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Inhibition of PDGF-Stimulated and Matrix-Mediated Proliferation of Human Vascular Smooth Muscle Cells by SPARC Is Independent of Changes in Cell Shape or Cyclin-Dependent Kinase Inhibitors

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Abstract Interactions among growth factors, cells, and extracellular matrix regulate proliferation during normal development and in pathologies such as atherosclerosis. SPARC (secreted protein, acidic, and rich in cysteine) is a matrix-associated glycoprotein that modulates the adhesion and proliferation of vascular cells. In this study, we demonstrate that SPARC inhibits human arterial smooth muscle cell proliferation stimulated by platelet-derived growth factor or by adhesion to monomeric type I collagen. Binding studies with SPARC and SPARC peptides indicate specific and saturable interaction with smooth muscle cells that involves the C-terminal Ca^{2+}-binding region of the protein. We also report that SPARC arrests monomeric collagen-supported smooth muscle cell proliferation in the late G1-phase of the cell cycle in the absence of an effect on cell shape or on levels of cyclin-dependent kinase inhibitors. Cyclin-dependent kinase-2 activity, p107 and cyclin A levels, and retinoblastoma protein phosphorylation are markedly reduced in response to the addition of exogenous SPARC and/or peptides derived from specific domains of SPARC. Thus, SPARC, previously characterized as an inhibitor of platelet-derived growth factor binding to its receptor, also antagonizes smooth muscle cell proliferation mediated by monomeric collagen at the level of cyclin-dependent kinase-2 activity. J. Cell. Biochem. 84: 759–771, 2002.

Key words: extracellular matrix; monomeric collagen; cell cycle; vascular biology

An interplay among growth factors, cells, and extracellular matrix (ECM) is believed to modulate proliferation in normal development and under certain pathological conditions. Cell cycle progression is coordinately regulated by growth factors, cell anchorage, the ECM, and cytoskeletal structure [Folkman and Moscona, 1978; Assoian and Zhu, 1997; Huang and Ingber, 1999]. Adherent cells irreversibly commit to cell cycle progression at a point late in G1 that correlates with hyperphosphorylation of retinoblastoma protein (pRb). Phosphorylation of pRb is mediated by sequential action of the G1 phase cyclin/cyclin-dependent kinase (cdk) complexes cyclin D-cdk4 and cyclin E-cdk2. Growth factors and ECM are required for induction and expression of cyclin D1, and forced expression of cyclin D1 in suspended cells can rescue pRb phosphorylation and cell cycle progression [Böhmer et al., 1996; Zhu et al., 1996]. While cyclin E levels do not change significantly during cell cycle traverse, cyclin E-cdk2 activity is induced in late G1 phase as a result of growth factors and ECM regulation of the $p21^{Cip1/Waf1}$ family of cdk inhibitors [Fang et al., 1996; Koyama et al., 1996]. However, upon interacting with specific ECM components, some cell types fail to progress through G1 in the presence of otherwise stimulatory concentrations of growth factors. For example, human smooth muscle cells (SMC) plated on polymerized type I

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collagen fail to respond to platelet-derived growth factor (PDGF) or serum, while they proliferate normally on the monomeric form of the same collagen [Koyama et al., 1996]. This ECM-associated cell cycle arrest appears to be mediated primarily through an increase in the cdk inhibitor $p27^{Kip1}$ and decreased cyclin E-cdk2 kinase activity. Thus, certain forms of ECM are dominant.

A small group of non-homologous, secreted glycoproteins with counteradhesive properties, termed matricellular proteins [Bornstein, 1995] have been shown to interact with cell-surface receptors, growth factors, and matrix components, but do not function as structural components of the ECM [Lane and Sage, 1994; Maurer et al., 1995; Brekken and Sage, 2000]. SPARC, also known as osteonectin and BM-40, a prototype for the family of matricellular proteins, consists of three distinct structural modules that regulate various aspects of tissue remodeling and repair, morphogenesis, and vascular growth in vivo [Engel et al., 1987; Holland et al., 1987; Bornstein, 1995; Iruela-Arispe et al., 1995; Hohenester et al., 1996]. High levels of expression of SPARC have been associated with the regulation of cellular proliferation, migration, and differentiation [for review, see Lane and Sage, 1994]. In cultured bovine aortic endothelial cells and fibroblasts, addition of soluble SPARC inhibits serum-stimulated cell cycle progression from G1 to S and disrupts focal adhesions [Funk and Sage, 1991; Murphy-Ullrich et al., 1995].

Primary mesenchymal cells isolated from SPARC-null mice exhibit increased rates of proliferation, express increased levels of cyclin A, and have a more flattened cell shape in comparison with their wild-type controls [Bradshaw et al., 1999]. SPARC also has been shown to bind the PDGF-AB and -BB dimers, potent mitogens and chemoattractants of mesenchymal cells, and inhibits binding to their cell-surface receptor [Raines et al., 1992]. Thus, SPARC can abrogate cell cycle progression by altering cellular reponses, including cell shape, and by binding and inhibiting growth factors.

In this study, we demonstrate that SPARC inhibits human SMC proliferation stimulated by PDGF or by plating on monomeric type I collagen. We report that SPARC arrests human SMC in the late G1-phase of the cell cycle through modulation of a unique set of cell cycle molecules: diminishment of (i) cyclin E-associated cdk2 activity, (ii) p107 and cyclin A levels, and (iii) phosphorylation of Rb protein, without effects on the cdk inhibitors. Furthermore, binding studies with SPARC and SPARC peptides indicate specific and saturable interaction with the surface of SMC. Unlike the previously-reported effects of SPARC on other cell types, the G1 arrest occurs in the absence of changes in cell shape and attachment.

