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Fibroblast Growth Factor Receptor-1 Mediates the Inhibition of Endothelial Cell Proliferation and the Promotion of Skeletal Myoblast Differentiation by SPARC: A Role for Protein Kinase A

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Abstract The role of the matricellular protein SPARC (secreted protein, acidic and rich in cysteine) in modulation of vascular cell proliferation is believed to be mediated, in part, by its ability to regulate the activity of certain growth factors through direct binding. In this study, we demonstrate that SPARC does not bind to basic fibroblast growth factor (bFGF/FGF-2) or interfere with complex formation between FGF-2 and its high-affinity FGF receptor-1 (FGFR1), yet both native SPARC and a peptide derived from the C-terminal high-affinity Ca^{2+}-binding region of protein significantly inhibit ligand-induced autophosphorylation of FGFR1 (>80%), activation of mitogen-activated protein kinases (MAPKs) (>75%), and DNA synthesis in human microvascular endothelial cells (HMVEC) stimulated by FGF-2 (>80%). We also report that in the presence of FGF-2, a factor which otherwise stimulates myoblast proliferation and the repression of terminal differentiation, both native SPARC and the Ca^{2+}-binding SPARC peptide significantly promote (>60%) the differentiation of the MM14 murine myoblast cell line that expresses FGFR1 almost exclusively. Moreover, using heparan sulfate proteoglycan (HSPG)-deficient myeloid cells and porcine aortic endothelial cells (PAECs) expressing chimeric FGFR1, we show that antagonism of FGFR1-mediated DNA synthesis and MAPK activation by SPARC does not require the presence of cell-surface, low-affinity FGF-2 receptors, but can be mediated by an intracellular mechanism that is independent of an interaction with the extracellular ligand-binding domain of FGFR1. We also report that the inhibitory effect of SPARC on DNA synthesis and MAPK activation in endothelial cells is mediated in part (>50%) by activation of protein kinase A (PKA), a known regulator of Raf-MAPK pathway. SPARC thus modulates the mitogenic effect of FGF-2 downstream from FGFR1 by selective regulation of the MAPK signaling cascade. J. Cell. Biochem. 90: 408–423, 2003.

Key words: SPARC; matricellular; fibroblast growth factor receptor-1; endothelial cell; proliferation; protein kinase A

The concerted action of growth factors and the extracellular matrix (ECM) provides signaling cues that regulate cellular processes such as proliferation, migration, and differentiation [Damsky and Werb, 1992; Eliceiri, 2001]. Matricellular proteins, a family of secreted glycoproteins that modulate cell–matrix interactions but do not function as structural components, have been implicated in transient regulation of cellular signaling events within the ECM microenvironment [Murphy-Ullrich, 2001; Bornstein and Sage, 2002]. SPARC, also known as osteonectin and BM-40, is a

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matricellular protein that inhibits the proliferation of a variety of cells, primarily through modulation of cell adhesion, growth factor activity, and cell cycle progression from G1 to S phase [for review see Brekken and Sage, 2000]. Direct binding of SPARC to platelet-derived growth factor (PDGF)-AB or -BB has been shown to inhibit the proliferation of human fibroblasts through suppression of ligand binding to cognate cell-surface receptors [Raines et al., 1992]. Studies on human microvascular endothelial cell (HMVEC) have shown that SPARC, and a peptide derived from the C-terminal high-affinity Ca^{2+}-binding region of the protein (peptide 4.2), inhibit cellular proliferation and MAPK activity stimulated by vascular endothelial growth factor ($VEGF_{165}$), primarily through a direct binding interaction and suppression of VEGF association with its cell surface receptors [Kupprion et al., 1998]. In contrast, inhibition of the proliferative properties of fibroblast growth factor (FGF)-2 by SPARC in bovine aortic endothelial cell (BAEC) has been shown to occur in the absence of a direct physical interaction between the two molecules, to be independent of the inhibition of ligand binding to its high-affinity FGFR1, and to require a serum factor [Hasselaar and Sage, 1992]. The exact mechanism through which SPARC inhibits endothelial cell proliferation stimulated by FGF-2 is not known and was, therefore, investigated in this study.

FGF-2 is a potent stimulator of growth and differentiation in cells of mesodermal origin [for review see Bikfalvi et al., 1997; Szebenyi and Fallon, 1999; Nugent and Iozzo, 2000] which exerts its functions through interactions with both high-affinity tyrosine kinase FGFRs and low-affinity heparan sulfate proteoglycans (HSPGs). Four distinct but structurally related FGFRs (FGFR1/*flg*, FGFR2/*bek*, FGFR3, and FGFR4) form a subfamily among transmembrane receptor tyrosine kinases. FGFRs are comprised of an extracellular ligand-binding domain containing two or three immunoglobulin-like repeats, a transmembrane region, and a split intracellular kinase domain. A trimeric complex between FGF-2, FGFR, and HSPG is believed to facilitate receptor dimerization and activation of the kinase domain, followed by autophosphorylation of the receptor and its association with downstream signaling effector molecules [Kan et al., 1993; Klint and Claesson-Welsh, 1999]. Autophosphorylated tyrosine residues in the receptor sequences are believed to serve as docking sites for binding and activation of Src homology-2 (SH2) domain-containing proteins like Grb2/Sos, known regulators of the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway [Szebenyi and Fallon, 1999]. The signaling pathways activated by FGFR1 have been studied extensively and have been implicated in mitogenesis, plasminogen-activator expression, cellular migration, and differentiation [Kan et al., 1993; Klint and Claesson-Welsh, 1999].

