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SPARC Inhibits Endothelial Cell Adhesion But Not Proliferation Through a Tyrosine Phosphorylation-Dependent Pathway

Kouros Motamed and E. Helene Sage*

Department of Biological Structure, School of Medicine, University of Washington, Seattle, Washington 98195

Abstract SPARC, a counteradhesive matricellular protein, inhibits endothelial cell adhesion and proliferation, but the pathways through which these activities are blocked are not known. In this study, we used inhibitors of major signaling proteins to identify mediators through which SPARC exerts its counteradhesive and antiproliferative functions. Pretreatments with the general protein tyrosine kinase (PTK) inhibitors, herbimycin A and genistein, protected against the inhibitory effect of SPARC on bovine aortic endothelial (BAE) cell spreading by more than 60 %. Similar pretreatments with PTK inhibitors significantly blocked the diminishment of focal adhesions by SPARC in confluent BAE cell monolayers, as determined by the formation of actin stress-fibers and the distribution of vinculin in focal adhesion plaques. Inhibition of endothelial cell cycle progression by SPARC and a calcium-binding SPARC peptide, however, was not affected by PTK inhibitors. Inhibition of DNA synthesis by SPARC was not reversed by inhibitors of the activity of protein kinase C (PKC), or of cAMP-dependent protein kinase (PKA), but was sensitive to pertussis (and to a lesser extent, cholera) toxin. The counteradhesive effect of SPARC on endothelial cells is, therefore, mediated through a tyrosine phosphorylation-dependent pathway, whereas its antiproliferative function is dependent, in part, on signal transduction via a G protein-coupled receptor. J. Cell. Biochem. 70:543–552, 1998.

Key words: SPARC; endothelial cell; cell spreading; focal adhesion; actin; vinculin; PTK inhibitors

Interactions between cells and their extracellular matrix are the major determinants of cell shape, cell cycle progression, migration, and differentiation [Hay, 1981; Sims et al., 1992; Adams and Watt, 1993; Juliano and Haskill, 1993]. As an adhesive substratum, the extracellular matrix facilitates attachment, spreading, and formation of focal adhesions through the action of a variety of cell surface receptors that include integrins [Woods et al., 1984; Woods and Couchman, 1988; Smith and Cheresh, 1988; Hynes, 1992; Clark and Brugge, 1995; Ruoslahti, 1996]. Focal adhesions are specialized sites of cell attachment that couple specific components of the extracellular matrix with the actin cytoskeleton [Burridge et al., 1988, 1992]. Alternatively, some components of the extracellular matrix can promote destabilization of focal adhesions, cell rounding, and detachment. SPARC (secreted protein acidic and rich in cysteine; also known as osteonectin and BM-40) is a prototype for the recently identified class of extracellular matrix-associated proteins with counteradhesive properties [Sage and Bornstein, 1991; Lane and Sage, 1994; Motamed et al., 1996]. Together with thrombospondin 1 [Bornstein, 1995; Murphy-Ullrich et al., 1995] and tenascin C [Erickson, 1993; Chiquet-Ehrismann, 1995; Crossin, 1996], they comprise a non-homologous functional group of secreted matricellular proteins that interact with cell-surface receptors, growth factors, and extracellular matrix but do not function as stuctural components of the extracellular matrix.

SPARC consists of three distinct structural modules which serve functional roles in tissue remodeling and repair, morphogenesis, and vascular growth in vivo [Engel et al., 1987; Lane and Sage, 1994; Maurer et al., 1995; Hohenester et al., 1996]. Previous studies have indicated several functional properties of this glycopro-

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*Correspondence to: E. Helene Sage, Department of Biological Structure, Box 357420, School of Medicine, University of Washington, Seattle, WA 98195–7420.

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tein: (1) inhibition of spreading of newly plated cells and promotion of rounding of spread cells in vitro [Sage et al., 1989; Lane and Sage, 1990]; (2) reversible dissolution of focal adhesions in spread endothelial cells [Murphy-Ullrich et al., 1995]; (3) promotion of gap formation and barrier dysfunction in endothelial monolayers [Goldblum et al., 1994]; (4) regulation of protein expression and proteolytic activities involved in morphogenesis and tissue remodeling [Hasselaar et al., 1991; Lane and Sage, 1994]; and (5) inhibition of proliferation of endothelial, smooth muscle, and fibroblast cell cycles in mid G1, independently of discernible changes in cell shape [Funk and Sage, 1991, 1993; Raines et al., 1992; Sage et al., 1995].

For the most part, these diverse bioactivities of SPARC have been associated with the presence of unique sequences within the follistatin-like module (domain II) and/or the collagen and extracellular Ca^{2+}-binding module (EC domain) of SPARC [Maurer et al., 1995]. To understand how SPARC exerts these pleiotropic effects on cells, we have begun to define the signaling pathways through which SPARC functions. Although both SPARC and a Ca^{2+}-binding SPARC peptide (termed peptide 4.2) have been shown to bind to BAE cells [Yost and Sage, 1993] and human smooth muscle cells (Funk et al., unpublished results), neither a receptor for SPARC nor the intracellular signaling mechanism(s) transduced by SPARC have been identified. We report here that SPARC exerts its proximal, counteradhesive properties through a protein tyrosine kinase-mediated pathway. Inhibition of the endothelial cell cycle by SPARC, however, appears to be mediated through a different mechanism. Through the use of several inhibitors of major signaling pathways, we show that inactivation of only pertussis toxin (PT)-sensitive, and to a lesser extent, cholera toxin (CT)-sensitive, heterotrimeric G proteins provided a minor rescue of the cell cycle inhibition mediated by SPARC. Thus, we propose that a separation of proximal and distal effects of SPARC exists at the signaling level, which can be distinguished functionally as counteradhesive and antiproliferative.