MATERIALS AND METHODS

Growth Factors and Antibodies

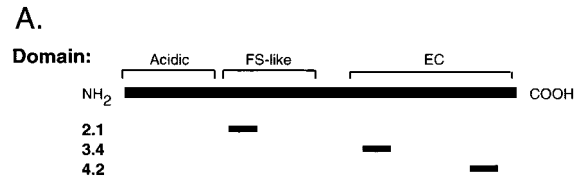
PDGF-AB was purified from outdated human platelet-rich plasma [Hart et al., 1990]. Polyclonal antisera against cyclin A and $p27^{Kip1}$ were a gift from J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA). Monoclonal antibodies against cyclin E (HE172 and HE12) were generously provided by E. Harlow (MGH Cancer Center, Charlestown, MA). Monoclonal antibody against $p21^{Cip1/Waf1}$ (Ab-5) was purchased from Oncogene Research Products (Manhasset, NY). Polyclonal antibodies against cyclin D1 (H-295), cyclin E (M-20), cyclin H (C-18), Cdc 25 A (F-6), Cdk2 (M-2), Cdk 4 (C-22), Cdk 7(C-19), p15 (C-20), p16 (C-20), p27 (F-8), p57 (C-20), Rb (C-15, IF-8), and p107 (C-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

SPARC Protein and Synthetic Peptides

Murine SPARC was purified from the conditioned medium of parietal yolk sac cells as previously described [Sage et al., 1989]. SPARC peptides were synthesized and purified by high pressure liquid chromatography by the Department of Molecular Pharmacology (University of Washington) and by Peptide Express (Colorado State University), and were solubilized as described by Lane and Sage [1990]. The levels of endotoxin in SPARC and peptide 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). The peptides used in this study are shown in Figure 1.

Cell Culture

Human SMC, isolated from thoracic aortas as described [Koyama et al., 1996], were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), non-



B.

Peptide	Sequence	Location
2.1	CQNHHCKHGK VCELDESNT	54-72
2.1scr	CVQENCLHDH ECPKSHNGTK	54-73
3.4	NEKRLEAGDH PVELLARDFE	184-203
4.2	TCDLDNDKYI ALEEWAGCFG	254-273
4.2AA	**TA**DLDNDKYI ALEEWAG**AF**G	254-273

Fig. 1. Diagram of the protein SPARC with the sequence and location of synthetic peptides. **A**: Peptides are named with reference to the predicted domain structure of murine SPARC (15). ▬ = 20 amino acids. Peptide 2.1 is from the follistatin (FS)-like domain, whereas peptides 3.4 and 4.2 are from the extracellular Ca^{2+}-binding (EC) domain. **B**: Sequences of synthetic peptides used in this study. Location of peptides, with amino acids numbered from the N-terminus after the removal of the signal peptide, is indicated at right. Sequences were derived from the amino acid sequence predicted from murine SPARC cDNA. Substituted residues in peptide 4.2 are shown in bold. Scr, scrambled.

essential amino acids (Sigma), sodium pyruvate (Gibco, Grand Island, NY), penicillin G (50 U/ml), streptomycin sulfate (50 μg/ml), and amphotericin B (2.5 μg/ml) (Sigma). All growth assays were performed on Corning tissue culture plasticware (Acton, MA). For coating with monomeric collagen (Vitrogen 100, Celtrix Corp., Palo Alto, CA), plates were soaked in 0.5% acetic acid for 20 min at 60°C, rinsed with distilled water, incubated with 0.1 mg/ml of collagen solution in 0.1 M acetic acid for 3 h at room temperature, washed, and stored in serum-free DMEM.

Measurement of DNA Synthesis and Proliferation

Thymidine incorporation was assayed as described [Funk and Sage, 1991]. Briefly, cells were plated at subconfluent density ($\sim 10^5$ cells/well) in 24-well plates. Forty-eight hours later, the cultures were incubated in growth-arrest medium containing 1% human plasma-derived serum (PDS). After an additional 48–72 h, the cells were stimulated with PDGF and SPARC or SPARC peptides. Twenty hours later, the cells were pulse-labeled with 2 μCi/ml [^{3}H]-thymidine (Amersham, Arlington Heights, IL) for 2–3 h and were subsequently fixed in ice-cold 5% trichloroacetic acid (TCA). TCA-insoluble material was solubilized in 0.4 N NaOH and was assayed in a liquid scintillation counter. Long-term growth was determined by cell counts as follows: 10^4 cells were plated in 6-well plates. On Day 1, cells were removed from 3 wells by brief exposure to trypsin, fixed in 3.7% formaldehyde containing 0.5% NaCl and 1.5% Na_2SO_4, and counted in a cell zone cell counter (Particle Data, Inc., Elmhurst, IL) to establish plating efficiency or number of cells on Day 0. Remaining cells were fed with growth-arrest medium (1% PDS in DMEM) plus 2 ng/ml PDGF and SPARC peptides. On days 2, 4, and 6, triplicate wells were treated with trypsin and were fixed for cell counting. Remaining cells were refed with the test medium.

LDH Assay

Conditioned medium was assayed for lactate dehydrogenase (LDH) activity with a kit from Bio-Analytics (Palm City, FL). Culture medium was removed from wells after 20 h and was centrifuged to remove cellular debris. Twenty-five microliters of the supernate was placed into a 96-well microtiter plate; 100 μl of enzyme reagent was added to test wells and to wells containing an LDH standard (Sigma) diluted to a range of concentration from 0.195–12.5 U/ml. After 5 min, the reaction was stopped with 0.2 M acetic acid, and the colored product was quantified in an ELISA reader at 490 nm.

Iodination of SPARC and Peptide 4.2

Protein (35–150 μg) in 100 mM phosphate buffer or phosphate-buffered saline (PBS) was iodinated to a specific activity of 35–50 cpm/fmole with 1–2 mCi [^{125}I] sodium iodide (Amersham) and Iodo-Beads^{TM} (Pierce, Rockford, IL) according to the manufacturer's protocol. The protein was separated from free nuclide on a BioGel (BioRad, Hercules, CA) P-2 (for peptides) or P-6 (for SPARC) desalting column that had been pre-conditioned with 0.1% gelatin and equilibrated in PBS. Protein concentration was determined by absorbance at 280 nm, and the labeled protein was visualized by autoradiography of samples resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE).

Binding Studies

Cell-surface binding experiments were performed as described previously [Bowen-Pope and Ross, 1985; Yost and Sage, 1993]. Briefly, confluent monolayers grown in 24-well plates

were rinsed twice with ice-cold binding medium (DMEM containing 25 mM HEPES (Sigma) and 2.5 mg/ml bovine serum albumin (BSA; Sigma)) and were incubated with gentle rocking at 4°C for 2.5 h with increasing concentrations of iodinated SPARC or peptide 4.2. As controls for nonspecific binding, parallel cultures were incubated with a 200-fold molar excess of unlabeled peptide 4.2. This peptide has been demonstrated to compete effectively with both iodinated peptide 4.2 and intact SPARC for binding to bovine aortic endothelial cells [Kupprion et al., 1998]. Cells were subsequently washed three times with ice-cold binding medium and were solubilized in 1% Triton X-100 containing 1 mg/ml BSA for 4 min on a rotary shaker. Parallel cultures incubated without iodinated peptide were released by trypsin digestion and were counted in an electronic counter to determine cell number. Bound [^{125}I] cpm were measured in a gamma counter and were converted to fmoles bound per 2×10^5 cells, the average number of cells per well.