In this study, we confirmed our previous findings that SPARC does not bind to FGF-2. Moreover, our results indicated that SPARC does not interfere with FGF-2–FGFR1 complex formation, yet it suppresses ligand-induced autophosphorylation of the receptor, extracellular-regulated kinase (ERK) activation, and DNA synthesis in HMVEC in a serum-independent manner. To ensure that suppression of FGF-2 activities by SPARC was mediated mainly through FGFR1, we used the murine myoblast cell line MM14 since FGF family members are the only growth factors known to repress their myogenesis, and they express FGFR1 almost exclusively [Templeton and Hauschka, 1992]. Withdrawal of FGF-2 leads to suppression of proliferation, concomitant activation of muscle differentiation-specific genes, and terminal differentiation to myocytes. The exact mechanism through which FGF-2 both stimulates myoblast proliferation and represses differentiation is not understood, but it is believed to involve both ERK-dependent and ERK-independent pathways [Campbell et al., 1995; Kontaridis et al., 2002]. Our results indicated that addition of SPARC and SPARC peptide 4.2 to myoblasts in the presence of FGF-2 significantly promoted their differentiation to myocytes.

Using HSPG-deficient myeloid cells and porcine aortic endothelial cells (PAECs) expressing full-length FGFR1 or chimeric PDGFRα/FGFR1, we demonstrated that: (i) the antiproliferative effect of SPARC on cells stimulated with FGF-2 did not require the presence of HSPG co-receptors, (ii) SPARC inhibited FGFR1-mediated ERK activation but not Akt phosphorylation, a downstream effector of phosphatidylinositol 3-kinase (PI3-K), suggesting that SPARC preferentially targets the MAPK signaling cascade, and (iii) SPARC antagonized FGFR1 signaling via an intracellular mecha-

nism of inhibition downstream from the receptor. Our results also indicate that inhibition of DNA synthesis and ERK activation in HMVEC stimulated by FGF-2 is, in part, mediated by activation of protein kinase A (PKA). To our knowledge, these findings provide the first evidence for an intracellular mechanism of inhibition of FGFR1 signaling by a matricellular protein.

MATERIALS AND METHODS

Growth Factors, Antibodies, and Inhibitors

Recombinant bovine FGF-2 was purchased from R&D Systems (Minneapolis, MN). Purified recombinant human (rh) FGF-2 for MM14 myoblast differentiation studies was provided to S. Hauschka by Zymogenetics, Inc. (Seattle, WA). Polyclonal antibodies against FGF-2, FGFR1, and PDGF-BB were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against human SPARC/osteonectin were from Chemicon (Temecula, CA) and Haematologic Technologies (Essex Junction, VT), respectively. Polyclonal antibodies against pan-MAPK, phospho-specific MAPK, pan-Akt, and phospho-Akt, were from Cell Signaling (Beverly, MA). Mouse anti-human phosphotyrosine IgG was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibody against α-enloase was a gift from Dr. A. Redlitz (Schering Pharmaceuticals, Berlin, Germany). Monoclonal antibody against β-tubulin was from Sigma (St. Louis, MO). Heparin (from porcine intestinal mucosa) was from Sigma. Forskolin, H-89, KT-5720, and calphostin C were from Calbiochem (La Jolla, CA), and LY294002 was from Biomol Research Laboratories (Plymouth Meeting, PA).

SPARC Protein and Synthetic Peptides

Murine SPARC was purified from the conditioned medium of murine parietal yolk sac carcinoma cells as previously described [Sage et al., 1989]. rh-SPARC was prepared in SF9 cells by the use of a baculoviral protein expression system and was collected in serum-free medium, as previously reported [Bradshaw et al., 1999]. SPARC peptides were synthesized and purified by HPLC by the Department of Molecular Pharmacology, University of Washington and by Peptide Express (Colorado State University, Fort Collins, CO) and were solubilized as described by Lane and Sage [1990]. Peptide 4.2 (TCDLDNDKYIALEEWAGCFG; amino acids 254–273) and peptide 3.4 (NEKRLEAGDHPVELLARDFE; amino acids 184–203) are from the extracellular Ca^{2+}-binding (EC) domain of human SPARC. The levels of endotoxin in SPARC and peptide preparations used in this study were below 0.1 EU/mg, as determined by the *Limulus* amebocyte lysate (LAL) gel clot assay (Associates of Cape Cod, Woods Hole, MA).

Cell Culture

Low passage (5–9) HMVEC (Clonetics, San Diego, CA) were grown in 75 cm^2 flasks coated with 2% gelatin (Sigma) in MCDB-131 medium (Sigma) containing 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA), 50 μg/ml endothelial cell growth supplement (Biomedical Technologies, Inc., Stoughton, MA), penicillin G (50 U/ml), streptomycin sulfate (50 μg/ml), 10 μg/ml heparin (Sigma), and 2 mM L-glutamine (Sigma). PAECs (PAEC/FGFR1 and αR/FR; a gift from Dr. Lena Claesson-Welsh, Uppsala, Sweden) were grown in Ham's F-12 medium (Life Technologies, Inc.) containing 10% fetal bovine serum, penicillin G, and streptomycin sulfate as above. HSPG-deficient murine myeloid cells expressing FGFR1 (BaF3/32D*flg*, a gift from Dr. Patrizia Dell'Era, Brescia, Italy, and BaF3/FR1c11, a gift from Dr. David Ornitz, St. Louis, MO) were grown in RPMI 1640 (Life Technologies, Inc.) containing 10% calf serum (Life Technologies, Inc.), 4 mM L-glutamine, 1% penicillin G, and streptomycin sulfate, 0.0035% β-mercaptoethanol (Sigma), and 10% WEHI3 (a murine macrophage-like IL-3-producing cell line)-conditioned medium, as described previously [Allen et al., 2001].

