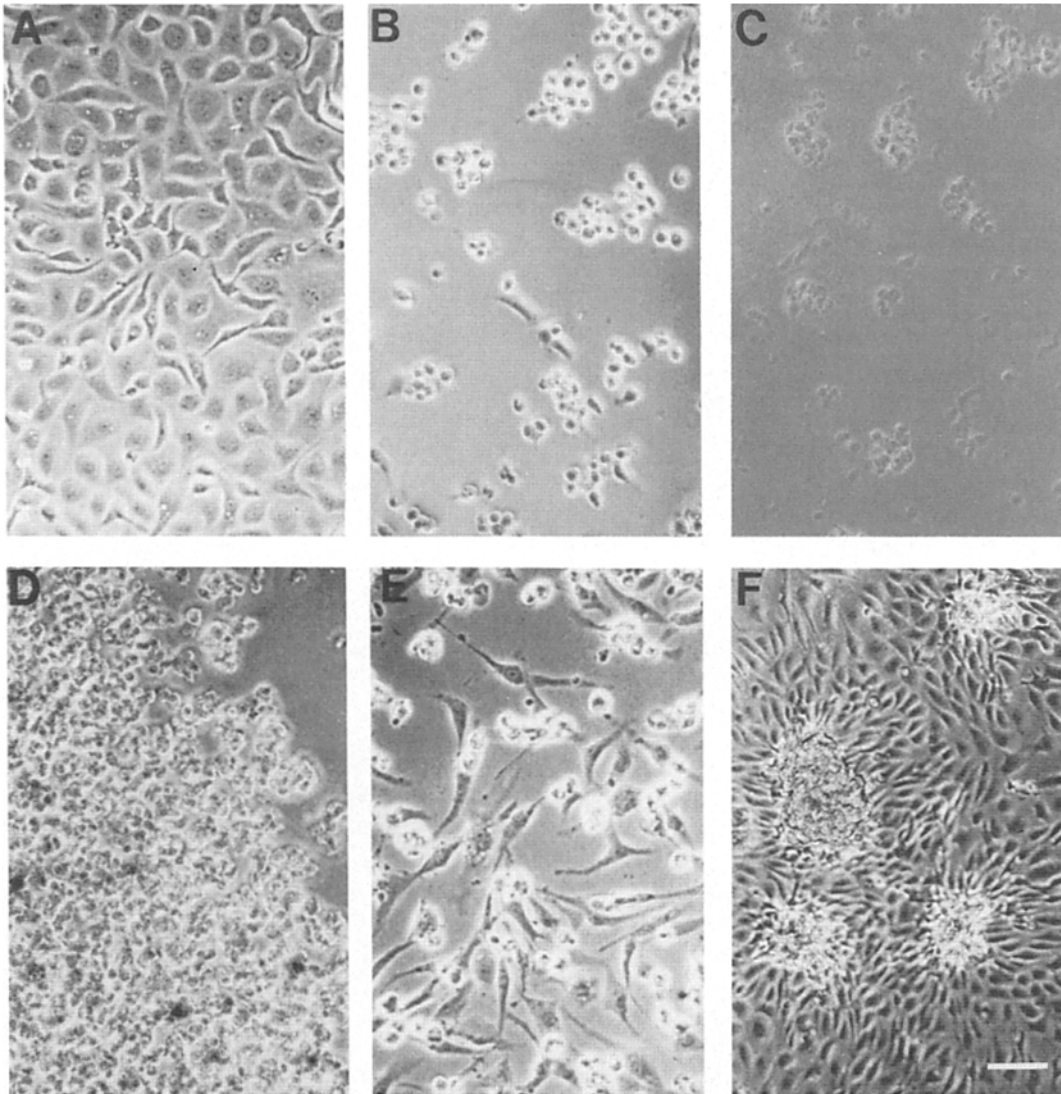
Figure 4. SPARC affects endothelial cell morphology and growth. 0.5 µg/ml SPARC in PBS was added to confluent BAE cells in DME containing 5% FCS. (A) Control receiving PBS alone; (B) 24 h after SPARC addition. Round cells in the presence of SPARC were detached with a stream of medium and were replated in fresh DME containing 10% FCS in the absence of SPARC. (C) 24 h after replating; (D) culture shown in C after 1 wk. A subconfluent BAE culture in 2.5% FCS received 0.25 µg/ml of highly purified native SPARC. (E) 6 h after addition ∼10% of the cells were detached; (F) 3 d after replating of detached cells in E, ∼50% of the cells were attached. Differences observed between the cells in D and F are largely due to the amount of SPARC initially added to the cells, as well as the duration of treatment with SPARC before replating after its removal. Bar, 50 µm.