Protein Extraction, Western Blotting, and Immunoprecipitation

For preparation of lysates, cells were washed in cold PBS, scraped off the dish, and sonicated in 300 μl lysis buffer (50 mM Tris-HCl, pH 7.5; 0.5% NP-40; 150 mM NaCl; 1 mM ethylene diamine tetraacetic acid (EDTA); 1 mM NaF; 0.5 mM sodium orthovanadate (Sigma), 10% glycerol; complete protease-inhibitor cocktail™ (Boehringer Mannheim, Indianapolis, IN)). Subsequently, extracts were centrifuged at 10,000*g* for 10 min, and the total protein content in supernates was determined by the Bradford protein assay (BioRad, Hercules, CA). Equal amounts of total protein (50–200 μg) were resolved by SDS–PAGE and were transferred to Immobilon-P™ membranes (Millipore, Marlborough, MA). Non-specific binding sites were blocked by incubation for 1–3 h at room temperature with PBS containing 0.05% Tween-20 (PBST; Sigma) and 5% nonfat dry milk. Membranes were exposed to antibodies in PBST-0.5% milk for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham) and was quantified by densitometry. For assessment of differences in protein loading, the membranes were incubated at 50°C for 30 min with 2-mercaptoethanol (100 mM) and SDS (2%) in Tris-HCl (62 mM, pH 6.7) (stripping buffer), blocked, and incubated with rabbit anti-α-enolase IgG as described previously [Kupprion et al., 1998]. For $p27^{Kip1}$ immunoprecipitation studies, 200 μg of total protein extract, precleared with immobilized protein A-Sepharose™ beads (Pierce), was incubated with 4 μg/ml of anti-$p27^{Kip1}$ rabbit polyclonal antibody in 200 μl of lysis buffer for 2–4 h on ice. The immune complexes were precipitated for 2 h with 50 μl of a 50% slurry of protein A-Sepharose at 4°C, and 30 μl of the supernates was used for immunodepletion studies. Immunoprecipitated complexes were washed three times with lysis buffer containing 0.5% NP-40, resuspended in 2× SDS sample buffer [Laemmli, 1970], boiled for 5 min, and resolved by SDS–PAGE. Subsequent immunoblots were performed with polyclonal antibodies against cyclin E and $p27^{Kip1}$ (Santa Cruz Biotech). Quantification of bands on autoradiograms was performed by densitometry and results from at least three independent experiments were shown as percentage of controls. None of the average densitometric values reported varied more than 8% in 3–5 independent experiments.

Histone H1 Kinase Assays

Total protein extract (200–500 μg), precleared with immobilized protein G-Sepharose™ beads (Pierce), was incubated with 4 μg/ml of anti-cyclin E monoclonal antibody in 200 μl of lysis buffer for 2–4 h on ice. The immune complexes were precipitated for 2 h with 50 μl of a 50% slurry of Protein G-Sepharose at 4°C. Immunoprecipitated complexes were washed three times with lysis buffer containing 0.5% NP-40 and twice with kinase buffer (50 mM HEPES, pH 7.5; 10 mM $MgCl_2$; 2.5 mM ethylene-bis (oxyethylenenitrilo) tetraacidic acid (EGTA); 10 mM β-glycerophosphate; 1 mM NaF; 0.1 mM sodium orthovanadate), and were resuspended in 30 μl of kinase buffer containing 1 μg histone H1 (Boehringer Mannheim) and 20 μM [γ-^{32}P] ATP (Amersham). Reactions were incubated at 30°C for 30 min, terminated by the addition of 2× SDS sample buffer, boiled for 5 min, and resolved by SDS–PAGE. Phosphorylation of histone H1 was detected by autoradiography and was quantified by densitometry.

Cell Spreading Measurements

These studies were performed as previously described [Lane and Sage, 1990]. Briefly, SMC maintained for 2 days in 1% PDS/DMEM were replated at subconfluent densities ($1-2 \times 10^4$ cells/ml) onto monomeric collagen-coated 24-wells plates in the presence or absence of SPARC (0.6 μM) or peptide 4.2 (0.2 mM). One or more fields from a single culture well were photographed at two different time points per experiment, 4 and 24 h after plating. The extent of cell spreading Rounding Index (RI) was determined quantitatively according to the formula $RI = (1A + 2B + 3C)/A + B + C$, where A, B, and C represent the total number of cells exhibiting fully spread (index of 1), partially spread (index of 2), and completely round (index of 3), respectively. Experiments were performed in triplicate, and the average values of three independent experiments were determined.

Immunocytochemistry

SMC maintained for 2 days in 1% PDS/DMEM were replated at subconfluent density and were cultured in 1% PDS/DMEM on monomer collagen-coated coverslips, in the presence or absence of native SPARC or SPARC peptide 4.2, for up to 24 h. Cells were washed with 37°C PBS, fixed in 3.7% paraformaldehyde for 20 min at room temperature, and rendered permeable in 0.5% Triton X-100/PBS for 10 min. Subsequently, cells were washed in PBS, blocked in 4% goat serum/1% BSA/PBS for 1 h, and incubated with mouse anti-vinculin primary antibody (Sigma) in 1% goat serum/1%BSA/PBS for 1 h, and in fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (UBI, Lake Placid, NY, used at 0.5 μg/ml) for 20 min in the dark. Labeling for F-actin was performed by staining with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, used at 2×10^{-7} M) for 20 min at room temperature. Singly-labeled cells were photographed under epifluorescence microscopy.

Image Processing

Immunocytochemistry slides and autoradiograms were converted to digital computer files with a Nikon LS-2000 slide scanner™ and an EPSON Expression 800 flatbed scanner™, respectively, and Adobe Photoshop software™. Files were processed and analyzed by NIH Image software™ and are presented as composite figures.