MATERIALS AND METHODS

Cell Culture

BAE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with low glucose (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) by volume, 1% penicillin G, and 1% streptomycin sulfate, as previously described [Funk and Sage, 1991]. All experiments in this study were performed with previously characterized strains of low-passage (< 10) BAE cells [Sage et al., 1989]. The levels of endotoxin in SPARC and peptide 4.2 preparations used in this study never exceeded 0.1 EU/mg, as determined by the *Limulus* amebocyte lysate (LAL) gel-clot assay (Associates of Cape Cod, Woods Hole, MA).

Reagents

All the signal transduction pathway inhibitors used in this study were purchased from Calbiochem-Novabiochem Intl. (La Jolla, CA). Native SPARC was purified from the culture media of murine parietal yolk sac (PYS-2) carcinoma cells by chromatography on diethylaminoethyl (DEAE)-cellulose and Sephadex G-200 as previously described [Sage et al., 1989]. SPARC peptides were synthesized and purified by the Peptide Core facility (Department of Pharmacology, University of Washington, Seattle) as previously described [Lane and Sage, 1990].

Cell Spreading Measurements

Confluent monolayers of low-passage BAE cells were pretreated with 1 μM herbimycin A for 16 h. The cells were treated with trypsin, resuspended in DMEM-1% FBS, and plated onto 24-well tissue-culture plastic substrata at densities of 1–2 X 10^4 cells/ml. SPARC (or an equivalent volume of PBS) and herbimycin A (0.1 μM) were added after cells were allowed to attach (5–10 min after plating), and cultures were photographed with an inverted phase-contrast microscope (Carl Zeiss, Inc., Thornwood, NY). One or more fields were recorded from a single culture well at two different time points per experiment, 2 and 4 h after plating. The extent of cell spreading was determined quantitatively by calculation of a "rounding index" (RI) [Lane and Sage, 1990] for each culture condition. Cells were scored at different stages of spreading according to the equation: $RI = (1A + 2B + 3C)/A + B + C$, where A, B, and C represent the total number of cells exhibiting fully-spread (index of 1), partially spread (index of 2), and completely rounded cell morphology (index of 3), respectively. Experiments were performed in triplicate, and the averaged

values of independent experiments were reported.

Immunocytochemistry

Confluent monolayers of BAE cells on glass coverslips were pretreated with 1 μM herbimycin A for 16 h. The cells were treated with SPARC (0.6 μM) or media alone in the presence or absence of herbimycin A (0.1 μM) for 4–6 h under serum-free conditions. The monolayers were fixed in 3.7% formaldehyde for 20 min, rendered permeable in 0.5% Triton X-100 in PBS for 10 min, blocked in 2% goat serum, and incubated with mouse monoclonal primary antibody against vinculin (Sigma, St. Louis, MO, used at 1 mg/ml in PBS containing 2% goat serum) for 1 h at room temperature. Cells were subsequently treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (UBI, Lake Placid, NY, used at 0.5 mg/ml) for 30 min at room temperature in the dark. Double labeling for F-actin was performed by subsequent staining with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, used at 2×10^{-7}M) for 20 min at room temperature. The stained monolayers were photographed by epifluorescence microscopy.

Cell Proliferation Assay

Contact-inhibited cultures of BAE cells were incubated in serum-free DMEM for 72 h. The quiescent cells (>90% in G_0) were subsequently replated in 24-well tissue culture plates (Costar, Cambridge, MA) at a density of approximately 50,000 cells/well in DMEM containing 2% FBS. Most inhibitors of known signaling pathways used in this study inhibited the cell cycle as a function of concentration. Therefore, we determined an IC_{50} for each compound, defined as the concentration at which it inhibited specifically the activity of the target protein by more than 90% (determined in separate assays) but did not inhibit [^{3}H]-thymidine incorporation by more than 50%. SPARC or SPARC peptide 4.2 (0.1 to 0.3 mM) and inhibitors were added to subcultured cells 5–10 min following replating to allow time for attachment of >90% of the cells. Controls included cells that received equivalent volumes of PBS and inhibitor solvent (water or DMSO). After an incubation of 18–20 h, cells were pulsed for 2 h with [^{3}H]-thymidine at 2 μCi/ml, washed once with PBS, incubated with cold 10% trichloroacetic acid for 1 h, and solubilized in 300 μL of 0.2 M NaOH. Incorporated counts were quantified by liquid-scintillation counting in 2 ml of Ecolume (ICN, Irvine, CA). The results of at least 3 independent experiments performed in triplicate were reported as mean ± S.D.

RESULTS

Inhibition of Endothelial Cell Spreading by SPARC Involves a Tyrosine Phosphorylation-Dependent Pathway

Previously we demonstrated that SPARC and several SPARC peptides promoted a partial detachment (rounding) of spread endothelial cells and inhibited the spreading of newly plated cells [Sage et al., 1989; Lane and Sage, 1990]. In this study, we asked through which signaling pathway(s) SPARC imparted its counteradhesive activity on endothelial cells and used the broad-spectrum inhibitors of protein tyrosine kinases (PTK), herbimycin A and genistein, in an attempt to abrogate the effect of SPARC. For assessment of the extent of BAE cell spreading in the presence of SPARC, cells were plated at low densities and allowed to attach (5–10 min) prior to addition of SPARC. Pretreatment with PTK inhibitors alone did not appear to affect the plating efficiency or degree of cell spreading (Fig. 1A). Two to four hours after plating, cells were rinsed with PBS and were photographed. The effect of the inhibitors on the anti-spreading effect of SPARC is demonstrated morphologically in Figure 1A. Determinations of rounding indices from the photographed fields revealed that pretreatment of endothelial cells with herbimycin A protected against SPARC-induced cell rounding by more than 60% (Fig.1B). Genistein was found to be equally effective at 30 μg/ml, whereas pretreatment with the PKC inhibitor chelerythrine (1 μM for 16 h) was ineffective against the anti-spreading effect of SPARC (data not shown). Therefore, inhibition of endothelial cell spreading by SPARC appeared to involve a tyrosine phosphorylation-dependent pathway.