In Vitro Binding Studies

rhSPARC or murine SPARC was biotinylated according to a protocol and with reagents provided by Pharmacia Corp. (Peapack, New Jersey). Biological activity of the biotinylated SPARC, as determined by DNA synthesis, was equivalent to that of unlabeled preparations (data not shown). Biotinylated SPARC (34 kDa) was incubated with FGF-2 (18 kDa) at 1:1, 3:1, and 1:3 molar ratios in 100–200 μl of binding buffer (Hank's balanced salt solution (Life Technologies, Inc.); 0.5% tryptone (Sigma); 0.1% Tween-20 (Sigma)) for 4 h on a rotary shaker at 4°C. SPARC complexes were precipitated after a 2–4 h incubation with 50 μl of a slurry of Ultralink Immobilized NeutrAvidin™

beads (Pierce, Rockford, IL). Supernates were collected and precipitated complexes were washed four times with 250–500 μl wash buffer (80% binding buffer/20% radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Non-idet P-40 (Sigma), 0.5% deoxycholic acid (Sigma), 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.5), resuspended in 80 μl of 2× SDS sample buffer [Laemmli, 1970], and boiled for 5 min. Forty microliters of supernates and precipitated complexes were resolved by SDS–PAGE, and subsequent immunoblots were performed with antibodies against FGF-2 and SPARC. To assess whether SPARC suppresses FGF-2 binding to FGFR1, we incubated FGF-2 (89.4 ng) with rhFGFR1α (IIIb)/Fc chimera (66 kDa, R&D Systems) and SPARC (at equimolar or at a 1:1:3 molar ratio), in the presence or absence of heparin (10 nM), under assay conditions similar to that used in the aforementioned binding studies with FGF-2. FGF-2/FGFR1 complexes formed in 100–200 μl of binding buffer were precipitated with 25–50 μl of a slurry of Ultralink Immobilized Protein G-Sepharose™ beads (Pierce), resuspended and boiled in 80 μl of 2× SDS sample buffer. Forty microliters of the precipitated complexes and the unbound fractions from supernates were resolved by SDS–PAGE and were immunoblotted either independently or sequentially with antibodies specific for FGF-2, SPARC, and FGFR1. FGFR1 was immunoblotted with either an anti-human Fc HRP-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or a polyclonal antibody specific for FGFR1. Heparin binding plate assays were performed as previously described [Allen et al., 2001]. FGF-2 (10–30 nM) was immobilized on 96-well plates coated with heparin (10 nM) in the absence or presence of SPARC (1.2 μM) for 1 h at room temperature. Soluble IIIc splice variant of FGF receptor alkaline phosphatase (FRAP) fusion protein (FR1cAP) (30–100 nM) was added to the heparin-immobilized FGF-2 in the presence or absence of SPARC (1.2 μM) for 1 h at room temperature. After removal of unbound receptors with PBS washes (3×), 50 μl of AP assay mix (1 M diethanolamine, 0.5 mM $MgCl_2$, 10 mM homoarginine, 6 mM *p*-nitrophenyl phosphate; all from Sigma) was added to each well. Binding of FRAP to heparin-bound FGF-2 was determined by recorded absorbance readings at 405 nm. Specificity of FRAP binding was verified by the observed minimal absorbance readings after exclusion of FGF-2 from binding studies.

Protein Extraction, Western Blotting, and Immunoprecipitation

Cells were sonicated in Non-idet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5; 0.5% NP-40; 150 mM NaCl; 1 mM 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 bicinchoninic acid (BCA) protein assay (Bio-Rad, Hercules, CA). Equal amounts of total protein (50–100 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) and 5% non-fat 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 Supersignal West Dura™ chemiluminescence substrate according to the manufacturer's instructions (Pierce) and was quantified by densitometry. For assessment of differences in protein loading, the membranes were incubated at 50°C for 45 min with 2-mercaptoethanol (100 mM) and SDS (2%) in Tris-HCl (62 mM, pH 6.7) (stripping buffer), blocked, and incubated with an anti-pan-ERK IgG or an anti-α-enolase IgG as previously described [Kupprion et al., 1998]. For FGFR1 immunoprecipitation studies, 200 μg of total protein extract was precleared with immobilized protein A Sepharose beads (Pierce) and was incubated with 4 μg/ml anti-phosphotyrosine mouse monoclonal antibody (UBI) in 200 μl lysis buffer for 4 h on ice. The immune complexes were precipitated for 4 h with 50 μl of a 50% slurry of Protein A Sepharose at 4°C, and 30 μl aliquots of supernates were used for immunodepletion studies. Immunoprecipitated complexes were washed three times with lysis buffer containing 0.5% Non-idet P-40, resuspended in 2× SDS sample buffer, boiled for 5 min, and resolved by SDS–PAGE. Subsequent immunoblots were performed with polyclonal antibodies against FGFR1 (Santa Cruz Biotechnology).