RESULTS

SPARC Inhibits PDGF-Stimulated and Extracellular Matrix-Supported Proliferation of Human SMC

When growth-arrested human SMC monolayers were stimulated with PDGF in the presence of SPARC, we observed an inhibition of DNA synthesis at concentrations between 0.06 and 0.9 μM (Fig. 2A). SPARC at 0.6 μM (equivalent to 20 μg/ml) was associated with an approximately 50% decrease in [^{3}H]-thymidine incorporation, in comparison to controls, as previously reported for other cell types [Funk and Sage, 1991; Raines et al., 1992; Kupprion et al., 1998]. Peptide 2.1, derived from a cysteine-rich sequence in the follistatin-like domain, and the Ca^{2+}-binding peptide 4.2, from the EC domain (for amino acid sequences see Fig. 1), were also inhibitory at concentrations between 0.05–0.4 mM (Fig. 2B). Incubation with 0.2 mM peptide 2.1 or 4.2 decreased thymidine incorporation by ~50 and 70%, respectively, whereas scrambled peptide 2.1 or mutated peptide 4.2AA had minimal or no inhibitory effect (Fig. 2B). The inhibitory effects

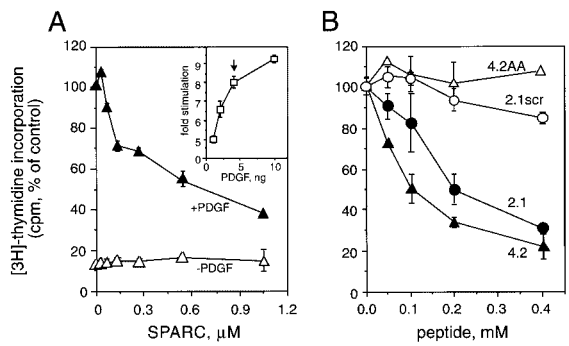


Fig. 2. SPARC and SPARC peptides 2.1 and 4.2 inhibit proliferation of PDGF-stimulated hSMC. Growth-arrested, subconfluent monolayers of human SMC were stimulated by the addition of 2–5 ng/ml PDGF-AB and increasing concentrations of murine SPARC (▲) **(A)**, or peptides 2.1 (●) and 4.2 (▲) **(B)**. Control wells were treated with SPARC without PDGF(△) (A) or peptide 4.2AA (△) and scrambled peptide 2.1 (○) (B). After an overnight incubation, cells were pulse-labeled with 2 μCi/ml [^{3}H]-thymidine for 3 h, and were subsequently fixed for scintillation counting. (A: inset), arrow indicates the concentration of PDGF used in A and B. Data are expressed as a percentage of PDGF-stimulated cells which generally ranged between 5×10^3 and 1×10^4 cpm/well. Curves shown represent the mean ± SD of triplicate wells and are from 1 experiment, which was representative of at least 3 separate experiments.

of SPARC, peptide 2.1, and peptide 4.2 were independent of the concentration of PDGF between 0.5 and 15 ng/ml (data not shown). The observed decrease in [^{3}H]-thymidine incorporation was not the result of cell death, as no measurable LDH was released into the culture supernate after 20 h of exposure to SPARC or SPARC peptides (data not shown). Little or no inhibition of [^{3}H]-thymidine incorporation was associated with the addition of peptides 4.2 (Fig. 3) and 2.1 (data not shown) to cells after S-phase had begun (12–15 h after stimulation). Cells treated with 0.2 mM peptide 4.2 at the time of release from growth-arrest (0 h) had approximately 65% fewer cpm than controls, as did cells treated at 3 and 6 h post-stimulation. When peptide 4.2 was added to cultures in excess of 12 h after the addition of PDGF, thymidine was incorporated at the same level as that of controls (Fig. 3).

Plating of growth-arrested SMC on monomeric collagen also supports DNA synthesis in the absence of PDGF [Koyama et al., 1996]. Both SPARC (0.6 μM) and peptide 4.2 (0.2 mM) diminished [^{3}H]-thymidine incorporation by as much 3- and 4-fold (at 18 h), respectively, relative to PBS controls (Fig. 4). Similar levels of inhibition of proliferation were observed with peptide 4.2 (0.2 mM) and 2.1 (0.2 mM) up to 40 h after plating of human SMC on monomeric collagen, whereas peptide 3.4 (0.2 mM), from the EC domain of SPARC, was not inhibitory (data not shown). The suppressive effects of SPARC and SPARC peptides were maintained for up to 40 h following plating. Analysis of 3-day growth assays and cell cycle distribution by cell sorting confirmed that the cells were arrested in G1 (data not shown), as reported previously for bovine aortic endothelial cells [Funk and Sage, 1991].

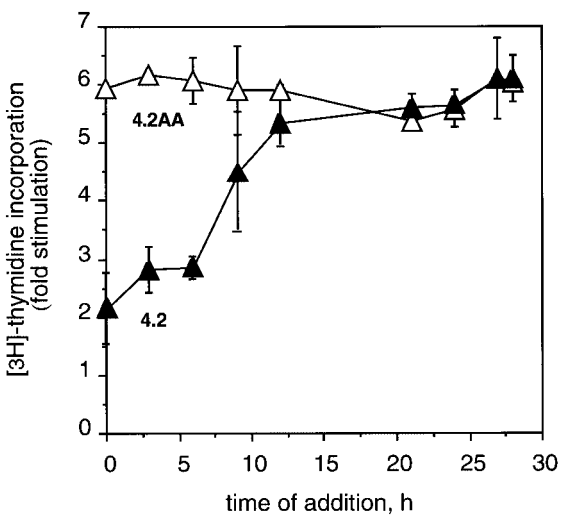


Fig. 3. Peptide 4.2 inhibits PDGF-stimulated proliferation only when added prior to the beginning of S-phase. Quiescent cultures were stimulated as described in Figure 2, but peptides 4.2 (▲) or 4.2AA (△) were added to triplicate wells at 3–4 h intervals after the addition of PDGF-AB. After 28 h, all wells were labeled with [^{3}H]-thymidine and were fixed 2 h later. Curves shown represent the mean ± SD of triplicate wells from 1 experiment, which was representative of 4 separate experiments.

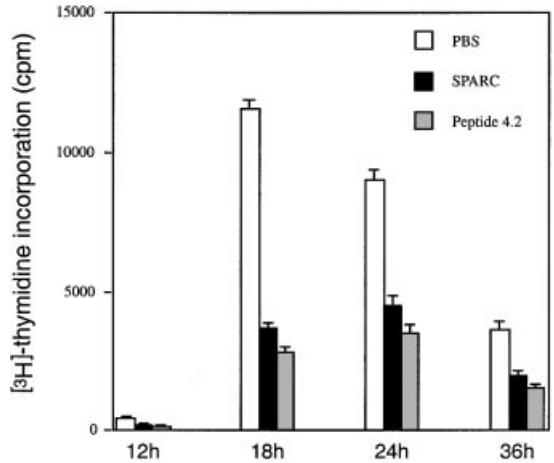


Fig. 4. SPARC and SPARC peptides inhibit DNA synthesis in human SMC following adhesion to monomeric collagen. Human SMC were synchronized in 1% PDS for 48 h and were plated on monomeric collagen in the absence (PBS) or presence of SPARC (0.6 μM) or peptide 4.2 (0.2 mM) for 12–36 h. [^{3}H]-thymidine was present during the last 3 h of the respective incubation times. Columns shown are from 1 experiment, which was representative of 3 separate experiments.