PTK Inhibitors Abrogate SPARC-Mediated Focal Adhesion Disassembly

By interference reflection microscopy and immunofluorescence staining techniques, SPARC and SPARC peptides 2.1 (amino acids 54–73) and 4.2 (amino acids 254–273) promoted a loss of focal adhesion plaques in BAE cells [Murphy-Ullrich et al., 1995]. However, unlike thrombo-

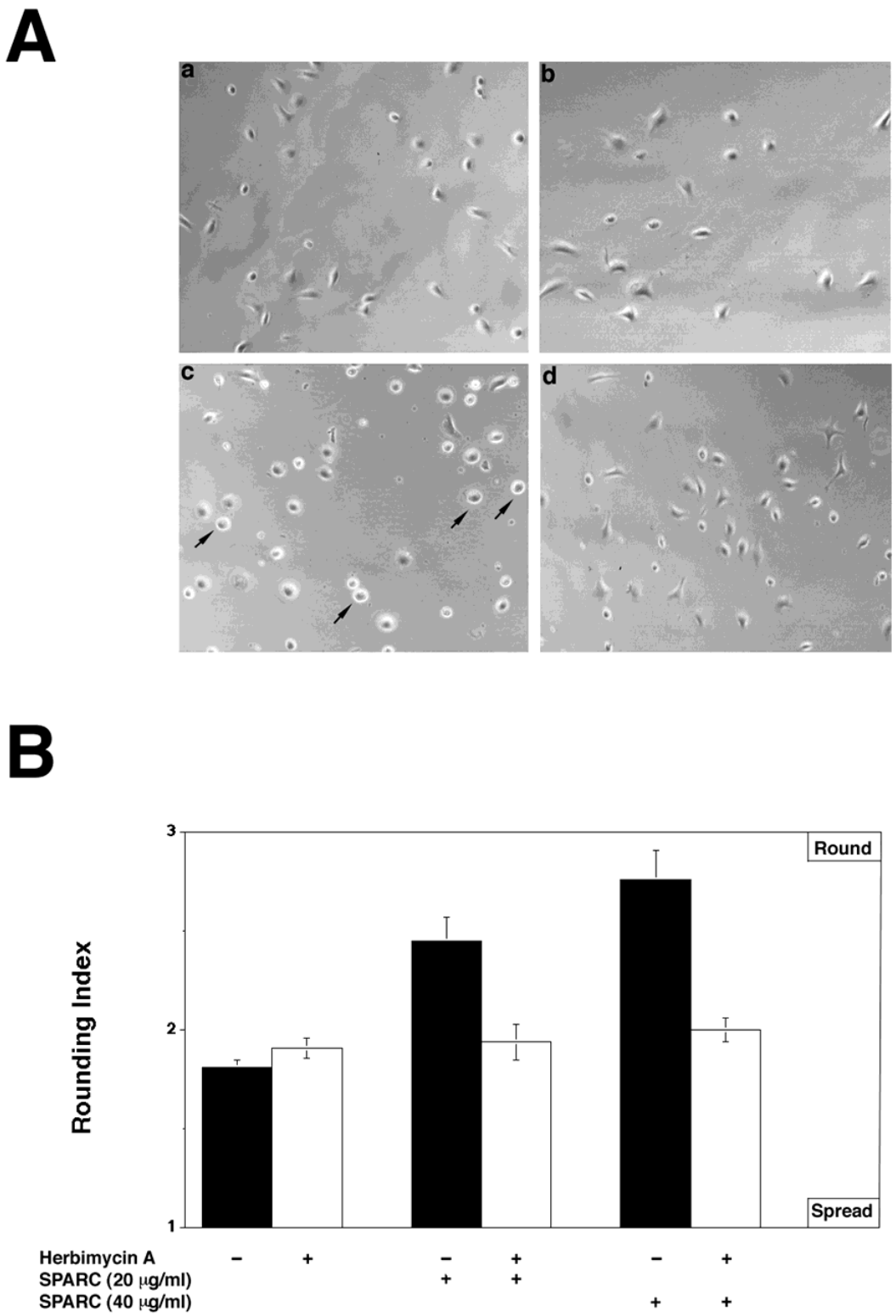

Fig. 1. **A:** Confluent cultures of control and BAE cells pretreated with herbimycin A (1 μM for 16 h) were plated in 1% FBS/DMEM at 5 x 10^4 cells/ml, with or without herbimycin A (0.1 μM) and SPARC (20 or 40 μg/ml, 0.6 or 1.2 μM, respectively) for 2–4 h. Cells were photographed at 2 h following plating without herbimycin A or SPARC (**a**), with herbimycin A only (**b**), with SPARC (40 μg/ml) (**c**), and pretreated with herbimycin A in the presence of SPARC (40 μg/ml) (**d**). Arrows in c indicate rounded cells (scored as 1). **B:** Graphic representation of a "rounding index" determination (see Materials and Methods) of experiment in A. Experiments were performed in triplicate, and the data are representative of two independent experiments.

