Functional Mapping of SPARC: Peptides from Two Distinct Ca\(^{++}\)-binding Sites Modulate Cell Shape

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Abstract. Using synthetic peptides, we have identified two distinct regions of the glycoprotein SPARC (Secreted Protein Acidic and Rich in Cysteine) (osteonectin/BM-40) that inhibit cell spreading. One of these sites also contributes to the affinity of SPARC for extracellular matrix components. Peptides representing subregions of SPARC were synthesized and antipeptide antibodies were produced. Immunoglobulin fractions of sera recognizing an NH\(_2\)-terminal peptide (designated 1.1) blocked SPARC-mediated anti-spreading activity. Furthermore, when peptides were added to newly plated endothelial cells or fibroblasts, peptide 1.1 and a peptide corresponding to the COOH terminal EF-hand domain (designated 4.2) inhibited cell spreading in a dose-dependent manner. These peptides exhibited anti-spreading activity at concentrations from 0.1 to 1 mM. The ability of peptides 1.1 and 4.2 to modulate cell shape was augmented by an inhibitor of protein synthesis and was blocked by specific antipeptide immunoglobulins. In addition to blocking cell spreading, peptide 4.2 competed for binding of \(^{[125]}\)SPARC and exhibited differential affinity for extracellular matrix molecules in solid-phase binding assays. The binding of peptide 4.2 to matrix components was Ca\(^{++}\)-dependent and displayed specificities similar to those of native SPARC. These studies demonstrate that both anti-spreading activity and affinity for collagens are functions of unique regions within the SPARC amino acid sequence. The finding that two separate regions of the SPARC protein contribute to its anti-spreading activity lead us to propose that multiple regions of the protein act in concert to regulate the interactions of cells with their extracellular matrix.

Although our understanding of the molecular bases of cell attachment and spreading has increased appreciably, the processes that regulate these phenomena are complex and less well defined. It is clear that cellular adhesion is a dynamic phenomenon. Migratory or transformed cells have fewer focal contacts and exhibit reduced spreading as compared to stationary or nontransformed cells. In nontransformed cells, focal contacts disassemble during mitosis and cell migration, with a resultant temporary change in cell shape (Burridge et al., 1988; Couchman and Rees, 1979; Woods and Couchman, 1988). Molecules that have been implicated as regulators of cell shape because of their effects on focal contacts include proteases, growth factors, phorbol esters, and agents that increase intracellular cAMP (Burridge et al., 1988; Herman and Pledger, 1985; Herman et al., 1987; Schliwa et al., 1984). In addition, several extracellular glycoproteins have recently been described as anti-spreading factors; i.e., proteins that regulate the adhesive characteristics of cells by altering contacts between cells and their extracellular matrix.

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1. Abbreviations used in this paper: BAEC, bovine aortic endothelial cell; CHX, cycloheximide; FCLF, fetal calf ligament fibroblast; SPARC, secreted protein acidic and rich in cysteine; Tsp, thrombospondin.
sive properties that may be cell-specific (Chiquet-Ehrismann et al., 1988; Kruse et al., 1985; Lahav, 1988; Lahav et al., 1987; Tuszynski et al., 1987). Recently, unique adhesive and anti-adhesive regions have been identified in tenasin with fragments of the recombinant protein (Spring et al., 1989). The effects of Tsp, which contains an RGD sequence (Lawler and Hynes, 1986), are apparently mediated via specific cell surface integrins (Lawler et al., 1988). Although tenasin has no RGDS sequences, both Tsp and tenasin interfere with the binding of fibronectin to cells (Chiquet-Ehrismann et al., 1988; Lahav et al., 1987). In contrast, SPARC has no demonstrated adhesive characteristics for cells and disrupts spreading in a variety of cultured cell strains. This anti-spreading activity of SPARC appears to act by an integrin independent pathway (Sage et al., 1989b). That Tsp binds to SPARC with a $K_d$ of 0.7 nM (Clezardin et al., 1988) suggests the possibility of an integrated regulatory pathway.

Synthetic peptides have now been used by a number of investigators to identify functional regions of complex proteins (Stewart and Young, 1984). The identification of adhesive regions of fibronectin (Ruoslahti and Pierschbacher, 1986; Ruoslahti and Pierschbacher, 1987; Dufour et al., 1988) and laminin (Graf et al., 1987a,b; Tashiro et al., 1989; Kleinman et al., 1989) demonstrated the feasibility of peptide reagents for studying cell-substrate adhesion. Important contributions have also been made in the identification of receptor binding sites (Morris et al., 1990) and in the analysis of specific amino acids involved in ligand binding sites (Parraga et al., 1990; Reid, 1990). In addition, peptide immunogens have proven valuable for the production of antisera against molecules for which sequence information was available (Tamura et al., 1983; Kamboj et al., 1989).

Here we report that antibodies raised against a peptide from the NH$_2$-terminal region of SPARC block SPARC-mediated anti-spreading activity. In addition, both the same NH$_2$-terminal peptide and a peptide derived from the COOH-terminal Ca$^{++}$-binding site inhibit cell spreading specifically. These results support the idea that SPARC acts via a Ca$^{++}$-dependent mechanism to modulate cell attachment (Sage et al., 1989b). We propose that this anti-spreading activity results from disruption of cell–substrate contacts and the subsequent reorganization of cytoskeletal elements. The synthesis and release of SPARC may be one of the options available to cells in the process of diminishing adhesive interactions with their substrates. The functional consequences of such actions include the regulation of cell shape, migratory potential, and the ability to divide.