SPARC Does Not Inhibit Proliferation of Human SMC by Alteration of Cell Shape

In fibroblasts and endothelial cells, SPARC and SPARC peptide 4.2 have been shown to inhibit spreading of newly-plated cells and to promote rounding of spread cells in vitro through dissolution of focal adhesions [Sage et al., 1989; Lane and Sage, 1990; Murphy-Ullrich et al., 1995]. We asked whether suppression of DNA synthesis in human SMC by SPARC was the result of changes in cell shape. The extent of human SMC spreading on monomeric collagen was determined quantitatively by a "rounding index" (RI) measurement (see Methods) in the presence or absence of exogenous SPARC (0.6 μM) or peptide 4.2 (0.2 mM). Averaged RI values of three independent experiments did not reveal a significant

difference between the control (1.36 ± 0.7) and SPARC-treated cells (1.38 ± 0.9) at 24 h. Similar results were obtained with peptide 4.2 (data not shown).

In the absence of changes in cell shape, we asked whether addition of SPARC to human SMC plated on monomeric collagen resulted in changes in the actin cytoskeleton. Comparison of more than 100 human SMC plated on monomeric collagen in the presence or absence of SPARC did not reveal any differences in the abundance of cortical actin or transcytoplasmic actin stress fibers in the majority ($> 98\%$) of the cells studied (Fig. 5). Moreover, determination of the abundance and distribution pattern of the punctate vinculin staining, a marker of focal adhesions, revealed no discernable differences in more than 97% of the control and SPARC-treated cells studied (Fig. 5).

[^{125}I]-SPARC and Peptide 4.2 Bind to Human SMC Monolayers

Iodinated SPARC and SPARC peptide 4.2 bound specifically to confluent human SMC monolayers. Binding of [^{125}I]-SPARC and peptide 4.2 was maximal at concentrations of approximately 40 nM (1.3 μg/ml, shown at 20 pmol in Fig. 6A) and 7 μM (15.8 μg/ml, shown at 3,500 pmoles in Fig. 6B), respectively. Binding of [^{125}I]-SPARC and of [^{125}I]-peptide 4.2 could be inhibited competitively by unlabeled SPARC ($ED_{50} \approx 52$-fold molar excess) and peptide 4.2 ($ED_{50} \approx 600$-fold molar excess), respectively, but not by peptide 4.2 AA (Fig. 6C,D).

SPARC Suppresses Cell Cycle Progression on Monomeric Collagen and Blocks Hyper-phosphorylation of Rb

To understand the mechanism through which SPARC decreases monomeric collagen-stimulated proliferation of human SMC, we asked whether the expression and/or the activities of G1-specific cell cycle proteins were regulated by SPARC. Levels of cyclin D1 and its associated cdk (cdk4) were not affected by addition of SPARC or peptide 4.2 (Fig. 7A), consistent with our previous findings that SPARC affects the cell cycle in late G1 [Funk and Sage, 1991]. We have previously shown that plating of synchronized human SMC on monomeric collagen increases cyclin E-associated cdk2 activity by approximately 350% at 12 h [Koyama et al., 1996]. SPARC or peptide 4.2 decreased cyclin E-associated cdk2 activity as early as 12 h (175%) and as late as 24 h (240%), relative to control (Fig. 7A).

Cdk2 activity can be modulated at multiple levels: (1) changes in cyclin and/or cdk levels; (2) regulatory phosphorylation of conserved residues at threonine (T)-14 or tyrosine (Y)-15 (inhibitory) and at T-161 (activating); and (3) interactions with two families of cdk inhibitors

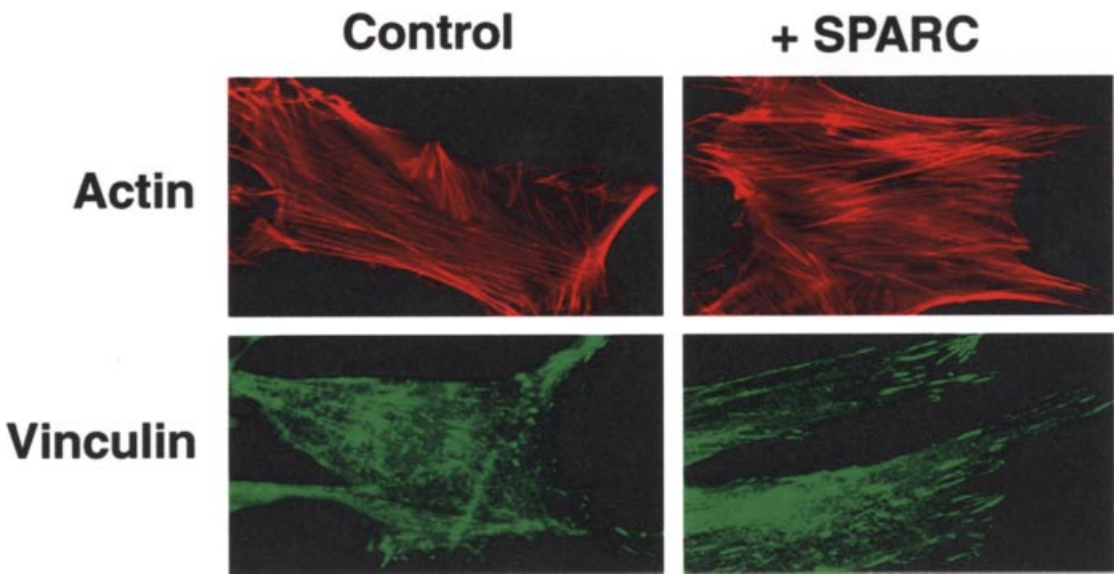


Fig. 5. SPARC does not alter the distribution of actin and vinculin. Human SMC were synchronized in 1% PDS for 48 h and were plated on monomeric collagen in the absence (control) or presence of SPARC (0.6 μM) for 24 h. Cells were fixed, rendered permeable, and stained with rhodamine-labeled phalloidin (actin) or FITC-labeled anti-vinculin IgG. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