(Table II and Fig. 8). When a complex between thrombo-spondin and type V collagen was formed in the presence of EDTA (Galvin et al., 1987), and tested in the assay with ^{125}I-SPARC, binding was generally greater for the complex than for each component alone (not shown). However, the results were variable and were found to be extremely sensitive to the concentration of Ca^{+2} in the binding buffer (data not shown). We found minimal binding of SPARC to laminin; in addition, background levels (similar to those obtained with BSA-coated and blocked wells) were observed with fibronectin, tissue plasminogen activator, GRGDSP and GRGESP (Fig. 8 A), fibrinogen (Table II), type I procollagen, types VI and VIII collagen, heparan sulfate proteoglycan, and plasminogen (data not shown). Binding to dena-

tured collagens, including gelatin as previously reported (Sage et al., 1984), was also low.

Fig. 8 B illustrates the effect of Ca^{+2} concentration on the binding of SPARC to types I, III, and V collagen. An increased association of ^{125}I-SPARC with these collagens was seen between Ca^{+2} concentrations of 18 and 37 mM. The dissociation constant (K_d) for the affinity of SPARC for type III collagen was calculated from Scatchard analyses of the data shown in Fig. 8 B to be ∼0.5 nM.

The ability of SPARC to elicit endothelial cell rounding could be mediated by its association with type III collagen. To examine this possibility, we performed the experiment shown in Fig. 9. BAE cells plated on type III collagen attached and spread readily within 24 h (Fig. 9 A), and prolif-
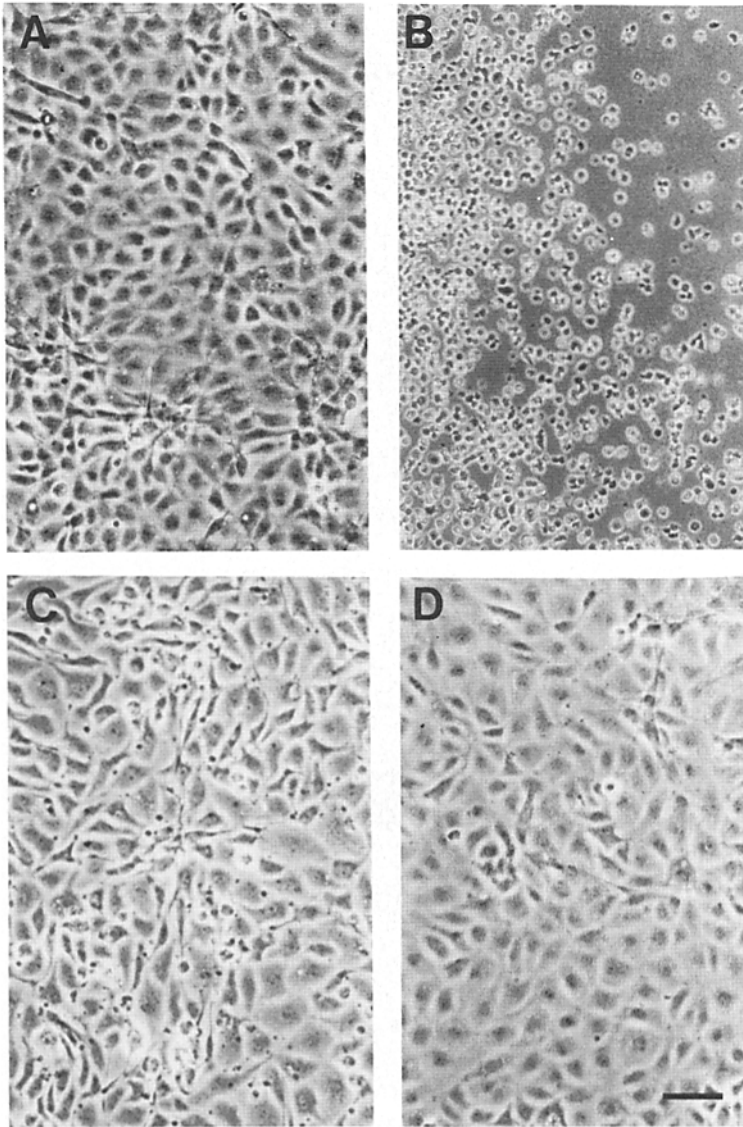
Figure 5. The rounding effect elicited by SPARC is inhibited by denaturation and by anti–SPARC IgG. Cultures of subconfluent BAE cells in DME containing 5% FCS were incubated with 0.5–0.75 μg/ml murine SPARC that was purified on Sephadex G-200 under native conditions (*B*). A fraction containing laminin from the same chromatographic procedure was added to replicate cultures (∼50-fold Molar excess over the concentration of SPARC) (*A*). Heat denaturation of the SPARC sample used in *B* (5 min at 70°C) produced an inactive protein as shown in *C*. In *D*, confluent BAE cells received 20 μl rabbit anti–mouse SPARC IgG (∼35-fold Molar excess), in the presence of 0.75 μg SPARC that had been preincubated with the antibody. Cultures were photographed 24 h later. Bar, 50 μm.

erated to near confluence after 4 d (Fig. 9, *B–D*). In contrast, cells plated on substrates of type III collagen which had been preincubated with SPARC were round after 24 h (Fig. 9 *E*) and failed to spread at the end of 4 d (Fig. 9, *F–H*). In the presence of SPARC, however, proliferation was not diminished (Fig. 9, compare *D* and *H*). This result suggests that the antispreading activity of SPARC could be amplified by its association with an extracellular matrix protein. Moreover, SPARC was fully functional when complexed with type III collagen.