**Materials and Methods**

**Peptide Synthesis and Labeling**

A series of 20-mers representing regions of the mouse SPARC amino acid sequence were synthesized by Dr. Patrick Chou (Howard Hughes Medical

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**Table I. Diagrammatical Representation of the SPARC Protein and the Location of Synthetic Peptides with Their Relative Anti-Cell Spreading Activities**

| Domain | I Ca$^{++}$-Binding | II Cysteine Rich | III Helical Domain | IV Ca$^{++}$-Binding |
|--------|---------------------|------------------|-------------------|---------------------|
|        | CHO                 | COOH             |                   |                     |
| NH$_2$ |                     |                  |                   |                     |
| 1.1    |                     |                  |                   |                     |
| 1.3    |                     |                  |                   |                     |
| 2.1    |                     |                  |                   |                     |
| 2.3    |                     |                  |                   |                     |
| 3.2    |                     |                  |                   |                     |
| 3.4    |                     |                  |                   |                     |
| 4.2    |                     |                  |                   |                     |

**Anti-spreading** + - - - ND - - - +

**B**

| Peptide | Sequence | Sequence location | E$_1$ (1% at 1 cm) |
|---------|----------|-------------------|--------------------|
| 1.1     | QTEVAEEIVE EETVVEETGV | 5-23 | 1.28* |
| 1.3     | QVEMGFEFDG AEETVEEVA | 31-50 | 1.28* |
| 2.1     | QNHHCNHKG KVCELDESNTP | 54-73 | 0.36 |
| 2.3     | TLEGTKGHKH LHLHLDYG | 113-130 | 1.28 |
| 3.2     | KNLVTLTLYER DEGNNLLTEK | 154-173 | 1.28 |
| 3.4     | NEKRLAEGDI PVELLARDFE | 184-203 | 1.28* |
| 4.2     | TDLDNDKITY ALEEWAGCPG | 254-273 | 7.21 |

*Peptides are named with reference to the predicted domain structure of murine SPARC (Engel et al., 1987). (+) Inhibition of spreading; (-) no inhibition observed; (ND) not determined. Bars indicate 20 amino acids. (B) Sequences of synthetic peptides used in these experiments. Sequences were derived from the predicted amino acid sequence of murine SPARC (Mason et al., 1986a). To determine peptide concentration, extinction coefficients (E$_1$ (1% at 1 cm)) were calculated based on amino acid composition (Gill and von Hippel, 1989). Cysteine residues are underlined. (*) Extinction coefficients derived for peptides synthesized with an additional NH$_2$-terminal tyrosine residue. The single letter amino acid code has been used.
Antibody Production and Characterization

Peptides were selected for antibody production based on our interest in their Ca++-binding properties (1.1, 1.3, and 4.2) or based on predicted antigenicity. Antigenicity was assessed by the hydrophilicity algorithm of Hopp and Woods (1981). The sequence YERDEG (aa 161-166) contained within peptide 1.3 is predicted to have high Ca++ binding capacity. Peptides were conjugated to keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO) with carbodiimide (Story Chemical Corp., Princeton, NJ) and purified immunoglobulin fraction from each antiserum (see above). BAEC were trypsinized, washed in 10% FCS/DME, washed in 1% FCS/DME, and subsequently plated on 24- or 48-well plates of tissue culture plastic (Costar, Cambridge, MA) in the presence of SPARC (1-40 μg/ml) or peptides (0.1-2 μg/ml) as described in the figure legends. In some cases, antibodies were preincubated with 500 μg/ml of a mixture of N-KLH, C-KLH, and free peptide (1:1:1) in PBS. Antibodies were added and immune complexes, was then added to BAEC and cell morphology was monitored for 24 h.

Effects of SPARC Peptides on Cellular Morphology

BAEC and FCLF were cultured as described above. Morphological effects of SPARC and peptides were determined by two procedures. (a) Cells were trypsinized, washed in 10% FCS/DME, washed in 1% FCS/DME, and subsequently plated on 24- or 48-well plates of tissue culture plastic (Costar, Cambridge, MA) in the presence of SPARC (1-40 μg/ml) or peptides (0.1-2 mM). For some experiments, peptides were preincubated with anti-peptide antibodies. Control experiments showed that spreading was not inhibited by antibodies alone. (b) Trypsinized cells were plated for 24 h on glass chamber slides (Lab-Tek, Nunc, Inc., Naperville, IL) in 10% FCS/DME. Cells were washed in serum-free DME and peptides added as above. Media were replaced with fresh 10% FCS/DME containing 5 μg/ml cycloheximide (CHX/DME; Sigma Chemical Co., St. Louis, MO) for 1 h after which peptides diluted in CHX/DME were added in fresh media to the cells. Cell morphology was assessed by visual inspection using an inverted phase photomicroscope (Carl Zeiss Inc.) and the images recorded on Ektachrome film (ET 135, 160 ASA Kodak). Cells surrounding untreated cells plated under similar conditions, were classified as "rounded." For quantitation of anti-spreading activity of peptides on freshly plated cells (procedure 1), a scoring system was devised to characterize the degree of spreading within a group of cells. Unattached cells were removed by washing and the culture was photographed as above. Cell counts were determined and cells were scored as (a) spread, flattened cells with diminished cellular refractivity; (b) unspread, rounded cells projecting short processes in the initial stages of spreading; and (c) round, highly refractile cells with no apparent processes. Cells representative of these three groups are indicated in Fig. 4. The number of cells in each group was then converted into a "rounding index" by the formula: rounding index = [(1 × a) + (2 × b)]
and were visualized by fluorescent autoradiography. Arrow indicates the location of the SPARC doublet.