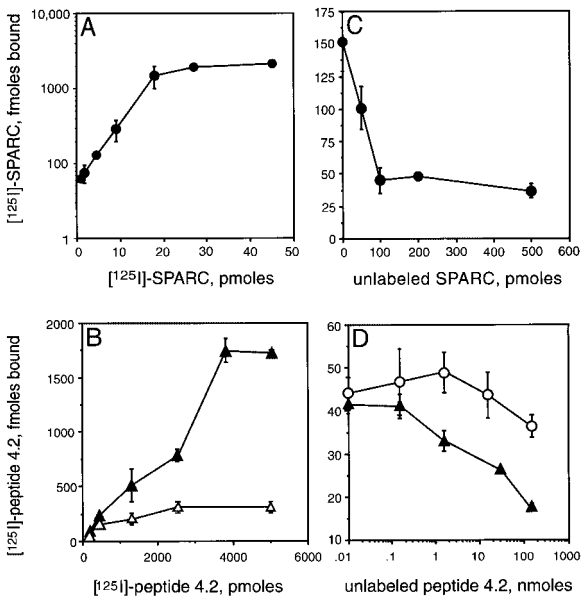


Fig. 6. Iodinated SPARC and peptide 4.2 bind to confluent hSMC monolayers. **A**: Saturable binding of iodinated SPARC to human SMC monolayers. Cells grown in 24-well plates were incubated at 4°C for 2.5 h with increasing concentrations of iodinated SPARC (●), expressed in pmoles per well (0.5 ml) (1 pmole ≈ 33 ng). Bound cpm are expressed as fmoles bound per 2×10^5 cells. Points shown represent the mean ± SD of triplicate wells from 1 experiment, which was representative of 4 separate experiments. **B**: Saturable binding of iodinated SPARC peptide 4.2 to human SMC monolayers. Cells grown in 24-well plates were incubated in 0.5 ml binding medium with increasing amounts of iodinated peptide 4.2 (▲), or iodinated peptide 4.2 plus a 500-fold molar excess of unlabeled 4.2 (△) for the determination of nonspecific binding. Peptide concentration is shown as pmoles per well (0.5 ml) (1 pmole ≈ 2.26 ng). Bound cpm are shown as fmoles bound per 2×10^5 cells. **C**: Competition for binding of [^{125}I]-SPARC with unlabeled SPARC. Cells layers were incubated with 1.35 pmoles of iodinated SPARC and increasing amounts of unlabeled competitor. Points shown represent the mean ± SD of triplicate wells from 1 experiment that was representative of 3 separate experiments. **D**: Competition for binding of [^{125}I]-peptide 4.2 with unlabeled peptides 4.2 (▲), and 4.2AA (○). Cell layers were incubated with 60 pmoles of iodinated peptide 4.2 and increasing amounts of unlabeled competitor. Points shown represent the mean ± SD of triplicate wells from 1 experiment, which was representative of 3 separate experiments, and are expressed as fmoles bound per 2×10^5 cells.

(CKIs) [for review, see Hunter and Pines, 1994; Peter and Herskovit, 1994; Morgan, 1995; Sherr and Roberts, 1995]. In the first category, SPARC decreased cyclin E-associated cdk2 kinase activity without suppression of the protein levels of cyclin E or cdk2 (Fig. 7A). Phosphorylation of cdk2 on T-160 by cdk-activating kinase (CAK) results in the activation of cdk2 with changes in its electrophoretic mobility [Gu et al., 1992]. This activated form of cdk2 appears as

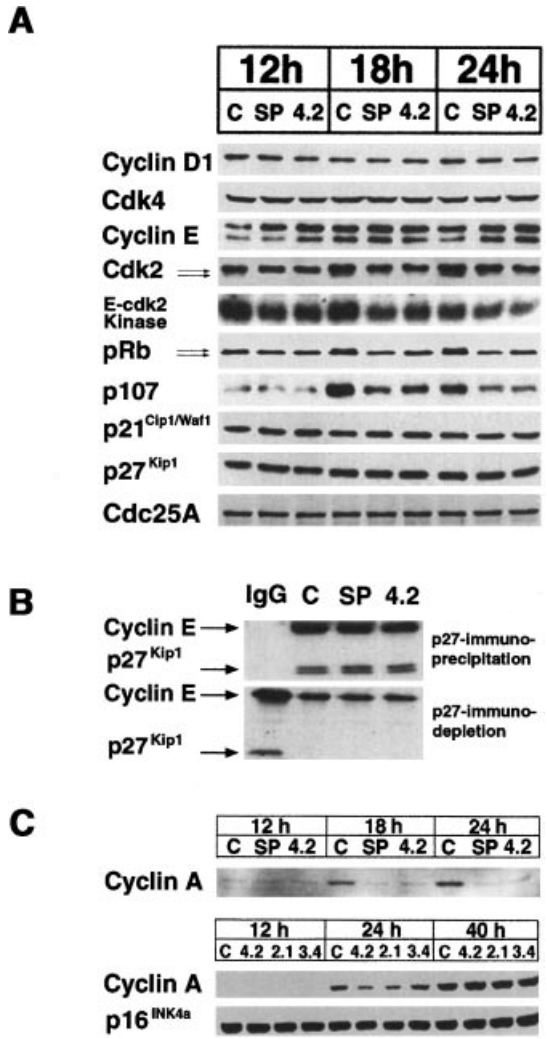


Fig. 7. SPARC diminishes cyclin E-cdk2 kinase activity, cyclin A protein level, and Rb phosphorylation. Quiescent human SMC were plated on monomeric collagen for 12–24 h in the presence or absence (C, control) of native murine SPARC (0.6 μM) or peptide 4.2 (0.2 mM). Equal amounts of cell lysates (25–100 μg) were separated by SDS–PAGE and analyzed for cyclin E-associated kinase activity, Rb phosphorylation, levels of cyclin D1, cdk4, cyclin E, cdk2, p21$^{Cip1/Waf1}$, p27^{Kip1}, and cdc25A by immunoblotting (**A**). Levels of p27^{Kip1} inhibitor associated with cyclin E complexes were determined in the presence or absence (C, control) of SPARC (0.6 μM) or peptide 4.2 (0.2 mM) 18 h after plating (**B**). p27^{Kip1} – immunoprecipitated extracts were subsequently immunoblotted with cyclin E and p27^{Kip1} antibodies (B, top panel). Rabbit IgG (IgG) was used as a control antibody to test the specificity of p27^{Kip1} immunoprecipitation. p27^{Kip1} – immunodepleted extracts were immunoblotted with cyclin E and p27^{Kip1} antibodies to ensure equal loading (B, bottom panel). Levels of cyclin A were determined 12–24 h after addition of SPARC and 4.2 as in A (**C**, top panel). Levels of cyclin A and p16^{Ink4a} were determined 12–40 h after the addition of 0.2 mM peptide 2.1, 4.2, or 3.4, as described in A (C, bottom panel). Results shown are from 1 experiment, which was representative of at least 4 independent experiments. Variability between experiments was less than 8% for every protein assayed.