Discussion

From the preceding experiments, we have made several observations concerning the properties and function(s) of SPARC: (*a*) the protein induced a rounded morphology in confluent monolayers of bovine SMC, fibroblasts, and endothelial cells; (*b*) SPARC maintained the rounded morphology of newly plated (trypsinized) cells and inhibited their spreading; (*c*) rounded cells retained viability, synthesized

protein, and exhibited a capacity for proliferation subsequent to replating in the absence of SPARC; (*d*) the effect of SPARC was specific, concentration dependent, and required native structure; (*e*) SPARC did not inhibit cellular aggregation; (*f*) BAE cells, rounded in the presence of GRGDSP, began to spread after 10 h, while cells treated with SPARC did not; (*g*) SPARC was associated with BAE cell plasma membranes; and (*h*) SPARC bound specifically, and in a Ca^{+2}-dependent manner, to types III and V collagen.

These results, and experimental data from prior studies in vascular biology, support the following hypothesis for the activity of SPARC in the endothelial extracellular space. We have shown (Sage et al., 1986) that injury to endothelial cells results in an increase in the secretion of SPARC. Thrombospondin and type V collagen, two major proteins of the extracellular matrix and subendothelium (Sage et al., 1981*a*; Wight et al., 1985), form a complex in low concentrations of Ca^{+2} (Mumby et al., 1984*b*; Galvin et al., 1987); dissociation occurs when the Ca^{+2} concentration is >30 μM (Galvin et al., 1987). Based on their relative affinities for each