Figure 1. Anti-peptide 1.1 IgG precipitates SPARC from tissue culture media. PYS cells were labeled for 12 h with [35S]Met, and the media were centrifuged and treated with protease inhibitors. Proteins were resolved by SDS-PAGE of (lane 1) unfractionated media; (lane 2) anti-SPARC immunoprecipitate; (lanes 3 and 4) anti-peptide 1.1 immunoprecipitate, serum from two different rabbits; (lanes 5 and 6) anti-peptide 4.2 immunoprecipitate, serum from two different rabbits. Proteins were resolved in the presence of DTT and were visualized by fluorescent autoradiography. Arrow indicates the location of the SPARC doublet.

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\frac{+ (3 \times c)}{(a + b + c)}, \text{ where } a, b, \text{ and } c \text{ are the number of cells in each group. An index of 1 thus represents a culture with only spread cells. A culture with increasing numbers of unspread and round cells would approach the maximum rounding index of 3. Values were calculated for two independent cultures and graphed as the average \pm SE.}
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Binding to Purified Extracellular Proteins

Solid-phase binding assays were conducted in polystyrene microtiter wells as previously described (Sage et al., 1989b). BSA (Pentex grade; Miles Laboratories, Inc., Naperville, IL) and ovalbumin (Sigma Chemical Co.) were solubilized in TBS. Native collagen types I (lathyritic rat skin), II (bovine cartilage), III (bovine skin), IV (bovine placenta), V (human amnion), and VIII (bovine Descemet's membrane) were isolated as described (Sage et al., 1989b). SPARC and Tap were prepared as described (Sage et al., 1989b). Collagens were solubilized in 0.1 N acetic acid, and dialyzed against TBS. Solubilized proteins were coated onto microtiter wells for 4-12 h (Remowell strips, Immulon; Dynatech Laboratories Inc., Alexandria, VA).

To assay for peptides that competed for the binding of SPARC to collagens, collagen types I and III were coated on microtiter wells and additional binding sites were blocked with Hepes binding buffer (20 mM Hepes, 130 mM NaCl, 1 mM CaCl₂, 3 mM KCl, 1 mM MgCl₂, 0.1 mM CuSO₄) containing 0.1% ovalbumin (Bade et al., 1989). [125I]-labeled SPARC was added to wells containing substrate-adsorbed proteins for 4-12 h, at 4°C. Wells were washed three times with Hepes binding buffer and bound radioactivity was measured by a gamma counter (Beckman Instruments Inc., Palo Alto, CA). Assays were done in triplicate and values graphed as the mean \pm SD. To determine specific binding, excess unlabeled ligand was mixed with labeled ligand before addition to substrate-coated wells. In competition assays, label was mixed with individual peptides before addition to wells.

To determine whether peptides would bind extracellular matrix components directly, purified proteins were plated in microtiter wells and excess binding sites blocked with TBS containing 1% ovalbumin. [125I]-labeled peptides were then added for 4-12 h, at 4°C with or without 5 mM CaCl₂; unbound radioactivity was removed with TBS containing 5 mM CaCl₂ and 1% ovalbumin. Quantitation of substrate protein bound to plates was performed by amino acid analysis of material solubilized by constant boiling HCl at 110°C. Bound radioactivity was corrected for pmoles of substrate on each well. Assays were done in triplicate, and values were graphed as the mean \pm SD.

Results

Anti-peptide Antibodies Recognize SPARC in a Conformation-specific Manner

In preliminary studies, affinity for immunizing (homologous) peptide and for native SPARC were assessed by ELISA (data not shown). All sera reacted with homologous peptides (titers of 10⁻³-10⁻⁴) but not with heterologous peptides. Furthermore, sera did not react with BSA, ovalbumin, or 70 K protein (a serum protein often bound to native SPARC). Only anti-peptide 1.1 antibodies reacted with native SPARC in these assays. Antibodies displayed negligible cross-reactivity with heterologous peptide-rabbit serum albumin conjugates. Reactivity to SPARC depended on the peptide immunogen and did not depend on the immunized animal (n = 2).