Measurement of DNA Synthesis and Proliferation

Thymidine incorporation was assayed as described [Funk and Sage, 1991]. Briefly, cells were plated at subconfluent density ($5-10 \times 10^4$ cells/well) in 24-well plates. Twenty-four hours later, the cultures were starved in respective media devoid of serum and growth supplements (MCDB-131 for HMVEC, RPMI-1640 + 0.5% WEHI3-conditioned medium for BaF3/32D*flg*, and Ham's F-12 for PAEC/FGFR1 and α-R/FR) for 24–48 h, stimulated with FGF-2 (20 ng/ml) for 15–17 h, and pulse-labeled with 4 μCi/ml [^{3}H]-thymidine (50 Ci/mmol, Amersham, Arlington Heights, IL) for 4 h. Material precipitated in ice-cold 10% trichloroacetic acid was solubilized in 0.4 N NaOH and was subsequently assayed in a liquid scintillation counter. Three-day proliferation assays of BaF3/FR1c11 myeloid cells were performed as described previously [Allen et al., 2001]. Cells were washed in media lacking WEHI3-conditioned medium and were plated in 96-well plates at 5×10^3 cells/well in a final volume of 100 μl. FGF-2 (10 nM) and heparin (50 nM) were added in the presence or absence of SPARC (1.2 μM), and cells were incubated at 37°C in 95% humidity and 7.5% CO_2 for 72 h. For quantification of relative cell numbers, 20 μl Cell Titer 96 AQueous One Solution™ reagent (Promega, Madison, WA) was added to each well, and absorbance readings were taken at the rate of 490 nm, after a 1–4 h incubation at 37°C.

Myoblast Differentiation Assay

Mass cultures of MM14 cells were grown on gelatin-coated plates in Ham's F-10 medium (Life Technologies, Inc.) containing 15% horse serum (Life Technologies, Inc.), calcium (1.26 mM), and FGF-2 (2 ng/ml) as described previously [Olwin and Hauschka, 1988]. Clonal assays of differentiation were performed as described previously [Clegg et al., 1987]. Briefly, exponentially growing MM14 cells were removed from dishes with trypsin, and approximately 100 cells were plated into 60 mm tissue culture dishes in the presence of FGF-2 (0.5 ng/ml) alone (control) or FGF-2 with SPARC (0.15 and 0.3 μM) or SPARC peptide 4.2 (50 and 150 μM). After approximately 3 days of growth, cultures were fixed, and stained with MF20 (Sigma), an antibody to myosin heavy chain, a marker of fully differentiated skeletal muscle [Clegg et al., 1987].

Image Processing

Autoradiograms were converted to digital computer files with an EPSON Expression 800 flatbed scanner™ and Adobe Photoshop software™. Files were processed and analyzed by NIH Image software™ and are presented as composite figures.

Statistical Analysis

The data presented are from at least three independent experiments and are reported as means ± SEM. Statistical significance was assessed with Student's *t*-test for paired comparisons, relative to control.

RESULTS

SPARC Does Not Bind to FGF-2 or Inhibit FGF-2 Binding to FGFR1

It was previously shown by slot blot analysis that there was no interaction between [^{125}I]-murine SPARC (1 μg/ml, specific activity 23.5 μCi/μg) and FGF-2 (500 ng), whereas SPARC bound to type III collagen (5 μg) and PDGF-AB (50 ng) [Hasselaar and Sage, 1992]. The lack of interaction between FGF-2 and SPARC was also verified by ligand blotting studies using [^{125}I]-FGF-2 [Hasselaar and Sage, 1992]. We wanted to verify that biotinylated rhSPARC or murine SPARC would also fail to interact with FGF-2 in solution precipitation assays. Our results revealed that biotinylated rhSPARC levels as low as 50 ng can be efficiently precipitated (>80%) with NeutrAvidin beads (Fig. 1A, top panel). This binding was shown to be specific, since addition of a 100-fold molar excess of unlabeled SPARC competed effectively (>85%) for binding (Fig. 1A, top panel). Using this assay system, and consistent with previous reports [Hasselaar and Sage, 1992; Gohring et al., 1998], we were able to show direct binding of SPARC to PDGF-BB at a 1:1 molar ratio (Fig. 1A, bottom panel). Addition of NeutrAvidin beads to this mixture resulted in a significant coprecipitation (>70%) of PDGF-BB with biotinylated rhSPARC (Fig. 1A, bottom panel). Effective competition (>75%) for binding with a 100-fold molar excess of unlabeled SPARC (Fig. 1A, bottom panel), and concentration-dependent increases in binding at higher molar ratios of SPARC to PDGF (data not shown),