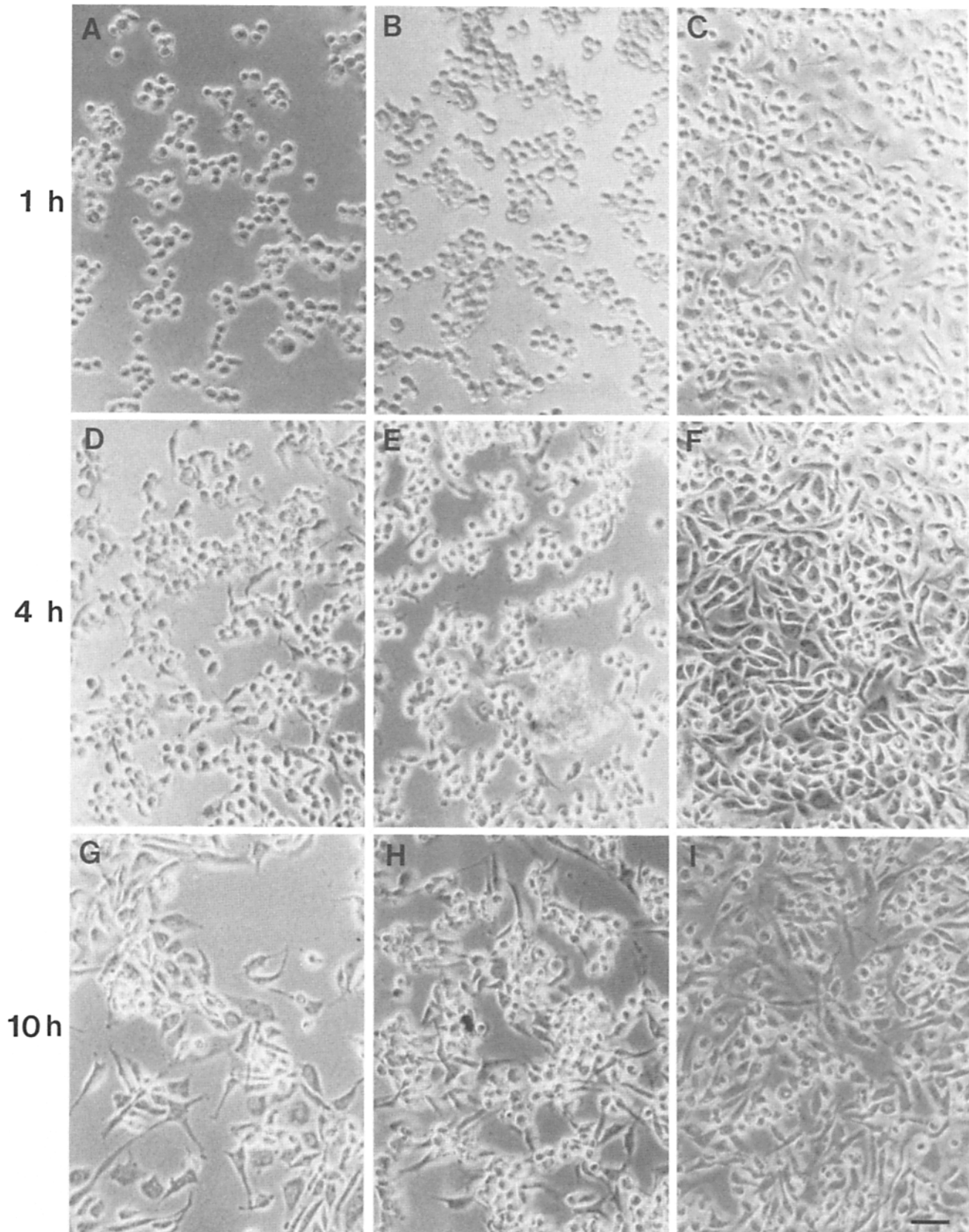
Figure 6. SPARC and a peptide GRGDSP inhibit endothelial cell spreading by independent mechanisms. Freshly trypsinized BAE cells were plated on plastic in DME containing 5% FCS. 10 μg GRGDSP, 10 μg GRGESP, and/or 0.5 μg SPARC were added in a total volume of 200 μl for the indicated times. Cultures shown in *A*, *D*, and *G* received RGD; those in *B*, *E*, and *H* received RGD and SPARC; and *C* and *I* received RGE and SPARC. The cells in *F* were cultured in RGE alone. While both SPARC and RGD caused a similar rounded morphology, after 10 h the effect of RGD was diminished (*G*). In contrast, cells in the presence of RGD plus SPARC remained largely unspread after 10 h (*H*). Bar, 50 μm.
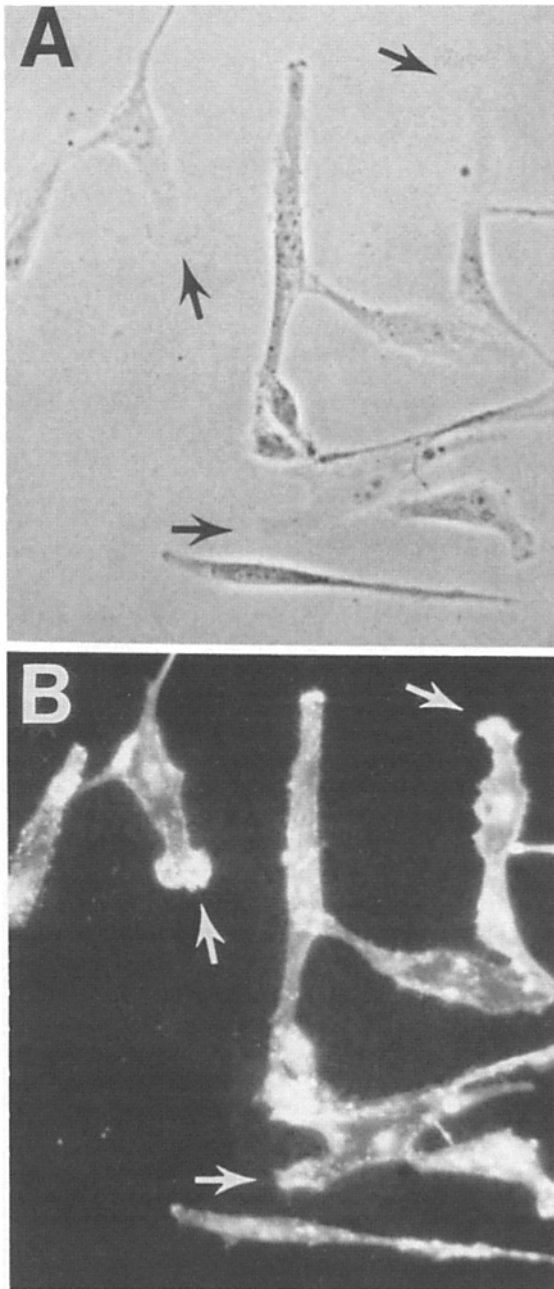
Figure 7. Immunofluorescence localization of SPARC on the endothelial cell surface. To freshly trypsinized cells was added 0.5 μg SPARC; 21 h later, the cells were fixed after several washes and exposed sequentially to rabbit anti-mouse SPARC IgG and FITC-goat anti-rabbit IgG. (*A*) Phase-contrast image of the same field shown as immunofluorescence image in *B*. Arrows indicate areas of cell membrane that are barely visible in *A* but that label strongly with the anti-SPARC antibody in *B*. Bar, 30 μm.