To confirm these results, radioimmune precipitations were performed on media from PYS cells. Anti-SPARC (Fig. 1, lane 2) and anti-peptide 1.1 antibodies from two different rabbits (Fig. 1, lanes 3 and 4) precipitated SPARC as a doublet (due to differential glycosylation) from these cultures. Antibodies generated to other peptides did not precipitate the 43-kD species (Fig. 1, lanes 5 and 6). Radioimmune precipitations of [125I]-labeled SPARC were also performed. Precipitations with anti-SPARC and anti-peptide 1.1 antibodies precipitated 43-kD SPARC. Preincubation with anti-peptide 1.1 antibodies with 0.2 mM peptide 1.1 competed for this binding by >95%. Antibodies against peptide 3.2 had no reactivity and antibodies against peptide 4.2 precipitated only trace amounts of the [125I]-labeled SPARC (data not shown).

Upon reduction or certain types of denaturation, additional epitopes were exposed. Western blots of partially purified SPARC (Fig. 2 b) confirmed that unreduced SPARC was specifically recognized by anti-peptide 1.1 antibodies and to a lesser extent by anti-peptide 1.3 antibodies, but not by antibodies raised against other peptides. After reduction, SPARC was recognized by all antibodies (Fig. 2 b). These results, which are summarized in Table II, suggested that the anti-peptide antibodies showed conformational specificity and that denatured SPARC does not display the same epitopes present on the native molecule. Only the NH₂ terminus of native SPARC seemed to provide an antigenic surface similar to that of its homologous synthetic peptide (1.1). Because SPARC isolated from tissues is often proteolyti-
Figure 2. Anti-peptide antibodies recognize SPARC in a regional and conformation-specific manner. (a) Partially purified SPARC was subjected to limited proteolysis. Fragments were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antisera. (C) Reaction without enzyme; (T1) 0.1 μg trypsin; (T2) 2 μg trypsin. 43-kD SPARC (SP) is cleaved to 28-kD NH2-terminal fragment and a 10-kD COOH-terminal fragment (arrows). The 70-kD protein (70K) recognized by anti-SPARC (αSPARC) polyclonal sera is a serum protein structurally unrelated to SPARC (Sage et al., 1986). Replicate blots were probed with anti-SPARC (αSPARC) polyclonal sera, antipeptide 1.1 (α1.1); and anti-peptide 4.2 (α4.2). (b) Samples of SPARC were resolved by SDS-PAGE in the absence (−) or presence (+) of a reducing agent (DTT) and transferred to nitrocellulose. Blots were probed serially with sera, stripped with 0.2 M glycine (pH 2.2), and reprobed. Blots were exposed to film for 20 h, and stripped blots were exposed for 48 h before incubation with second antibody to ensure no carryover between probings.

Anti-peptide Antibodies Recognize SPARC

Peptide 1.1 antibodies blocked the anti-cell spreading activ-

Table II. Recognition of SPARC by Anti-peptide Antibodies is Dependent on Conformation

| Antibody reactivities | Immuneogen | Self | Native SPARC | Reduced SPARC | Block SPARC rounding |
|-----------------------|------------|------|--------------|---------------|---------------------|
|                       |            |      |              |               |                     |
| 1.                    | 1.1        | ++   | ++           | ++            | ++                  |
| 2.                    | 1.3        | ++   | ++           | ++            | ++                  |
| 3.                    | 2.1        | ++   | ++           | ++            | ++                  |
| 4.                    | 3.2        | ++   | ++           | ++            | ++                  |
| 5.                    | 3.4        | ++   | ++           | ++            | ++                  |
| 6.                    | 4.2        | ++   | ++           | ++            | ++                  |
| 7.                    | SPARC      | ++   | ++           | ++            | ++                  |

Anti-peptide antisera specific for different regions of the SPARC molecule were scored in three separate assays for their ability to bind the intact SPARC protein.

* Specific binding to each immunogen was measured by ELISA (Self).

Binding to native SPARC was measured by an immunoprecipitation assay.

Blocking to denatured SPARC was measured after reduction and Western blotting.

Ability to inhibit bioactivity of SPARC was measured by preincubating SPARC with antisera before adding to cells.

(+): Positive; (−): negative.
Figure 3. Anti-peptide 1.1 IgG blocks SPARC-mediated anti-spreading activity. BAECs were plated for 24 h on glass coverslips in 10% heat-inactivated FCS/DME. Cells were then washed in 1% heat-inactivated FCS/DME, and treated with 10 μg/ml SPARC for 4 h. (a) SPARC alone; (b) preincubated with anti-SPARC immunoglobulins; (c) preincubated with anti-peptide 1.1 immunoglobulins; (d) preincubated with anti-peptide 4.2 immunoglobulins. Cells incubated with PBS alone, or with immunoglobulins alone, were indistinguishable from untreated cells (data not shown).

ity of SPARC (Fig. 3). BAEC were plated for 24 h in media containing 10% heat-inactivated FCS. After treatment for 4 h with 10 μg/ml SPARC, most cells had assumed a rounded morphology (Fig. 3 a). Pretreatment of the SPARC with anti-SPARC antibodies (Fig. 3 b) or anti-peptide 1.1 antibodies (Fig. 3 c) blocked this activity. However, anti-peptide 4.2 antibodies were ineffective at blocking SPARC (Fig. 3 d). The fact that the anti-peptide 1.1 antibodies could neutralize SPARC activity suggested that the NH2 terminus of SPARC could participate in the anti-spreading activity of SPARC. To test whether peptide 1.1 or other peptides had a direct effect, peptides were added directly to cells in culture.