spondin 1 and tenascin C, the disassembly of focal adhesions by SPARC was shown not to be mediated by a cGMP-dependent protein kinase [Murphy-Ullrich et al., 1996]. We asked whether the PTK inhibitors that protected against the inhibition of cell spreading by SPARC could also abrogate the disassembly of focal adhesions in confluent BAE cells that is typically seen with SPARC, since a striking feature of focal adhesions is their high content of phosphotyrosinated proteins [Burridge et al., 1988]. Pretreatment of confluent monolayers of BAE cells with PTK inhibitors alone had no significant effect on the disassembly of focal adhesions, as determined by actin stress-fiber formation and vinculin distribution (Fig. 2). Exposure of cells to SPARC for 4–6 h resulted in a loss of vinculin-containing focal adhesion plaques and a concomitant reorganization of actin stress-fibers. As previously described, a subpopulation of SPARC-resistant cells was present in all the BAE cell strains tested [Murphy-Ullrich et al., 1995]. Preincubation of cells with PTK inhibitors prior to exposure to SPARC did not appear to be associated with actin cytoskeletal reorganization or a reduction in punctate staining for vinculin, in comparison to cells treated with SPARC alone (Fig. 2). Similar pretreatments used to diminish the activity of PKA with H-7 (3 μM), of PKC with chelerythrine (1 μM) or phorbol-12-myristrate-13 acetate (PMA) (5 μM), and of cyclic-GMP-dependent protein kinase (PKG) with KT-5823 (0.3 μM) did not protect against the abrogation of focal adhesions that was observed after addition of SPARC peptide 4.2 (Table 1). The disassembly of focal adhesions in BAE cells that is induced by SPARC thus appears to involve a tyrosine phosphorylation-dependent pathway.

Inhibitors of Major Signaling Pathways Do Not Block the Anti-Proliferative Effect of SPARC

Regulation of proliferation of endothelial cells is known to be mediated by several different signaling mechanisms. Activation of a PKC isoenzyme has been implicated in the inhibition of proliferation and cell cycle progression of rat capillary endothelial cells [Harrington et al., 1997]. Increases in intracellular concentrations of cAMP and concomitant activation of PKA activity have been reported to inhibit basal proliferation of BAE cells [Leitman et al., 1986], as well as growth factor-stimulated activation of the mitogen-activated protein kinase (MAPK) cascade and mitogenesis in bovine capillary endothelial cells [D'Angelo et al., 1997]. The results of our in vitro kinase assays failed to show significant differences in basal or growth factor-stimulated activity of PKC or PKA after the addition of SPARC or SPARC peptide 4.2 to BAE cell cultures (data not shown). Consistent with these findings, pretreatments with specific inhibitors of PKC (chelerythrine and long-term inactivation by PMA) or PKA (H-7) also failed to reverse the inhibition of [^{3}H]-thymidine incorporation by SPARC peptide 4.2 (Table 1). We also asked whether the antiproliferative effect of SPARC or SPARC peptide 4.2 on BAE cells was mediated by signaling pathways similar to those involved in its counteradhesive properties. The same PTK inhibitors that protected against the anti-spreading and focal adhesion disassembly functions of SPARC failed to reverse its antiproliferative effect on BAE cells in [^{3}H]-thymidine incorporation assays (Fig. 3). Other PTK inhibitors (tyrphostin A47 and lavendustin A, Table 1), or long-term pretreatments with herbimycin A or genistein were found to be equally ineffective (data not shown). From the array of inhibitors of other major signaling pathways that we tested, only PT (18%), and to a lesser extent CT (10%), provided minor rescue (reversal) of inhibition by SPARC peptide 4.2 (Table 1). The counteradhesive and anti-proliferative effects of SPARC, therefore, appear to be mediated through different signaling pathways.

DISCUSSION

A recurring theme underlying angiogenesis, reendothelialization, wound repair, and tumor metastasis is the ability of adherent endothelial cells to regulate their morphology by establishment of transient focal adhesions with the extracellular matrix. SPARC and SPARC peptides spanning its Ca^{2+}-binding sites have been shown to disrupt endothelial cell contacts with the extracellular matrix and thereby effect changes in cell shape [Sage et al., 1989, 1995; Lane and Sage, 1990], although the intracellular signaling mechanisms by which SPARC mediates its various functions are not known.

Protein phosphorylation has been shown to modulate different stages of cell adhesion [Turner et al., 1989; Burridge et al., 1992; Schaller et al., 1992; Juliano and Haskill, 1993]. Loss of actin stress fibers and disassembly of focal adhesions as a result of activation of cyclic