other and possibly for Ca^{+2}, SPARC and type V collagen might interact with a transfer of Ca^{+2}, leading to dissociation of type V collagen and thrombospondin. Alternatively, SPARC could bind to thrombospondin to dissociate the putative complex. Type V collagen could then be cleaved specifically by thrombin (Sage et al., 1981b), followed by a dissolution of the matrix that would facilitate endothelial cell

proliferation. Native thrombospondin, which binds up to 12 Ca^{+2} (with an overall low affinity of ~10^{-4} M) (Lawler and Hynes, 1986), has been shown to promote cell attachment and proliferation, and to function as a platelet lectin (Frazier, 1987). On the other hand, the selectively high affinity of SPARC for type III collagen, a major component of the subendothelium and tunica media of large vessels (Sage, 1986a, and references therein), is suggestive of another mechanism involving this collagen and/or other ligands.

After the experiments described in this report were completed, Clezardin et al. (1988) published similar observations regarding complex formation between thrombospondin and osteonectin purified from bovine bone and human platelets. EDTA-treated osteonectin was inactive, and a K_d of 0.86 nM was reported for binding of soluble thrombospondin to solid-phase–adsorbed osteonectin. Although in these respects our results are similar, Clezardin et al. (1988) were unable to demonstrate competitive binding between thrombospondin and type I collagen, type IV collagen, or BSA for solid-phase–adsorbed osteonectin. We have shown immunologic as well as structural differences between SPARC purified from platelets and cell culture (Mann, K., and H. Sage, unpublished experiments). Alternatively, SPARC adsorbed to microtiter wells is likely to exhibit different properties from that in solution, especially with respect to availability of binding sites that are in turn dependent on a native conformation. Although we have demonstrated binding of SPARC to thrombospondin, on a molar basis this level was low when compared to the affinity of SPARC for collagen, especially types III and V.

Since earlier studies had claimed that osteonectin selectively bound to the organic phase of bone (Termine et al., 1981) and to partially purified preparations of type I collagen (Romberg et al., 1985), we suggest that the binding observed by these investigators was due to these other collagens which have been shown to copurify with type I. Gelatin has been shown not to be a ligand for SPARC (Sage et al., 1984; Domenicucci et al., 1988), while EDTA-extracted SPARC also did not bind to native type I collagen (Domenicucci et al., 1988). This result is consistent with the Ca^{+2}-dependent binding properties of SPARC that we describe in this study.

We presently do not understand the extent or mechanism of Ca^{+2} binding by SPARC. Sequencing of the bovine and

*Table II. Binding of ^{125}I-SPARC to Immobilized Extracellular Proteins**

Protein	SPARC bound/protein
	pmol/pmol
Collagen type I	0.26
Collagen type II	0.62
Collagen type III	1.62
Collagen type IV	0.59
Collagen type V	0.96
BSA	0.022
Fibrinogen	0.11

* Proteins were coated at 5 μg/well; picomoles of protein adsorbed to each well were calculated by amino acid analysis. 7 × 10^4 cpm ^{125}I-SPARC were added per well (2.2 × 10^7 cpm/μg). Replicate determinations (2–4) (Sage et al., 1986; Nicosia and Madri, 1987; Rodgers, 1988) produced values ± 2–12% SEM.
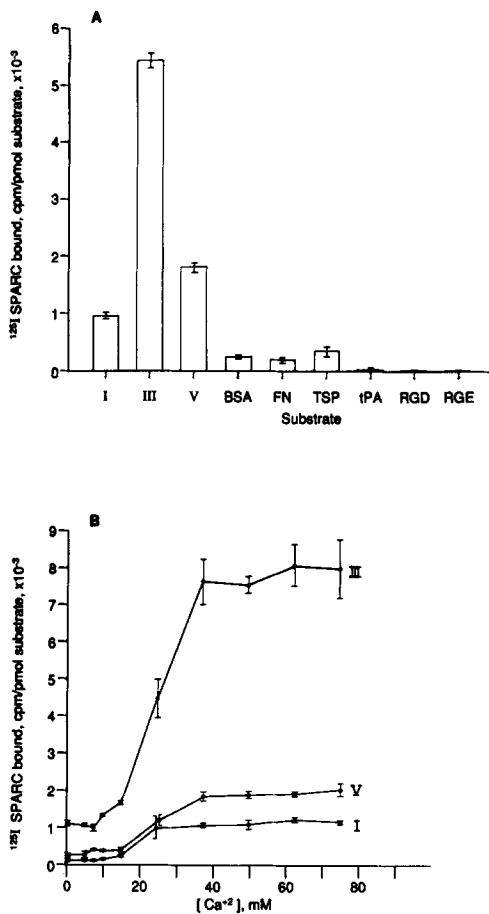
Figure 8. Binding of ¹²⁵I-SPARC to components of the vascular extracellular matrix. (A) Affinity of ¹²⁵I-SPARC for different ligands. Types I, III, and V collagen, BSA, fibronectin (FN), thrombospondin (TSP), tissue plasminogen activator (tPA), and the peptides GRGDSP (RGD) and GRGESP (RGE) were coated onto polystyrene wells at concentrations of 25–50 μg/ml. After additional reactive sites on the plastic were blocked with ovalbumin (1 mg/ml), wells received from 25,000 to 100,000 cpm ¹²⁵I-SPARC and were incubated 10–16 h at 4°C. Subsequent to several washes, each well was detached and counted in a gamma counter. Data shown are from two experiments but are representative of 25 experiments. Error bars show mean ± SEM (n = 2). (B) Ca⁺²-dependent binding of ¹²⁵I-SPARC to collagens. Types I, III, and V collagen were coated as described in A. After the blocking step, wells were washed twice with TBS containing 1 mM EDTA, or TBS containing from 1 to 75 mM CaCl₂. ¹²⁵I-SPARC was subsequently added in the appropriate buffer and allowed to bind from 10 to 16 h at 4°C. Washing was performed with the same binding buffer, and wells were measured individually for radioactivity in a gamma counter. Data (representative of five experiments) are shown from a single experiment (in triplicate) and are plotted as mean ± SEM (n = 3).