Peptides Corresponding to Ca++-binding Regions Inhibit Cell Spreading

BAEC and FCLF were trypsinized and plated on tissue culture plastic in the presence of 1% FCS and increasing amounts of peptide. Under these conditions, control BAEC assumed a spread morphology within 1 h (Fig. 4 a), and FCLF within 20 min (Fig. 4 c). In the presence of 1% FCS, peptide 1.1 at a concentration of 0.8 mM permitted attachment, but not spreading, of 70% of the BAEC after 3 h (Fig. 4 b). Peptide 4.2 had a similar effect on 85% of the cells (Fig. 4 d); i.e., 85% of the cells displayed a rounded phenotype. The effect was no longer apparent after 24 h (data not shown). Peptide 3.2 (Fig. 4 e), as well as peptides 1.3, 2.1, and 3.4 (data not shown), had no effect up to concentrations of 1.5 mM peptide.

FCLF were two to five-fold more sensitive with respect to the anti-spreading effect of peptides 1.1 and 4.2. When FCLF were plated on dishes coated with type I collagen, 0.3 mM peptide 1.1 or 4.2 was capable of inhibiting spreading in 80–90% of the cells (Fig. 4, f and h) 2.5 h after plating; in contrast, peptide 3.2 exhibited no effect on spreading (Fig. 4 g). Preincubation of 1 mM peptide with homologous anti-peptide immunoglobulins eliminated the anti-spreading effect of peptides 1.1 and 4.2 on both cell types. The use of heterologous antibodies did not block the anti-spreading effect. These observations argue strongly that the anti-spreading effects are due to specific actions of the peptides themselves and not to trace contaminants in the peptide preparations or to cytotoxicity.

The dose response of BAEC to peptides was assessed by scoring the degree of cell reattachment and spreading in newly plated cultures. BAEC displayed a dose-dependent response to peptides 1.1 and 4.2, but not to inactive peptides or to an equivalent amount of PBS carrier (Fig. 5). 3 h after plating, control cells had a rounding index of 1.12 (SE ± 0.033) consistent with cells that were completely spread. In contrast, cultures exposed to 0.6 mM peptide 1.1 had an index of 1.39 (+0.062) that was indicative of a significant decrease in cell spreading (p < 0.05). 1.0 mM peptide 1.1 still permitted cell spreading, but the process was delayed. By 3 h, 21.1% of cells exposed to 1 mM peptide 1.1 were scored as spread. By 24 h, the effect of the peptide was overcome. For comparison, only 7.1% of cells exposed to 1.0 mM peptide 4.2 were spread at 3 h. There was no statistical difference between cells treated with peptide 3.2 and control (PBS) at 3 or 24 h. The morphological effects of all peptides used in this study are summarized in Table I.

Serum Concentration and Preplating Diminished the Anti-spreading Activity

Initial studies had shown that BAEC, preplated 20 h in 10% FCS, did not change shape in response to peptides at a concentration of 0.8 mM in the presence of 2.5% FCS (see for
Figure 4. SPARC peptides 1.1 and 4.2 delay spreading of endothelial cells and fibroblasts. BAECs were plated on plastic dishes in 1% FCS/DME ± peptides (a, b, c, and d). Cells were photographed after 3 h. (a) PBS control; (b) 0.8 mM peptide 1.1; (c) 1.0 mM peptide 3.2; (d) 0.8 mM peptide 4.2. FCLFs were plated on collagen coated dishes in 1% FCS/DME ± peptides (e, f, g, and h). Cells were photographed after 2.5 h. (e) PBS control; (f) 0.3 mM peptide 1.1; (g) 0.3 mM peptide 3.2; (h) 0.3 mM peptide 4.2. Peptides derived from other regions of SPARC were inactive in this assay when added at concentrations up to 1.5 mM (data not shown). Open arrow (→) indicates a spread cell (group a); solid arrowhead (▲) indicates a cell that has begun to spread (group b); and the solid arrow (→) indicates an attached round cell that has not initiated spreading (group c).

Figure 5. The antispreading activity of peptides 1.1 and 4.2 is dose dependent. Peptides were added to BAECs released from their substrate by brief exposure to trypsin. After 3 h, cultures were photographed and scored for degree of cell spreading. A score of 3 indicates rounded cells that have not initiated spreading, while a score of 1 indicates fully spread cells. Further details of the scoring procedure are given in Materials and Methods. (●) Peptide 1.1; (▲) peptide 1.3; (●) peptide 3.2; (●) peptide 4.2; (▲) PBS control.