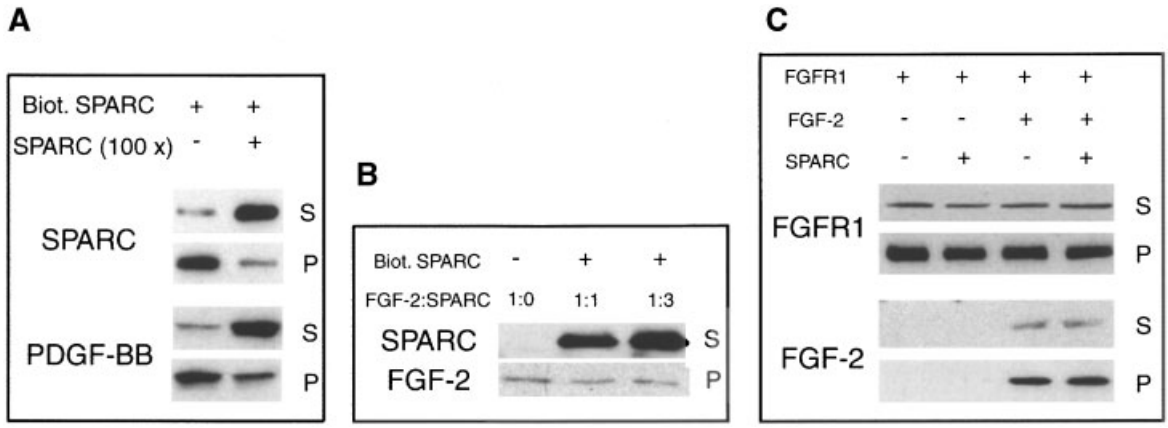

Fig. 1. SPARC does not bind to fibroblast growth factor (FGF)-2 or interfere with the binding of FGF-2 to FGFR1. **A**: Biotinylated rhSPARC (53 ng) alone (**top panel**) or preincubated with PDGF-BB at a 1:1 molar ratio (**lower panel**), was precipitated after a 4 h incubation with 40 µl of a slurry of NeutrAvidin™ beads at 4°C in the presence (+) or absence (−) of a 100× molar excess of unlabeled SPARC. Half of the precipitated complexes (P) and 40 µl of the supernates (S) were immunblotted with antibodies against SPARC (top panel) or PDGF-BB (lower panel). **B**: FGF-2 (29.8 ng) alone (1:0), or preincubated with biotinylated SPARC at 1:1 or 1:3 molar ratios, was precipitated as in (A). Half of the precipitated complexes (P) were immunblotted with an antibody against SPARC (**top panel**), and 40 µl of the supernates (S) were immunblotted with an antibody against PDGF-BB (**lower panel**). **C**: FGF-2 (89.4 ng) was incubated with FGFR1 and unlabeled SPARC at a respective 1:1:3 molar ratio in the presence of heparin (10 nM). Half of the complexes precipitated with Protein G-Sepharose™ beads (P) and 40 µl of the supernates (S) were immunblotted with antibodies against FGFR1 (**top panels**) and FGF-2 (**bottom panels**). The results shown are from one experiment that was representative of at least four independent experiments. Variability among experiments was less than 8% for each protein.

were indicative of a specific binding between the two molecules. Regardless of the molar ratios of FGF-2 and biotinylated rhSPARC used, or of the assay conditions, we did not detect a significant level of co-precipitation of FGF-2 with SPARC, further confirming the absence of a direct molecular interaction between the two molecules (Fig. 1B). Similar results were obtained in studies with biotinylated murine SPARC (data not shown).

To determine whether SPARC could suppress the binding of FGF-2 to FGFR1 in vitro, we first verified that FGF-2 would bind to a purified Fc chimera of recombinant human extracellular ligand-binding region of FGFR1 (FGFR1α (IIIb)/Fc chimera) in the presence of heparin. Under our assay conditions, which included 10 nM heparin in the binding reaction, there was optimal complex formation between the ligand and its high-affinity chimeric receptor at a 1:1 molar ratio. Complex formation between FGF-2 and FGFR1 was not inhibited to any significant extent when SPARC was added simultaneously at equimolar ratios (Fig. 1C) or at 5× higher molar ratios (data not shown). Preincubation of SPARC with either FGF-2 or FGFR1 for up to 2–4 h prior to the initiation of the assay also failed to suppress complex formation between the ligand and its high-affinity receptor (data not shown). Furthermore, the results of our plate assays (see Experimental Procedures) did not indicate any significant inhibition of FR1c-AP binding to heparin-immobilized FGF-2 by SPARC or SPARC peptide 4.2 (Allen et al., data not shown). Together, these results confirmed our earlier findings that there is no molecular interaction between SPARC and FGF-2. Moreover, consistent with our previous finding that SPARC did not interfere with the high-affinity binding of [^{125}I]-FGF-2 to BAEC [Hasselaar and Sage, 1992], our results demonstrated that SPARC did not appear to affect complex formation between FGF-2 and its cognate high-affinity receptor in vitro.

SPARC Inhibits DNA Synthesis, MAPK Activation, and FGFR1 Phosphorylation of HMVEC

SPARC has been shown to inhibit DNA synthesis of BAEC in the presence of serum [Olwin and Hauschka, 1988], as well as in the presence of plasma-derived serum [Hasselaar and Sage, 1992]. It was, therefore, hypothesized that the inhibition of DNA synthesis by SPARC in BAEC is dependent on the presence of a serum factor that is not released by platelets [Hasselaar and Sage, 1992]. Under serum-free

conditions, SPARC had no effect on the mitogenic function of FGF-2, but effectively antagonized its migratory and chemotactic properties in BAEC [Hasselaar and Sage, 1992]. Since FGF-2 is a potent inducer of capillary endothelial cell proliferation [Ornitz et al., 1996], we asked whether the anti-proliferative properties of SPARC in HMVEC also requires a serum factor. Our results showed that FGF-2 alone (20 ng/ml) stimulated DNA synthesis of these cells up to fivefold, and that SPARC (0.6 μM) and peptide 4.2 (0.2 mM), but not the control SPARC peptide 3.4 (0.2 mM), exerted their inhibition (>80%) in a serum-independent fashion (Fig. 2A). A 2 h preincubation of HMVEC with SPARC or peptide 4.2 (but not the control SPARC peptide 3.4) was shown to be required for maximal inhibition of ligand-induced autophosphorylation of FGFR1 (>85%) and MAPK phosphorylation (>75%), relative to controls (Fig. 2B,C). These results indicate that SPARC imparts its inhibitory effect on DNA synthesis in HMVEC stimulated by FGF-2 through a mechanism that is independent of serum and appears to be mediated through a significant suppression of FGFR1 autophosphorylation and MAPK activation.

SPARC Pretreatment Does Not Alter MAPK Activity

Whereas simultaneous addition of FGF-2 and SPARC resulted in significant suppression of DNA synthesis and proliferation of HMVEC, relative to controls, inhibition of ligand-induced FGFR1 phosphorylation, and MAPK activation was enhanced significantly by a 2 h incubation of cells with SPARC (Fig. 2B,C). Release of matrix-bound or cell-associated FGF-2 by SPARC pretreatment, and the concomitant transient activation of FGFR1 and MAPK, could be a potential mechanism for FGFR1 desensitization. To address this possibility, we examined the state of phosphorylation of MAPK during a 2 h timecourse of cellular incubation with SPARC. Our results indicated that such exposure to SPARC did not significantly affect the phosphorylation of MAPK in HMVEC (Fig. 3) or in αR/FR cells (data not shown). The apparent decreases in MAPK activity at 60 and 120 min were not considered significant, since similar fluctuations in the phosphorylation state of MAPK were observed for the corresponding timepoints of PBS-treated controls in parallel experiments (data not shown).