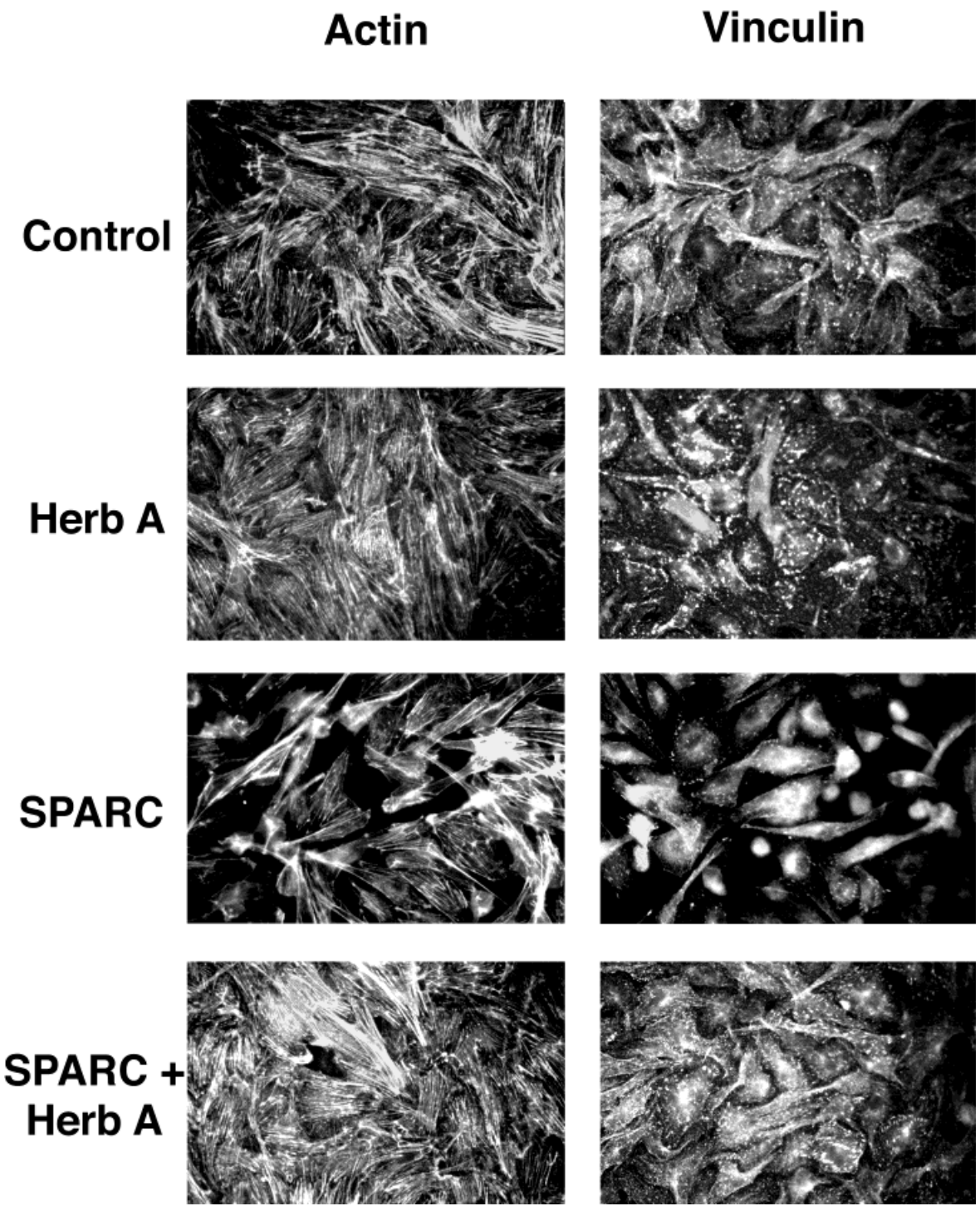

Fig. 2. Postconfluent cultures of BAE cells exposed to SPARC (20 μg/ml) in the presence or absence of herbimycin A (0.1 μM) for 6 h. The monolayers were fixed, rendered permeable, and stained with rhodamine-labeled phalloidin (actin), or FITC-labeled anti-vinculin IgG. Control and monolayers pretreated with herbimycin A had abundant transcytoplasmic F-actin fibers and punctate adhesion plaques that contained vinculin. Monolayers with SPARC exhibited significant gaps between cells, a peripheral distribution of F-actin, and a significant decrease in the number of vinculin-containing plaques. In contrast, monolayers pretreated with herbimycin A did not exhibit focal adhesion disassembly and appeared similar to control cells with respect to the actin cytoskeleton.

TABLE I. Heterotrimeric G-Protein Inhibitors Partially Reverse the Inhibition of DNA Synthesis Induced by SPARC in BAE Cells*

Inhibitor	IC_{50} (μM)	Target	% Reversal of inhibition
Herbimycin A[a]	1	PTK	0
Genistein[a]	140	PTK	0
Lavendustin A[a]	0.2	PTK	0
Tyrphostin A47	1	PTK	0
H-7[a]	3	PKA	0
H-7	6	PKA/PKC/PKG	0
H-89	0.06	PKA	0
KT-5823[a]	0.3	PKG	0
KT-5823	4	PKG/PKC	0
KT-5823	10	PKG/PKC/PKA	0
PMA[a]	5	PKC	0
Chelerythrine[a]	1	PKC	0
PT[a]	10	Gαi protein	18 ± 1.5
CT[a]	10	Gαs protein	10 ± 1.1
PD-98059[a]	30	MAPKK	0
U-73122	1	PLC	0
Thapsigargin	0.01	Ca^{2+} ATPase	0

*Quiescent cultures of BAE cells were plated in 1% FBS/DMEM in 0.1–0.3 mM SPARC peptide 4.2, and concentrations of inhibitors of known signaling pathways at which incorporation of [^{3}H]-thymidine was not inhibited by more than 50% (see Materials and Methods). Controls included cells that received equivalent volumes of PBS and/or inhibitor solvent (water or DMSO). After an incubation of 20 h, [^{3}H]-thymidine incorporation was determined as % of control (relative to PBS alone). % reversal of inhibition for each inhibitor was determined by subtraction of the % inhibition of proliferation resulting from SPARC peptide 4.2 and the inhibitor treatment from that of peptide 4.2 alone. The results of at least 4 independent experiments performed in triplicate were reported as mean ± S.D. PLC, phospholipase C.

[a]Sixteen-hour pretreatments with the inhibitor prior to exposure to SPARC peptide 4.2 resulted in similar % reversal of inhibition.

AMP-dependent protein kinase (PKA) have been reported in fibroblasts and mesangial cells [Lamb et al., 1988; Turner et al., 1989]. Activation of PKC was shown to enhance endothelial cell attachment and spreading , and inhibition of PKC by chelerythrine (a specific inhibitor of PKC that has little or no effect on other known protein kinases), but interestingly not by PMA, was shown to inhibit spreading of human umbilical vein endothelial cells [Yamamura et al., 1996]. The results of our in vitro kinase assays in BAE cells, however, did not reveal any significant changes in the activity of PKA or PKC in response to SPARC (data not shown). Recently, it was reported that cGMP-dependent protein kinase is necessary for abrogation of focal adhesions by either thrombospondin 1 or tenascin C, but not by SPARC [Murphy-Ullrich et al., 1996].