Example Fig. 6, c and e). However, when the peptides were presented in serum-free medium, cells rounded up and remained attached but unspread (not shown). This result suggested that serum factors, or a metabolic activity of the cells supported by serum, affected the anti-spreading activity of the peptides. To analyze the role of cellular metabolism on SPARC function, we presented the SPARC and peptides to cells pretreated with an inhibitor of protein synthesis, cycloheximide (CHX). CHX (5 μg/ml) pretreatment inhibited protein synthesis by >95%. SPARC or peptides were added to control BAEC or to CHX-pretreated BAEC in 2.5% FCS, plus or minus CHX. After 2 h, cells were fixed and photographed (Fig. 6). CHX markedly augmented the response to added SPARC (compare Fig. 6 a; 10 μg/ml SPARC) with Fig. 6 b (0.02 μg/ml SPARC). The activities of peptides 1.1 (Fig. 6 d) and 4.2 (Fig. 6 f) were also enhanced, since these peptides had little effect on preplated cells in the presence of serum (Fig. 6, c and e). CHX pretreatment had no effect on the morphology of control cells or on cells incubated with peptide 3.2 (not shown). These observations suggested that, while SPARC can affect metabolically active cells, it is significantly more active when protein synthesis is reduced. The failure of peptides 1.1 and 4.2 to round previously spread, metabolically active cells may be due to the diminished activities of these peptides when compared to native SPARC.
Figure 6. Antispreading activity of SPARC and peptides is augmented by inhibition of protein synthesis. BAECs were plated for 24 h on glass coverslips in 10% FCS/DME. Cells were then preincubated for 1 h with 2.5% FCS/DME alone (a, c, and e), or in the same media containing 5 μg/ml CHX for 1 h to block protein synthesis (b, d, and f). Media were then replaced with fresh media containing SPARC or peptides. After 2 h, cells were fixed and photographed. (a) 10 μg/ml SPARC; (b) 0.02 μg/ml SPARC; (c and d) 0.8 mM peptide 1.1; (e and f) 0.8 mM peptide 4.2.

Peptide 4.2 Competes for the Binding of SPARC to Purified Collagens and Binds to Collagens in a Ca²⁺-dependent Manner

To look more closely at the interactions of SPARC with proteins in the extracellular matrix, we iodinated SPARC and peptides and assayed their binding to various collagen substrates in solid-phase binding assays. In competition assays, peptide 4.2, but not peptide 1.1, competed with [¹²⁵I]SPARC for binding to type III collagen (Fig. 7a). 46% of the specific binding was competed by addition of a 50-fold molar excess of peptide 4.2 over SPARC, and addition of a 250-fold molar excess of peptide 4.2 competed for 59% of the specific binding. There was no additional competition, even when a 1,250-fold molar excess of peptide 4.2 over SPARC was added (Fig. 7a). Similar effects were seen on type I collagen even though the total binding was less than that observed for type III collagen (Table III). Addition of 1 mM EGTA (fourfold molar excess over available Ca²⁺) eliminated 100% of the specific binding of SPARC to these substrates. Nonspecific binding was 39% in this assay, determined with a 60-fold molar excess of unlabeled SPARC.

When peptides were iodinated directly, peptides 1.1 (with an NH₂-terminal Tyr residue added) and 3.2 failed to bind
Figure 7. Peptide 4.2 competes for binding of SPARC and binds to collagens in solid phase binding assays. [125I]Peptides and [125I]-SPARC were tested for binding to various purified collagens in solid phase binding assays. Test proteins were bound to plastic microtiter wells and the remaining sites were blocked with ovalbumin. (A) Wells were coated with type III collagen. Blocked wells were then exposed to [125I]SPARC, in the presence or absence of cold competitor SPARC (I), peptide 1.1 (O), or peptide 4.2 (0). Unbound counts were washed off and bound 125I was determined in a 3'–counter. Values were expressed as percent of control after correction for nonspecific binding. Values are the average of triplicate determinations. (B) Wells were coated with various collagens (Types I, II, III, IV, V, and VIII), ovalbumin (Ov), or BSA. Blocked wells were then exposed to [125I]peptide 4.2 in the absence of (black bars) or presence of (hatched bars) 5 mM CaCl2. Unbound counts were subsequently washed away with buffer containing 5 mM CaCl2. Bound 125I was determined in a γ-counter. Values were expressed as percent of control after correction for nonspecific binding. Values are the average of triplicate determinations, ± SD.

Table III. Specific binding of [125I]SPARC to Collagen Substrates is Inhibited by Peptide 4.2

| Substrate | Competitor | Specific binding (SD) % |
|-----------|------------|-------------------------|
| Type I Collagen | None | 100 (0.9) |
| | SPARC | 0.0 (1.3) |
| | Peptide 4.2 | 57.8 (35.2) |
| | 1 mM EGTA | -3.5 (5.0) |
| Type III Collagen | None | 100 (9.4) |
| | SPARC | 0.0 (8.5) |
| | Peptide 4.2 | 40.9 (6.4) |
| | 1 mM EGTA | -0.6 (5.0) |

Substrate proteins were plated on microtiter wells and nonspecific binding sites were blocked with 0.1% ovalbumin. [125I]SPARC was added in the presence or absence of cold competitor. Unbound radioactivity was washed away and bound [125I] counted in a γ counter. Measurements are averaged from three wells. In three independent experiments, the average nonspecific binding (NSB) was 35.6% on type I collagen and 39.1% on type III collagen.
binding domain, both inhibited cell spreading when added to primary cultures of endothelial cells or fibroblasts. The effect was dose dependent, augmented by the presence of CHX, and inhibited by addition of homologous antipeptide antibodies. Peptides from other regions of the molecule had no effect. Addition of FCS prevented rounding of previously spread cells by peptides, but this effect of serum could be overcome by pretreating the cells with CHX. We have performed other experiments that show that the peptides themselves do not markedly alter the levels of total protein synthesis, although there are alterations in the levels of mRNA and protein corresponding to specific macromolecules (T. F. Lane, M. L. Iruela-Arispe and H. Sage, manuscript in preparation); the activity is therefore apparently not the result of cytotoxicity.