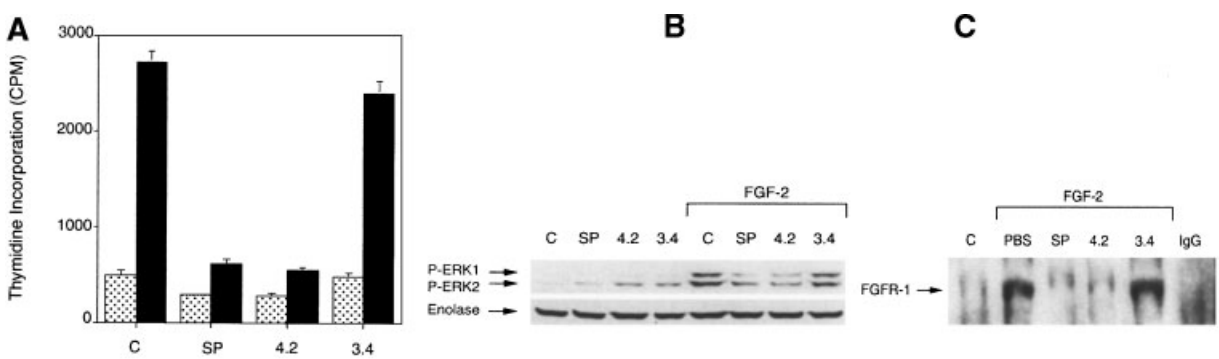

Fig. 2. SPARC inhibits DNA synthesis, mitogen-activated protein kinase (MAPK) activation, and FGFR1 phosphorylation in human microvascular endothelial cells (HMVEC) stimulated by FGF-2. **A**: HMVEC were grown to 80% confluence in fully supplemented medium. Cells were subsequently deprived of serum and growth supplements for 36–48 h, and were incubated for an additional 15–17 h with PBS (C), SPARC (0.6 μM), peptide 4.2 (0.2 mM), or control peptide 3.4 (0.2 mM) in the absence (stippled bars) or presence (closed bars) of FGF-2 (20 ng/ml). Cells were pulse-labeled with ^{3}H-thymidine for 4 h, and were subsequently fixed for scintillation counting. Data shown are from one experiment that was representative of four separate experiments. Variability among experiments was less than 10% for each condition shown. **B**: HMVEC were growth-arrested as described in (A), and were pretreated for 2 h with PBS (C), SPARC (SP), peptide 4.2 (4.2), or control peptide (3.4). Pretreated cells were incubated with or without FGF-2 (40 ng/ml) for 10 min, and cell extracts were immunoblotted with an antibody specific for the phosphorylated form of MAPK, and with an antibody against α-enolase as an internal control. **C**: Growth-arrested HMVEC were treated with or without FGF-2 (C), in the presence or absence of SPARC, peptide 4.2 or peptide 3.4 as in (B). Equal amounts of cell extracts were immunoprecipitated with either an anti-mouse IgG or a phospho-tyrosine-specific antibody, and were subsequently immunoblotted with an antibody specific for FGFR1. Equal protein loading was verified in parallel experiments in which cell extracts were immunoprecipitated and immunoblotted with an antibody specific for FGFR1 (data not shown). The results shown in (B) and (C) are from one experiment that was representative of three to four separate experiments. Variability among experiments was less than 10% for each protein.

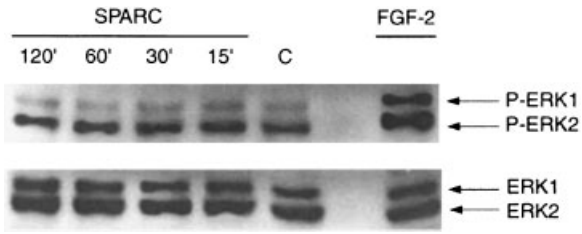

Fig. 3. Incubation with SPARC does not alter the basal level of MAPK activity in HMVEC. HMVEC, growth-arrested as in Figure 2, were incubated with SPARC up to 120 min in the absence of serum and growth supplements. Cell extracts were prepared at indicated timepoints, resolved by electrophoresis on duplicate gels, and immunoblotted with either an anti-phospho-specific MAPK IgG (P-ERK1/2, **top panel**) or an anti-pan-ERK IgG (ERK1/2, **bottom panel**). Extracts from cells incubated with PBS up to 2 h (data not shown), and those stimulated by FGF-2 (40 ng/ml for 10 min) were used as controls. The results shown are from one experiment that was representative of three independent experiments. Variability among experiments was less than 8% for each protein.

Suppression of FGFR1-Mediated DNA Synthesis and Proliferation by SPARC Is HSPG-Independent

To determine whether low-affinity FGF-2 receptors are necessary for the anti-proliferative function of SPARC, we used two independently isolated, non-adherent, interleukin (IL)-3-dependent murine myeloid cell lines, devoid of both heparan sulfate, and FGF receptors, that stably overexpress FGFR1 (BaF3/FR1c11 and BaF3/32D/*flg*). Overexpression of FGFR1 in the BaF3/FR1c11 cell line has been shown to obviate the IL-3 requirement when the cells are grown in the presence of FGF-2 and heparin [Ornitz et al., 1996]. Simultaneous addition of FGF-2 (10 nM), heparin (10 nM), and murine SPARC (1.2 μM) to BaF3/FR1c11 cells resulted in a significant decrease in the number of proliferating cells (greater than threefold), relative to controls stimulated with FGF-2 and heparin alone (Fig. 4). The observed anti-proliferative effect of SPARC was specific for FGF-2, since SPARC had no inhibitory effect on the proliferation of BaF3 cells stimulated with IL-3 (Fig. 4). Similar levels of inhibition of FGF-2 but not IL-3-stimulated proliferation by SPARC were found in BaF3/32D/*flg* cells (data not shown). Together, these results support the claim that the anti-proliferative effect of SPARC was not due to toxicity, was specific to cells stimulated with FGF-2, and did not require cell surface low-affinity FGF-2 receptors.