Clues to possible involvement of a tyrosine phosphorylation pathway effecting the counteradhesive functions of SPARC are provided by the following observations: (1) Many cytoplasmic proteins become tyrosine-phosphorylated in response to integrin-dependent adhesion [Turner et al., 1989; Burridge et al, 1992; Schaller et al., 1992; Juliano and Haskill, 1993; Defilippi et al., 1994; Chen et al., 1994, Vuori and Ruoslahti, 1995]; (2) v-*src*-transformed cells become anchorage-independent or form abnormally unstable focal adhesions in which many of the protein components (focal adhesion kinase $p125^{FAK}$, paxillin, tensin, and Crk-associated substrate $p130^{Cas}$) become hyper-tyrosine-phosphorylated [Burridge et al., 1992]; (3) Pretreatment with inhibitors of protein tyrosine phosphorylation (herbimycin A and genistein) appeared to protect against gap formation and barrier dysfunction properties of SPARC by inhibition of the hyper-phosphorylation of paxillin (a focal adhesion protein) and β-catenin (an adherens junction protein), in bovine pulmonary artery endothelial cells (Goldblum et al., unpublished data). Our studies indicate that similar pretreatments protected against the anti-spreading (Fig. 1) and focal adhesion disassembly functions (Fig. 2) of SPARC in BAE cells. The exact sequence of events leading to proper assembly of focal adhesion plaques is not fully understood. This process appears to involve tyrosine phosphorylation and sequential recruitment of several focal adhesion-associated proteins. A plausible mechanism through which SPARC mediates its counteradhesive properties could be the regulation of recruitment or activity level of one or more focal adhesion-associated proteins (e.g.,. $p125^{FAK}$, c-Src, tensin, paxillin, and $p130^{Cas}$). It is possible that hyper-tyrosine phosphorylation of paxillin and/or other focal adhesion components in response to SPARC results in improper assembly or disassembly of focal adhesions. Cells with a targeted disruption of a focal adhesion-associated protein, or cells expressing dominant negative mutations in one or more of these proteins, are currently being used to determine whether SPARC mediates its counteradhesive function by interaction with a specific component of focal adhesion plaques. Other potential targets of reorganization of actin cytoskeleton by SPARC

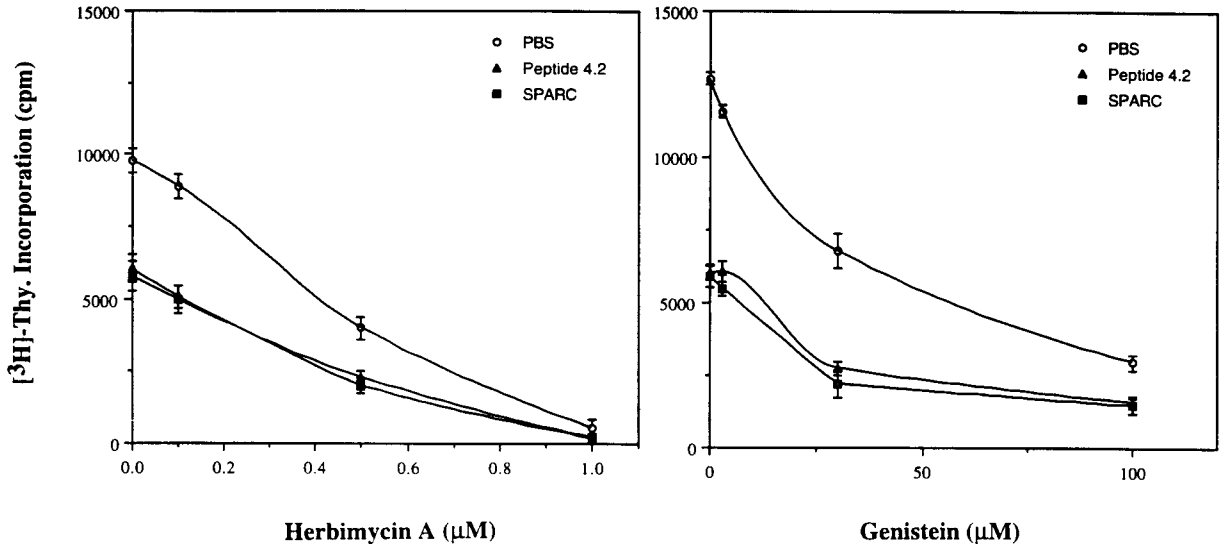

Fig. 3. Confluent, quiescent cultures of BAE cells were plated in 1% FBS/DMEM in 0.6 μM SPARC (squares), 0.2 mM SPARC peptide 4.2 (triangles), or PBS (circles) and the inhibitors herbimycin A and genistein for 20 h. DNA synthesis was assayed by incorporation of [^{3}H]-thymidine. Total incorporated cpm are plotted as a function of increasing concentrations of inhibitor, and are representative of at least 3 independent experiments performed in triplicate. Neither inhibitor reversed the inhibition of BAE cell proliferation mediated by SPARC or peptide 4.2.

are the lipid kinases (e.g., phosphoinositol-3 kinase) or small GTP-binding proteins (e.g., Rho), the activation of which also involves a tyrosine phosphorylation-dependent pathway [Flinn and Ridley, 1996; Stephens et al., 1993; Schwartz et al., 1995].