mouse genes revealed an unusual degree of hypervariability in exon 3, which codes for domain I, the glutamic acid–rich region purportedly responsible for most of the Ca⁺² bound by this protein (Findlay et al., 1988; McVey et al., 1988). At the present time the significance of this lack of conservation is not known. Despite the prevalence of glutamic acid repeats in domain I, we were unable to demonstrate either the presence of Gla or convincing similarities between SPARC and several vitamin K–dependent coagulation zymogens and

plasma proteins (Friedman and Przysiecki, 1987), matrix Gla protein (Price et al., 1987), or the consensus sequence for the β-hydroxyAsp or β-hydroxyAsn modification described in several coagulation and anticoagulant proteins (Öhlin et al., 1988). In contrast, SPARC domain IV contains a consensus E-F-hand sequence (Engel et al., 1987) which is similar to certain of the Ca⁺²-binding regions of the fibronectin receptor (Hynes, 1987) and thrombospondin (regions 1 and 2) (Frazier, 1987). These regions are referred to as "EF-elbows" and may confer a structural advantage to extracellular proteins which bind Ca⁺² or other metals.

The putative extracellular interaction of SPARC with proteins of the subendothelium could occur without invocation of Ca⁺², since it is possible that the cation subserves an exclusively structural role in the conformation of the SPARC protein. A potential problem with our hypothetical model is the micromolar concentration of Ca⁺² in the cytosol vs. its mM concentration outside the cell. If, as current beliefs pertain, cells are uniformly exposed to millimolar levels of Ca⁺², how can high-affinity binding sites (e.g., an EF-hand with K_d of 10⁻⁶–10⁻⁷ M) release or modulate their Ca⁺²? Although there are few data that directly address this point, we offer the following explanations for the feasibility of our model: (a) The activity of millimolar Ca⁺²-dependent protease found in sarcoplasmic reticulum (0.1–0.2 μM Ca⁺² in the resting state) is thought to depend on specific inhibitor levels (Otsuka and Goll, 1987); (b) microdomains of high density surface charges, created by membrane glycoproteins, are known to render cell surfaces nonuniform; (c) the Ca⁺²-sensitive α-subunit of the vitronectin cell surface receptor projects six EF-hands into the extracellular space, although their role is presently unclear (Hynes, 1987); (d) maximal aggregation of the basement membrane proteins laminin and nidogen (entactin) occurred at 50 mM Ca⁺², although the complex showed 16 binding sites for Ca⁺² with K_d's from 5 to 300 mM (Paulsson, 1988); (e) SPARC may bind another cation competitively (e.g., as Mg⁺² is bound by prothrombin (Deerfield et al., 1987); and (f) in a ternary complex the K_d for Ca⁺² may be affected by phospholipid or another protein (Deerfield et al., 1987; Lambers et al., 1987). A ternary complex involving thrombospondin, plasminogen, and histidine-rich glycoprotein has in fact been reported (Silverstein et al., 1985).