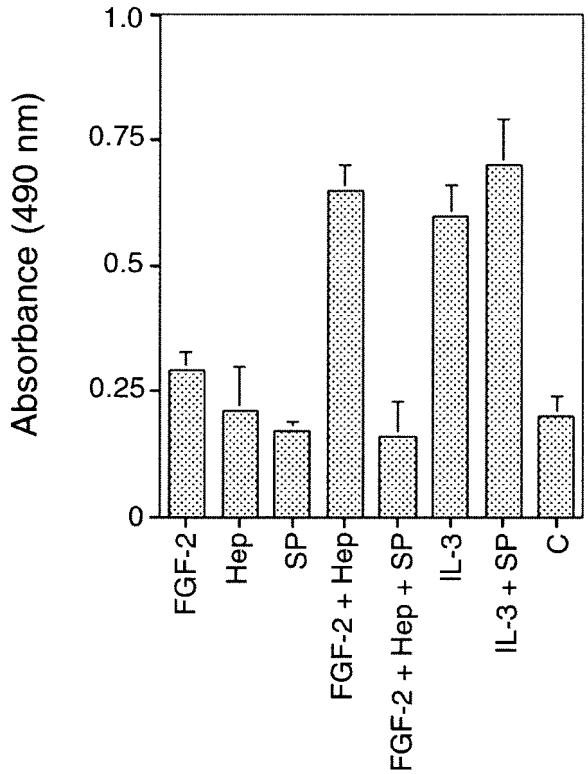

Fig. 4. Suppression of FGFR1-mediated proliferation by SPARC is HSPG-independent. HSPG-deficient BaF3/FR1c11 myeloid cells were washed in media lacking WEHI3-conditioned medium and were plated in 96-well plates at 5×10^3 cells/well. FGF-2 (10 nM) and heparin (Hep, 10 nM) were added in the presence or absence of SPARC (SP, 1.2 μM) for 72 h. Colorimetric quantification of cell numbers was achieved by the addition of Cell titer 96 Aqueous One Solution™ reagent and measurement of absorbance readings at the rate of 490 nm. PBS-treated (C) and IL-3-stimulated proliferation served as controls. The results shown are from one experiment that was representative of three separate experiments.

SPARC Promotes Differentiation of Skeletal Myoblasts

To confirm the inhibitory effect of SPARC on FGFR1-mediated signaling, we used MM14 murine myoblasts, a permanent cell line that expresses FGFR1 almost exclusively [Templeton and Hauschka, 1992]. In the presence of FGF-2, myoblasts proliferate, whereas withdrawal of FGF-2 leads to suppression of proliferation, concomitant activation of muscle differentiation-specific genes, and terminal differentiation from myoblasts to myocytes [Clegg et al., 1987]. In a 72 h clonal assay of myoblast growth and differentiation, the presence of SPARC (0.15 or 0.3 μM) or SPARC peptide 4.2 (50 or 150 μM) in the medium containing 0.5 ng/ml FGF-2,

TABLE I. SPARC Promotes Terminal Differentiation of MM14 Skeletal Muscle Myoblasts

	Clone size (cells/clone)	Percentage of terminally differentiated (cells/clone)	n[a]
Control (0.5 ng/ml FGF-2)	26.8 ± 2.0[b]	5.0 ± 2.3	61
(+) SPARC (0.15 μM)	15.1 ± 1.3*	47.4 ± 6.3*	57
(+) SPARC (0.3 μM)	7.2 ± 0.5*	71.5 ± 3.6*	61
(+) Peptide 4.2 (50 μM)	14.1 ± 1.0*	55.5 ± 3.6*	81
(+) Peptide 4.2 (150 μM)	9.2 ± 0.9*	66.4 ± 4.3*	48

Effects of SPARC (0.15 and 0.3 μM) or SPARC peptide 4.2 (50 and 150 μM) on terminal differentiation of MM14 were tested in a clonal assay of myoblast differentiation in the presence of fibroblast growth factor (FGF)-2 (0.5 ng/ml). Terminal differentiation of myoblasts was quantified by staining of myosin heavy chain.
[a]Number of clones scored.
[b]Three independent experiments were quantified. Numbers represent mean value ± SEM from a representative experiment.
*$P < 0.0001$, relative to control.

resulted in a significant decrease in clone size (up to threefold), and a substantial increase in the percent of differentiated cells per clone (up to 14-fold), as determined by immunostaining for the presence of myosin heavy chain (Table I). These findings are in agreement with the observed inhibition of FGFR1 signaling by SPARC in HMVEC.