Antibodies raised against peptide 1.1 blocked SPARC-mediated anti-spreading activity. This result suggested that the NH₂ terminus of the protein was required for biological activity. We were also interested in the structure of SPARC as revealed by antibodies directed against other regions of the molecule. However, the pattern of anti-peptide immunoreactivity was found to be highly dependent on the conformation of SPARC. In fact, out of six anti-peptide antibodies raised in this study, only those against peptide 1.1 recognized native SPARC in solution. Antibodies raised against other regions required reduction of SPARC to expose relevant epitopes. Interestingly, Mason et al. (1986a,b) described antibodies to a peptide corresponding to the COOH-terminal 11 amino acids of murine SPARC. The sequence of this peptide does not overlap with peptide 4.2. Unlike antibodies to peptide 4.2, the antibodies to the COOH-terminal peptide immunoprecipitated SPARC from cell culture media. The immunoreactivity data suggest that the NH₂-terminal Glu-rich region of SPARC is exposed to the solvent and maintains a conformation that is little affected by the remainder of the protein. Since peptide 4.2 competes for the binding of SPARC to collagens, it appears that this domain is exposed to solvent as well. Conservation of sequence and lack of immunogenicity in the EF-hand domain suggest, however, that the exposed portion is not the sequence recognized by the antibodies we have characterized to date.

Although the sequence represented by peptide 3.2 is predicted to be antigenic by hydrophilicity analysis (Hopp and Woods, 1981), antibodies raised to peptide 3.2 failed to recognize native SPARC and reacted poorly with the denatured protein. Tainer et al. (1984) have suggested that the cross-reactivity of anti-peptide antibodies with native protein is a function of the relative mobility of the corresponding site in the protein. Their study suggests that highly ordered regions would be poorly recognized by anti-peptide antibodies. If this analysis can be extended to SPARC, then one might predict that region 3 (peptide 3.2) and the EF-handlike domain (peptide 4.2) are well-ordered regions of SPARC, whereas the NH₂ and COOH termini are not. The conformational dependence shown by the antipeptide antisera used in this study would support a model of SPARC as a highly ordered protein. Most peptide epitopes were masked in the native structure but became exposed after reduction. A high degree of internal structure could help to explain the high percentage of conserved residues in SPARC. More precise structural studies will be required to understand the native conformation of the SPARC protein. Such studies will improve our understanding of how the Ca⁺⁺-binding sites function in the disruption of cell-substrate contacts.

The observation that anti-spreading activity is augmented by CHX is the first observation of its kind for SPARC. Interestingly, blockage of protein synthesis increases the activity of other proteins that have anti-spreading activity in endothelial cells, such as Tsp (Murphy-Ulrich and Höök, 1989). Pretreatment with CHX, followed by exposure to native SPARC, resulted in a 50-100-fold increase in anti-spreading activity when compared to cells in which protein synthesis was not affected. Similar effects were seen in serum-free medium in the absence of CHX, but addition of serum to CHX-treated cells did not alter the effect seen with CHX alone. These data suggest the involvement of newly synthesized proteins required for the processing of SPARC or for signal transduction. Specific models of how this effect is mediated could include the inactivation of SPARC by a newly activated protease, or in the turnover of cell surface and extracellular matrix components bound by SPARC. The synthesis of inhibitors of SPARC, or of a protease activity that inactivates SPARC, could be important factors regulating the concentration of SPARC needed to affect cells in vivo.

SPARC has been recognized for its ability to bind extracellular matrix components (Termine et al., 1981; Romberg et al., 1985; Clezardin et al., 1988; Sage et al., 1989b). On a molar basis, native SPARC binds preferentially to type III collagen, but also binds to other collagen types including I, II, IV, and V (Sage et al., 1989b). In the present study, we used the binding of SPARC to type III collagen as an assay to identify peptides that could affect this process. Peptide 4.2 competed for >50% of the binding of SPARC to type I and type I collagen. In addition, peptide 4.2 showed specific binding to collagens in a Ca⁺⁺-dependent fashion, but did not bind albumin, ovalbumin, SPARC, or fibronectin. While the intact SPARC protein discriminates among collagens, showing a preference for type III collagen (Sage et al., 1989b), peptide 4.2 did not appear to make this distinction. The ability of intact SPARC to discriminate among collagens may thus be dependent on its three-dimensional structure. Other regions of the protein that contribute to the specificity of the collagen binding site may be required. Ongoing studies are being carried out with larger peptides to test this hypothesis. Our present data suggest that a COOH-terminal region of SPARC, which includes the Ca⁺⁺-binding site represented by peptide 4.2, plays a role in the binding of SPARC to collagens. Continued work with the peptides may identify other regions of SPARC that interact with components of the extracellular matrix, and may lead to additional insights into the structure of active sites within the SPARC protein itself.