Inhibition of BAE cell proliferation by SPARC, however, appears to be a distal effect, i.e., subsequent to changes in tyrosine phosphorylation of focal adhesion proteins, and occurs independently of discernable changes in cell shape. As shown in Table 1, we were not able to identify unequivocally a signaling pathway by which SPARC exerted its inhibitory effect on DNA synthesis in BAE cells. Since our preparations of BAE cells respond minimally to basic fibroblast growth factor (bFGF), or vascular endothelial growth factor (VEGF), we had to use low concentrations of serum (2%) to stimulate proliferation of quiescent BAE cells in our studies. Other potential shortcomings associated with the use of signaling pathway inhibitors include: (1) the inhibitory effect of the majority of the compounds alone on the cell cycle, (2) the lack of complete specificity toward a single target protein, and (3) compensation of the inhibited pathway by components of another pathway. Nevertheless, the results of our inhibitor studies indicate a possible involvement of a PT-sensitive heterotrimeric G protein-mediated pathway in the inhibition of BAE cell proliferation induced by SPARC (Table 1). Receptor-mediated activation of the PT-sensitive Gαi has been shown to result in stimulation of the MAPK pathway, with distal targets of mitogenesis and gene expression [Rens-Domiano and Hamm, 1995; Craig and Johnson, 1996]. Receptors with tyrosine kinase activity have been known to activate MAPK via son of sevenless (SOS)-mediated accumulation of $p21^{ras}$-GTP, which in turn activates $p74^{raf-1}$, MAPK kinase (MAPKK), and MAPK [Davis, 1993; Marshal, 1995]. In contrast to the well-defined activation of the Ras-MAPK pathway, the mechanism used by PT-sensitive, G protein-coupled receptors is not clear. Recent results from work in COS-7 cells predict a model of MAPK activation wherein stimulation of Gi-coupled receptors results in activation of c-Src and a concomitant tyrosine phosphorylation of the adapter protein Shc, followed by recruitment of Ras guanine nucleotide exchange factor (GAP) and Ras activation [Lutrell et al., 1996]. Furthermore, studies in 3T3 cells have shown that the integrin-mediated activation of the p42 form of MAPK (also known as extracellular signal-regulated kinase, ERK-2) is Ras-dependent, but the Ras-MAPK pathway is not necessary for the formation of stress fibers and focal contacts [Clark and Hynes, 1996]. Recently, we have shown that SPARC significantly diminishes VEGF-induced activation of the two forms of MAPK

(ERK-1 and ERK-2) in human dermal microvascular endothelial cells [Kupprion et al., unpublished data]. However, in this study with BAE cells, we did not see a reversal of the antiproliferative effect of SPARC using the MAPKK inhibitor PD98059 (Table 1). This apparent discrepancy is likely due to inherent differences in the experimental systems: e.g., human microvascular cells, and stimulation specifically with VEGF in the absence of serum.

We thus speculate that the antiproliferative effect of SPARC on endothelial cells is, at least in part, mediated through inhibition of MAPK, independently of its regulation of counteradhesion. We have also shown that one of the proximal effects of SPARC is to diminish endothelial cell spreading and focal adhesion formation, a process that involves a protein-tyrosine kinase-mediated pathway. The antiproliferative function of SPARC we believe to be a more distal effect and is dependent, in part, on a signal transduction event mediated through a G protein-coupled receptor.

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REFERENCES

Adams JC, Watt FM (1993): Regulation of development and differentiation by the extracellular matrix. Development 117:1183–1198.

Bornstein P (1995): Diversity of function is inherent in matricellular proteins: An appraisal of thrombospondin 1. J Cell Biol 130:503–506.

Burridge K, Fath K, Kelly G, Nuckolls G, Turner C (1988): Focal adhesions: Transmembrane junctions between extracellular matrix and the cytoskeleton. Annu Rev Cell Biol 4:487–525.

Burridge K, Turner CE, Romer, LH (1992): Tyrosine phosphorylation of paxillin and pp 125 ^{FAK} accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. J Cell Biochem 119:893–903.

Chen QM, Kinch MS, Lin TH, Burridge K, Juliano RL (1994): Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J Biol Chem 269:26602–26605.

Chiquet-Ehrismann R (1995): Inhibition of cell adhesion by anti-adhesive molecules. Curr Opin Cell Biol 7:715–719.

Clark EA, Brugge JS (1995): Integrins and signal transduction pathways: The road taken. Science 268:233–239.

Clark EA, Hynes RO (1996): Ras activation is necessary for integrin-mediated activation of extracellular signal-regulated kinase 2 and cytosolic phospholipase A_2 but not for cytoskeletal organization. J Biol Chem 271:14814–14818.

Craig SW, Johnson RP (1996): Assembly of focal adhesions: progress, paradigms, and portents. Curr Opin Cell Biol 8:74–85.

Crossin KL (1996): The role of tenascin domains in developmental patterning, cell migration, and gene expression. In Crossin KL (ed.):"Tenascin and Counter-Adhesive Molecules of the Extracellular Matrix." Amsterdam: Harwood Academic Publishers, pp 23–46.

D'Angelo G, Lee H, Weiner RI (1997): cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. J Cell Biochem 67:353–366.

Davis RJ (1993): The mitogen-activated protein kinase signal trransduction pathway. J Biol Chem 268:1453–1456.

Defilippi P, Bozzo C, Volpe G, Romano G, Venturino M, Silengo L, Tarone G (1994): Integrin-mediated signal transduction in human endothelial cells: analysis of tyrosine phosphorylation events. Cell Adhes Commun 2: 75–86.

Engel J, Taylor W, Paulsson M, Sage H, Hogan B (1987): Calcium-binding domains and calcium-induced transition in SPARC (osteonectin, BM-40), an extracellular glycoprotein expressed in mineralized and nonmineralized tissues. Biochemistry 26:6958–6965.

Erickson HP (1993): Tenascin-C, tenascin-R, and tenascin-X: a family of talented proteins in search of functions. Curr Opin Cell Biol 5:869–876.

Flinn HM, Ridley AJ (1996): Rho stimulates tyrosine phosphorylation of focal adhesion kinase, $p130^{CAS}$ and paxillin. J Cell Sci 109:1133–1141.

Funk SE , Sage EH (1991): The Ca^{2+}-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. Proc Natl Acad Sci USA 88:2648–2652.

Funk SE , Sage EH (1993): Differential effects of SPARC and cationic SPARC peptides on DNA synthesis by endothelial cells and fibroblasts. J Cell Physiol 154:53–63.