early as 12 h following plating of human SMC on monomeric collagen and is predominant at 24 h (Fig. 7A). Decreases in cdc25A levels, a cdk2-activator phosphatase responsible for dephosphorylation of the inhibitory Y-15, did not appear to account for the diminution of cyclin E-cdk2 activity by SPARC (Fig. 7A). Furthermore, cdk2-activating CAK holoenzyme (cyclin H/cdk7) was found to be expressed abundantly in human SMC on monomeric collagen [Koyama et al., 1996] and was not significantly regulated by SPARC or peptide 4.2 (data not shown). Finally, SPARC and peptide 4.2 did not change the steady-state levels of the cdk2 inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (Fig. 7A), or that of $p57^{Kip2}$ (data not shown). $p16^{Ink4a}$, a specific inhibitor of cyclin D-dependent kinases, did not show cell cycle-dependent regulation and was not affected by SPARC or SPARC peptides (Fig. 7C). Moreover, cdk2 inhibitor immunodepletion studies failed to show significant differences in the redistribution of the total pool of $p27^{Kip1}$ (Fig. 7B) or that of $p21^{Cip1/Waf1}$ (data not shown) to cyclin E-associated complexes in the extracts of cells exposed to SPARC, relative to controls.

Hyperphosphorylation of Rb by activated cyclin A-cdk2 in late G1 has been implicated as the rate-limiting step for entrance into S phase [Resnitzky et al., 1995]. Analysis of the phosphorylation state of Rb 12–24 h after exposure to SPARC and peptide 4.2 revealed a decrease in the hyperphosphorylated form (upper band in Rb row) by as much as 250% at 18 h, relative to control (Fig. 7A). SPARC and peptide 4.2 also inhibited the expression of another Rb-like protein, the late G1-S-phase-specific p107, by as much as 310% at 18 h (Fig. 7A) without affecting levels of the related protein p130 (data not shown). Phosphorylation levels of p107 and p130 did not appear to be regulated by SPARC (data not shown). SPARC suppressed the expression of cyclin A protein as early as 18 h (210%), and maximally at 24 h (305%), after plating of the cells on monomeric collagen (Fig. 7C). Diminished cyclin A expression was also observed with 0.2 mM SPARC peptide 2.1 (210%) and 4.2 (275%), but not 3.4 (Fig. 7C). $p16^{Ink4a}$, a specific inhibitor of cyclin D-dependent kinases, did not show cell cycle-dependent regulation and was not affected by SPARC or SPARC peptides; therefore, it was used as an internal control (Fig. 7C). Densitometry values reported are from one experiment, which was representative of at least four independent experiments with less than 8% variability between experiments.

DISCUSSION

Our study demonstrates that treatment of human SMC with murine SPARC, as well as with SPARC peptides 2.1 and 4.2, results in an inhibition of PDGF- and ECM-stimulated DNA synthesis over a range of concentrations similar to those reported for endothelial cells and fibroblasts ($ED_{50} \approx 0.6$ μM for SPARC and $ED_{50} \approx 0.2$ mM for SPARC peptides) [Funk and Sage, 1991, 1993; Kupprion et al., 1998] (Figs. 2 and 4). Inhibition of ECM- and PDGF-stimulated proliferation could indicate that SPARC acts primarily on the SMC, with a minimal contribution from SPARC as a binding protein for PDGF [Raines et al., 1992]. However, our data do not rule out the possibility of different mechanisms of action at similar potencies. The disparity between the effective concentrations of native protein and peptides used in this study can be attributed to several factors: (i) peptides are inherently less stable than the corresponding native proteins, and in all likelihood do not adopt the appropriate tertiary structure required for optimal activity. (ii) Native SPARC imparts its antiproliferative activity through two distinct domains (follistatin-like and EC). It is therefore plausible that independent or synergistic action of these domains in the native protein is required for optimal activity. Juxtaposition of the two domains is indeed supported by X-ray crystallographic studies [Hohenester et al., 1996]. Disparities of over an order of magnitude between the ED_{50} of native protein and peptides have also been reported for the matricellular protein thrombospondin-1 [Kanda et al., 1999].

We observed that higher ED_{50} values of SPARC and SPARC peptides (15- and 28-fold, respectively) were required to inhibit proliferation of SMC in our system, compared to the concentrations that conferred maximal binding to the cell surface. Likely explanations for this disparity are the following: (i) the binding studies were carried out on confluent monolayers, whereas the proliferation studies utilized actively-dividing subconfluent cell populations grown on monomeric collagen or plastic. That SPARC binds to both collagen and plastic surfaces could, at least in part, account for the higher ED_{50} requirement of SPARC and

SPARC peptides in biological activity assays. (ii) As a consequence of purification-related denaturation, a significant population of SPARC molecules might retain the capacity to bind to the receptor but lack the necessary conformation to elicit downstream receptor-mediated events. (iii) Non-receptor-mediated events may partially account for the regulation of SMC proliferation by SPARC in our model system.

Inhibition of SMC Proliferation Occurs in the Absence of Changes in Cell Shape

Endothelial cells and fibroblasts, but not SMC, respond to SPARC with a change in cell shape that leads to cell rounding [Sage et al., 1989]. Cell shape has been shown to be coupled to DNA synthesis [Assoian and Zhu, 1997; Huang and Ingber, 1999]. Thus, the induction of cell rounding by SPARC could be a primary mechanism by which SPARC inhibits DNA synthesis in endothelial cells and fibroblasts. However, in SMC, inhibition of monomeric collagen-supported cell cycle traverse by SPARC was independent of changes in cell shape (Fig. 5) and was, at least in part, the result of significant diminution of cyclin E-cdk2 activity, p107 levels, cyclin A expression, and Rb phosphorylation (Fig. 7).