The formation of protein complexes may have relevance to endothelial cell behavior, particularly with respect to the association of enzymes with their inhibitors. Silverstein et al. (1986) have reported that the association of thrombospondin with plasminogen was enhanced by plasminogen activators. Urokinase-type plasminogen activator, a component of local adhesion plaques and migrating endothelium, has been implicated in cellular migration by "directional proteolysis" (Pepper et al., 1987; Pöllänen et al., 1988). The recent finding of Ca-dependent protease II, together with one of its substrates (talin) in adhesion plaques, supports the concept of an extracellular regulatory role for protease–protease inhibitor complexes (Beckerle et al., 1987). Both plasminogen activator–inhibitor and another soluble inhibitor of serine proteases, protease nexin-1, are associated with the extracellular matrix and are thought to control in part its proteolysis (Farrell et al., 1988; Knudsen and Nachman, 1988). Extensive homology searches have failed, however, to identify SPARC with any of these proteins, and activities resembling
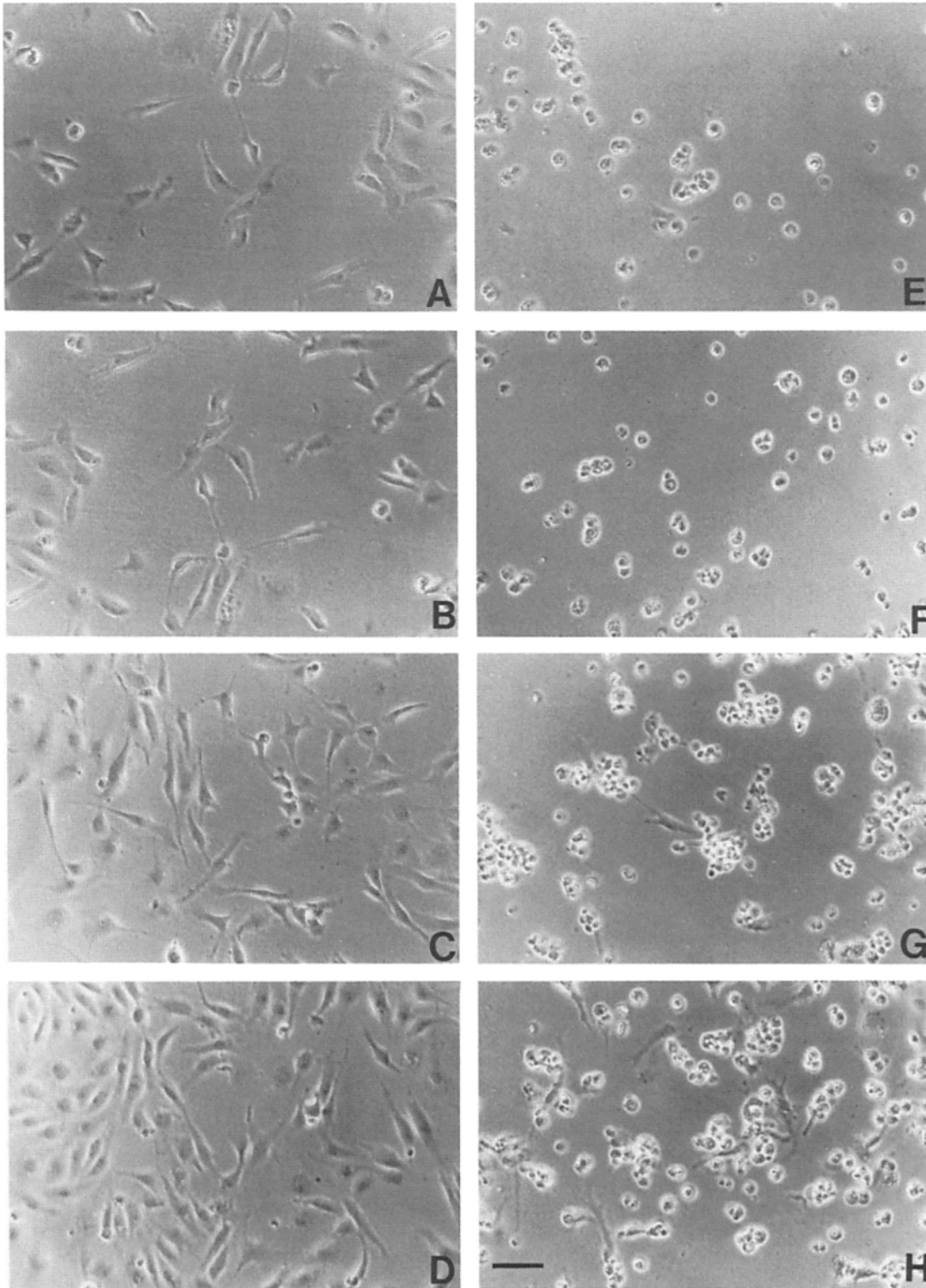
Figure 9. SPARC bound to type III collagen inhibits spreading of BAE cells. Plastic petri dishes were coated with native type III collagen in PBS (200 μg/well) overnight at 4°C, washed five times with PBS, and some dishes were incubated with SPARC (30–50 μg/well) overnight at 4°C. After several additional washes with PBS, trypsinized BAE cells resuspended in DME containing 5% FCS were plated at equal densities onto substrates of type III collagen alone (*A–D*) or SPARC bound to type III collagen (*E–H*). Photomicrographs were taken 24 (*A* and *E*), 48 (*B* and *F*), 72 (*C* and *G*), and 96 h (*D* and *H*) after plating. Bar, 50 μm.