While peptide 4.2 competed for the binding of SPARC to collagens at concentrations within one order of magnitude of those required by the intact protein, clearly much larger doses of peptides were required to mimic the effects of native SPARC on cell spreading. The apparent requirement for high concentrations of peptides, relative to those needed for activity of the native protein, is not unique to SPARC. Peptides derived from the cell binding region of fibronectin (RGDS) and laminin (YIGSR), at concentrations between 0.1 and 4 mM, inhibited attachment, while nanomolar concentrations of the native protein displayed similar efficiencies in the assay (DuFour et al., 1988; Chen et al., 1987; Kleinman et al., 1989). Studies that used peptides to map receptor bind-
ing sites claimed that concentrations 10^4-fold greater than that of the native competitor were necessary to inhibit binding (Morris et al., 1990; Longo et al., 1990). The requirement for such high concentrations of a fragmented protein is often attributed to the existence of multiple sites within the native protein that act synergistically to bind receptors (Dufour et al., 1988; Kleinman et al., 1989; Morris et al., 1990). In addition, peptides are likely to occur in several conformations in solution. We interpret the need for high amounts of peptide as a direct consequence of the loss of cooperative sites residing in other regions of the protein and the diminution of conformational constraints provided by the native protein environment.

Even with the limitations that protein fragmentation affords, the ability of specific peptides to modulate cell attachment is now widely appreciated. Anti-spread activity has been reported in peptides derived from fibronectin (Ruoslati and Pierschbacher, 1986; Dufour et al., 1988), laminin (Graf et al., 1987a, b; Kleinman et al., 1989) and in recombinant fragments of tenascin (Spring et al., 1989). In the case of fibronectin, peptides containing the sequence RGDs form the recognition site for cell surface receptors of the integrin class (for review, see Hynes, 1987; Ruoslati and Pierschbacher, 1987). Proteins such as von Willebrand factor (Dejana et al., 1989; Titani et al., 1986), fibrinogen (Cheresh et al., 1989), and Tsp (Lawler et al., 1988) also have functional RGD-containing sequences. Interestingly, while these RGD domains occur in various proteins, the proteins themselves mediate diverse spreading effects in various cells. These effects may be mediated by different cell surface receptors but are almost surely due to different properties of the intact proteins as well. For example, Tsp allows cell attachment by an RGD-dependent mechanism (Tuszynski et al., 1987) but prevents spreading (Lahav, 1988; Lawler et al., 1988; Murphy-Ullrich and Höök, 1989). Tenascin contains no RGD sequences but has been shown to prevent the interaction of fibronectin with cell surface integrins. It has been proposed that tenasin, by virtue of its size and complex hexabrachion structure, acts by imposing a physical barrier between cells and their substrates (Chiquet-Ehrismann et al., 1988).

It is possible that various extracellular proteins function to diminish the affinity or accessibility of cell–extracellular matrix interactions. Given the need for high concentrations of many extracellular proteins (required for tissue structure and hydrodynamics), as well as a requirement for cells to carry specific receptors for these proteins, it seems reasonable to propose that a class of proteins exists that modulates the ability of cells to interact with the extracellular matrix. Negative modulators of cell–matrix interactions could provide a mechanism for regulation of the overall level of cell–extracellular matrix binding without (or in concert with) the release of degradative enzymes or the reduction of cell surface receptors. We propose that SPARC is a new member of this class of modulators, which also includes Tsp (Murphy-Ullrich and Höök, 1989) and tenascin (Chiquet-Ehrismann et al., 1988). SPARC contains no RGD sequences and there are no data to indicate that it acts via an integrin-mediated mechanism. The nature of the cell surface binding site for SPARC remains to be determined.

The appearance of SPARC mRNA by day 9 of development in the mouse (Howe et al., 1988; Mason et al., 1986b), as well as the tissue distribution of SPARC mRNA and protein (Holland et al., 1987; Wewer et al., 1988; Sage et al., 1989), suggested to us that SPARC is associated with cellular events requiring tissue remodeling, cell movement, and/or proliferation. The presence of large amounts of SPARC in the peritoneal layer of bone after day 13 of mouse development (Nomura et al., 1988) and in certain invasive tumors (Mann et al., 1987) is consistent with this interpretation. The identification of anti-spreading activity in SPARC was the first function proposed that suggested a unified role for the protein in the various tissues and cultured cells in which it is seen (Sage et al., 1989b). Thus, cells in early somites, gut epithelium, or remodeling bone could all require SPARC to modulate their interactions with extracellular matrix. We have now proposed that the synthesis and release of SPARC is one of the options available to cells in the process of diminishing adhesive interactions with their substrate. The regulation of cell shape, migratory potential, and the ability to divide are only a few of the biological consequences. In the present study, we have confirmed the observation of anti-spread activity of SPARC for BAEC and FCLF cells. We have also shown that unique regions of the protein may contribute to this activity by binding extracellular matrix components and preventing cellular spreading. We are currently investigating the interaction of SPARC with membrane-bound molecules, to identify a pathway by which the presence of SPARC might be linked to the regulation of specific genes.

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