SPARC Modulates a Unique Set of Cell Cycle Molecules

Inhibition of SMC proliferation by SPARC fails to alter other cell cycle molecules targeted by cytoskeletal changes and ECM. For example, polymerized collagen fibrils also inhibit SMC proliferation through the increased levels and association of the cdk inhibitors $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ with the cyclin E-cdk2 complex. The lack of cyclin E-cdk2 activity in suspended cells also appears to be due primarily to increased expresssion of the cdk inhibitors [Fang et al., 1996]. However, we observed no change in total levels or cyclin E-cdk2-associated levels of the cdk inhibitors following treatment with SPARC or SPARC peptide 4.2 (Fig. 7A,B). Rather, our data identify a modulation of the Rb family by SPARC, and the downstream effects of the failure to inactivate the potent repressor activity of Rb.

A central role of the Rb family (Rb, p107, and p130) is their negative regulation of the activity of E2F factors that control the transcription of many cell cycle-regulated genes, including regulators of S-phase entry and genes required for DNA replication [for review, see Kaelin, 1999]. Rb inhibits transcription by the formation of complexes with transcription factors and has been shown to regulate gene expression through E2Fs by (a) inhibition of their transcriptional activity through sequestration, or (b) direct binding and recruitment of histone deacetylase repressor complex to E2F-regulated promoters [Brehm et al., 1998; Kaelin, 1999]. Phosphorylation of the C-terminal region of Rb by the cyclin D-cdk4/6 complex in early G1 was recently reported to initiate displacement of histone deacetylase from the protein-binding pocket region and to trigger its sequential phosphorylation and disruption by cdk2 [Harbour et al., 1999]. Conversely, the activity of histone acetyl-transferase has been shown to be maximal prior to S-phase entry and to be critical for activation of E2F [Ait-Si-Ali et al., 2000; Zhang et al., 2000]. Whether SPARC inhibits activation of E2F through modulation of histone acetylation in a cdk-independent manner is currently under investigation. In addition to the well-established role of Rb as a negative regulator of cyclin E and cyclin A expression through E2F, E2F-independent suppression of cdk2 activity in vascular SMC has also been reported [Sasaguri et al., 1996]. Lastly, physical interaction of Rb with prohibitin, a potential tumor suppressor, has been shown to inhibit the activity of E2Fs [Wang et al., 1999]. Since cell cycle-dependent nuclear localization of SPARC was recently reported [Gooden et al., 1999], a direct interaction with Rb as a potential mechanism through which SPARC inhibits Rb phosphorylation cannot be excluded.

Interestingly, SPARC also suppressed the protein levels of the Rb family member p107 to a significant degree, 18 h after the plating of human SMC on monomeric collagen (Fig. 7A). p107 is believed to be a potent inhibitor of cell cycle progression, and its overexpression was shown to suppress G1 to S cell cycle traverse (for review, see Smith et al., 1998; Zhu et al., 1993]. However, the exact molecular mechanisms of p107 function during growth suppression remains to be elucidated. Unlike Rb and p130, p107 is not present in quiescent cells, and its accumulation in proliferating cells during G1–S phase is regulated, for the most part, at the level of transcription. p107 has been reported to interact with E2F, forming a p107/E2F/cyclin E/cdk2 complex in the late G1-phase, and a p107/

E2F/cyclin A/cdk2 complex in the S-phase [Lees et al., 1992; Schwarz et al., 1993]. This latter complex was reported to have histone H1 kinase activity [Devoto et al., 1992]. The continued presence of these complexes in proliferating cells, as well as the E2F-mediated repression of the *p107* gene in quiescent cells, indicates a positive role for p107 in the regulation of cell proliferation [Leoncini et al., 1999; Timchenko et al., 1999]. Increased levels of p107 expression in vivo have also been reported in proliferating vascular SMC following balloon angioplasty [Garriga et al., 1998]. Conversely, suppression of p107 levels has been reported after treatment of cells with several antiproliferative agents, such as the atherosclerotic plaque-stabilizer 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor lovastatin, the antimetabolite 5-fluorouracil, and the cAMP-dependent protein kinase activator cyclic nucleotide dibutyryl cAMP [Jang et al., 1999]. Significant diminution of p107 expression in SMC by SPARC in our experimental system is in agreement with the hypothesis that p107 plays a positive role in S-phase entry, rather than acting as a tumor suppressor. In summary, our findings show that inhibition of monomeric collagen-supported cell cycle traverse by SPARC is independent of alterations in cell shape or the actin cytoskeleton (Fig. 5) and is, at least in part, the result of significant diminution of cyclin E-cdk2 activity, p107 levels, cyclin A expression, and Rb phosphorylation (Fig. 7).

Potential Effects of SPARC on SMC In Vivo

Elevated and coincident expression of both SPARC and PDGF-B chain has been reported after arterial injury and in advanced lesions of atherosclerosis, primarily in medial SMC and in cells of the neointima [Raines et al., 1992]. The seemingly paradoxical increase in the expression of both SPARC and PDGF-B chain could be considered yet another example of the balance between the function of activators and inhibitors within a given cell. Coincident expression of activators and inhibitors has been reported for matrix metalloproteinases and their tissue inhibitors, plasminogen activators and their inhibitors (PA/PAI), as well as for activators and inhibitors of angiogenesis (VEGF and FGF-2 vs. angiostatin and endostatin) in solid tumors [Marneros and Olsen, 2001; Pepper, 2001]. Thus, SPARC might contribute to the regulation of vascular human SMC proliferation in atherogenesis by its direct action on the SMC and by its inhibition of PDGF and vascular endothelial growth factor activity through direct binding [Raines et al., 1992; Kupprion et al., 1998]. Identification of Rb inactivation and the suppression of p107 levels by SPARC as a potential mechanism for inhibition of matrix-mediated SMC proliferation in the absence of changes in cell shape or cdk inhibitors is a novel function of this matricellular protein. Although both SPARC and the Ca^{2+}-binding peptide 4.2 have been shown to bind to bovine aortic endothelial cells [Yost and Sage, 1993] and human SMC (this study), neither a receptor nor a cell surface-binding component for SPARC has been identified. Recently, SPARC was shown to bind to a 150-kDa cell surface receptor in breast and prostate cancer cell lines [Koblinski et al., 2001]. Binding of SPARC to this component was manganese-dependent and was inhibited by RGD but not RGE. Moreover, it was speculated that tumor cell invasion and matrix metalloprotease-2 activation in these cell lines by SPARC is mediated through the action of this putative receptor. Definitive identification of SPARC receptor(s) or cell surface-binding component(s) will help decipher the exact mechanism of cell cycle arrest by this matricellular protein.

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