Goldblum SE, Ding X, Funk SE, Sage EH (1994): SPARC regulates endothelial cell shape and barrier function. Proc Natl Acad Sci USA 91:3448–3452.

Harrington EO, Loffler J, Nelson PR, Kent KC, Simons M, Ware JA (1997): Enhancement of migration by protein kinase C alpha and inhibition of proliferation and cell cycle progression by protein kinase C delta in capillary endothelial cells. J Biol Chem 272:7390–7397.

Hasselaar P, Sage EH (1992): SPARC antagonizes the effects of basic fibroblast growth factor on the migration of bovine aortic endothelial cells. J Cell Biochem 49:272–283.

Hay ED (1981): Extracellular matrix. J Cell Biol 91:205–223.

Hohenester E, Maurer P, Hohenadl C, Timpl R, Jansonius J N, Engel J (1996): Structure of a novel extracellular $Ca^{(2+)}$-binding module in BM-40. Nat Struct Biol 3:67–73.

Hynes RO (1992): Integrins: Versatility modulation and signaling in cell adhesion. Cell 69:11–25.

Juliano R, Haskill S (1993): Signal transduction from the extracellular matrix. J Cell Biol 120:577–585.

Lamb NJC, Fernandez A, Conti MA, Adelstein R, Glass DB, Welch WJ, Feramisco JR (1988): Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. J Cell Biol 106:1955–1971.

Lane TF, Sage EH (1990): Functional mapping of SPARC: Peptides from two distinct Ca^{++}-binding sites modulate cell shape. J Cell Biol 111:3065–3076.

Lane TF, Sage EH (1994): The biology of SPARC, a protein that modulates cell-matrix interactions. FASEB J 8:163–173.

Leitman DC, Fiscus RR, Murad F (1986): Forskolin, phosphodiesterase inhibitors, and cyclic AMP analogs inhibit proliferation of cultured bovine aortic endothelial cells. J Cell Physiol 127:237–243.

Lutrell LM, Hawes BE, van Biesen D, Lutrell DK, Lansing TJ, Lefkowitz RJ (1996): Role of c-Src tyrosine kinase in G protein-coupled receptor and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 217:19443–19450.

Marshal CJ (1995): Specificity of receptor tyrosine kinase signaling:transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-–185.

Maurer P, Hohenadl C, Hohenester E, Göhring W, Timpl R, Engel J (1995): The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV. J Mol Biol 253:347–357.

Motamed K, Bassuk J, Sage EH (1996): SPARC as a counteradhesive protein: Structural and functional correlates. In Crossin KL (eds.): "Tenascin and Counter-Adhesive Molecules of the Extracellular Matrix." Amsterdam: Harwood Academic Publishers, pp 127–144.

Murphy-Ullrich JE, Lightner VA, Aukhil I, Yan YZ, Erickson HP, Höök M (1991): Focal adhesion integrity is downregulated by the alternatively spliced domain of tenascin. J Cell Biol 115:1127–1136.

Murphy-Ullrich JE, Lane TF, Pallero MA, Sage EH (1995): SPARC mediates focal adhesion disassembly in endothelial cells through a follistatin-like region and the calcium-binding EF-hand. J Cell Biochem 57:341–350.

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

Rens-Domiano S, Hamm HE (1995): Structural and functional relationships of heterotrimeric G-proteins. FASEB J 9:1059–1066.

Ruoslahti E (1996): Integrin signaling and matrix assembly. Tumour Biol. 17:117–124.

Sage EH, Bornstein P (1991): Minireview: Extracellular proteins that modulate cell-matrix interactions: SPARC, tenascin, and thrombospondin. J Biol Chem 266:14831–14834.

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

Sage EH, Bassuk J , Yost JC, Folkman MJ , Lane TF (1995): Inhibition of endothelial cell proliferation by SPARC is mediated through a Ca^{+2} -binding E-F hand sequence. J Cell Biochem 57:127–140.

Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT (1992): pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 89: 5192–5196.

Schwartz MA, Schaller MD, Ginsberg MH (1995): Integrins: Emerging paradigms of signal transduction. Annu Rev Cell Dev Biol 11:549–599.

Sims JR, Karp S, Ingber DE (1992): Altering the cellular mechanical forces results in integrated changes in cell, cytoskeletal, and nuclear shape. J Cell Sci 103:1215–1222.

Smith JW, and Cheresh DA (1988): The Arg-Gly-Asp domain of the vitronectin receptor: Photoaffinity cross-linking implicates amino acid residues 61–203 of the beta subunit. J Biol Chem 263:18726–18731.

Stephens LR, Jackson TR, Hawkins PT (1993): Agonist-stimulated synthesis of phosphatidyl- inositol (3,4,5)-triphosphate: a new intracellular signaling system. Biochem Biophys Acta 1179:27–75.

Turner CE, Pavalko FM, Burridge K (1989): The role of phosphorylation and limited cleavage of talin and vinculin in disruption of focal adhesion integrity. J Biol Chem 264:11938–11944.

Vuori K, Ruoslahti E (1995): Tyrosine phosphorylation of $p130^{Cas}$ and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. J Biol Chem 270:22259–22262.

Woods A, Couchman JR (1988): Focal adhesions and cell matrix interactions. Collagen Rel Res 8:155–182.

Woods A, Höök M, Kjell'en L, Smith CG, Rees DA (1984): Relationship of heparan sulfate proteoglycans to the cytoskeleton and extracellular matrix of cultured fibroblasts. J Cell Biol 99:1743–1753.

Yamamura S, Nelson PR, Kent KC (1996): Role of protein kinase C in attachment, spreading, and migration of human endothelial cells. J Surg Res 63:349–354.

Yost JC, Sage EH (1993): Specific interaction of SPARC with endothelial cells is mediated through a carboxy-terminal sequence containing a calcium-binding E-F hand. J Biol Chem 268:25790–25796.