those of tissue factor, plasminogen activator, and plasminogen activator–inhibitor were absent from purified preparations of SPARC (S. Carson, University of Colorado, Denver, CO; and D. Loskutoff, The Research Institute of Scripps Clinic, La Jolla, CA; unpublished experiments). A limited region within SPARC domain II, on the other hand, was found to be related to sequences of the protease inhibitors ovomucoid (third domain), pancreatic secretory trysin–type protease inhibitor, and acrosin inhibitor I (Engel et al., 1987; Bolander et al., 1988). Although this similarity may indicate a role for SPARC as an inhibitor of extracellular proteolytic activity, the sequence conferring inhibitory activity to ovomucoid is not represented in SPARC.

The ability of extracellular matrix proteins to modulate cellular phenotypes and growth has now been amply demonstrated for several matrix constituents (Herman and Castellot, 1987; Ben-Ze'ev et al., 1988; Farrell et al., 1988). Fibronectin has been shown to stimulate the differentiation of neural crest cells into adrenergic cells, to inhibit the differentiation of myoblasts into myotubes, and to promote dedifferentiation of chondrocytes to mesenchymal cells (Ruoslahti, 1988). Laminin stimulates neurite outgrowth and neuronal differentiation (Martin and Timpl, 1987). Processes which regulate endothelial cell angiogenesis in vitro are modulated by components of the basement membrane as well as by a laminin-binding protein (Form et al., 1986; Yannariello-Brown et al., 1988). It is likely that these effects are mediated by cell surface receptors which, in the case of RGD receptors or integrins, function as transmembrane proteins that interact with cytoskeletal elements via talin and vinculin (Buck and Horwitz, 1987; Hynes, 1987). Distribution of these receptors in vitro is partially dependent on the type and accumulation of extracellular matrix (Singer et al., 1988). Occupancy of cell surface receptors may also result in metabolic changes, such as protein phosphorylation, which could, in turn, modulate gene expression (Yarden and Ullrich, 1988).

Our experiments suggest that the rounding effect elicited by SPARC on BAE cells occurred by a mechanism which did not disrupt intercellular adhesion nor RGD receptor-mediated cell–substratum adhesion (Ginsberg et al., 1988). BAE cells are responsive to certain of the components of extracellular matrix. Cheresh (1987) has shown that human endothelial cells synthesize an RGD-directed adhesion receptor that mediated cellular attachment to vitronectin, fibrinogen, and von Willebrand factor. Although the platelet glycoprotein complex GP IIb/IIIa, another member of the integrin family of receptors using the peptide sequence RGD, was shown to facilitate adhesion of human endothelial cells to the BAE extracellular matrix or to purified matrix proteins (Charo et al., 1987; Chen et al., 1987), antibodies to this complex did not affect initial cellular attachment (Chen et al., 1987).

Tenascin (also called cytotactin) (Jones et al., 1988), a secreted protein unrelated to SPARC, also produced a rounded morphology when added to fibroblasts and inhibited the attachment of these cells to RGD-containing proteins such as fibronectin (Chiquet-Ehrismann et al., 1988). In contrast, Herbst et al. (1988) have shown that type IV collagen selectively promoted RGDS-independent adhesion of BAE cells. Since SPARC bound selectively to components of the extracellular matrix which might be used by BAE cells as attachment and/or spreading factors, it is possible that

SPARC would disrupt the association and cause the cells to become rounded. It appears that endothelial cells can attach to substrata by mechanisms other than RGD-directed receptor(s). We propose that SPARC interferes with one or more of these alternate mechanisms.

We also propose that SPARC and thrombospondin share potentially similar roles in the regulation of endothelial cell behavior. Both proteins are secreted at increased levels by sparsely plated, growing cells (Mumby et al., 1984a; Sage et al., 1986). Thrombospondin undergoes receptor-mediated binding and degradation by endothelial cells (Murphy-Ullrich and Mosher, 1987) and has been implicated as an essential component in the growth of SMCs (Majack et al., 1988). Part of this mechanism may involve binding of thrombospondin to sulfated glycolipids of the plasma membrane (Roberts et al., 1985), to a cell surface receptor (Murphy-Ullrich and Mosher, 1987), to bona fide constituents of the extracellular matrix such as type V collagen and heparan sulfate proteoglycan (Mumby et al., 1984b; Frazier, 1987), or to SPARC (Clezardin et al., 1988 and this work). The conformational changes resulting from such interactions could contribute to dissolution of cell–matrix contacts as an autocrine or paracrine response to signals directing cellular migration or proliferation.

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