SPARC Suppresses FGFR1-Mediated Proliferation and MAPK Activation but not Akt Phosphorylation in the Absence of Ligand Binding

FGFR1 is believed to be the major signaling receptor for FGF-2-mediated proliferation of endothelial cells. It contains at least seven tyrosine (Y) autophosphorylation sites but to date only Y766 and Y463 have been shown to associate directly with the downstream signaling molecules phospholipase C (PLC)-γ and the SH2/SH3-containing adaptor protein Crk, respectively [Mohammadi et al., 1996; Larsson et al., 1999]. Whereas FGFR1-mediated mitogenesis in different cell types, including endothelial cells, has been shown to be independent of PLC-γ activity [Mohammadi et al., 1992; Cross et al., 2000], signaling through Crk has been implicated in mitogenesis of endothelial cells via activation of extracellular signal-regulated kinase (ERK) and Jun kinase [Larsson et al., 1999]. PAEC lack endogenous PDGFRs and express low levels of endogenous FGF receptors [Westermark et al., 1990]. To delineate the mechanism through which SPARC antagonizes FGFR1 signaling in the absence of direct binding, we used PAEC lines that express high levels of stably integrated full-length FGFR1 (PAEC/FGFR1), or a chimeric FGFR1 bearing the extracellular and transmembrane domains of PDGFR-α fused to the intracellular domain of wild-type FGFR1 [denoted αR/FR; Landgren et al., 1998]. PDGF-BB has been shown to mimic faithfully the activation of FGFR1 downstream signaling in this chimeric construct [Kanda et al., 1996], and we obtained similar results with PDGF-AA, previously reported not to bind to native SPARC [Raines et al., 1992]. Our findings indicated that SPARC and SPARC peptide 4.2 inhibit FGF-2 and PDGF-AA-stimulated DNA synthesis in PAEC/FGFR1 and αR/FR by as much as 74 and 55%, respectively (Fig. 5A). Consistent with these findings, PDGF-AA-stimulated activation of MAPK in αR/FR was also diminished significantly (>75%) in the presence of SPARC (Fig. 5B) and peptide 4.2 (data not shown). As with HMVEC, maximal diminution of FGFR1-mediated MAPK activity, but not DNA synthesis, in αR/FR required a 2 h pretreatment with SPARC or peptide 4.2 (Fig. 5A,B).

Lastly, to test whether SPARC inhibits the activation of signaling pathways downstream from FGFR1 phosphorylation other than MAPK, we used PAEC/FGFR1 cells in which FGF-2 has been shown to stimulate PI3-K activation to significant levels [Cross et al., 2000]. Addition of SPARC to PAEC/FGFR1 did not result in significant changes in the levels of FGF-2-stimulated phosphorylation of Akt, a downstream target of PI3-K activity (Fig. 5C), but significantly inhibited DNA synthesis (Fig. 5A) and MAPK activation (data not shown). Collectively, these findings are in agreement with published reports that SPARC antagonizes the activity of PDGF-AA and FGF-2 in the absence of a direct molecular interaction

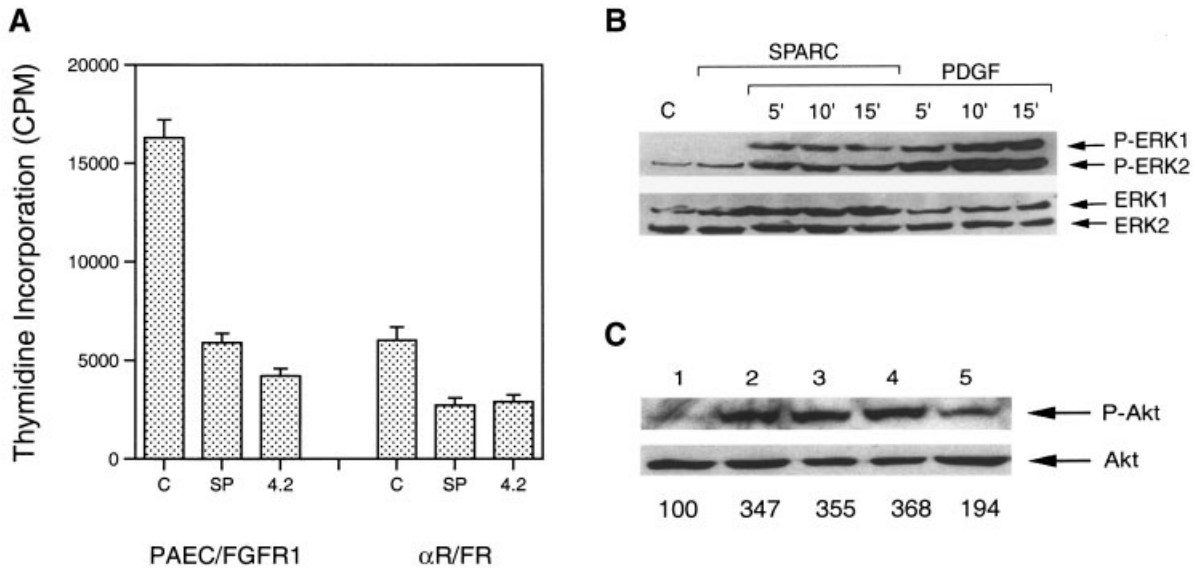

Fig. 5. FGFR1-mediated DNA synthesis and MAPK activation but not Akt phosphorylation in porcine aortic endothelial cells (PAECs) are inhibited by SPARC. **A**: PAEC expressing full-length FGFR1 (PAEC/FGFR1) and a chimeric line expressing both the extracellular and the juxtamembrane domains of PDGF-AA fused to the intracellular domain of FGFR1 (αR/FR), were growth-arrested for 48 h prior to stimulation with FGF-2 (20 ng/ml) and PDGF-AA (50 ng/ml), respectively, in the presence of PBS (C), SPARC (SP, 0.6 μM), or SPARC peptide 4.2 (4.2, 0.2 mM) for 17–20 h prior to a 4 h pulse with [^{3}H]-thymidine. Cells were subsequently fixed for scintillation counting. Data shown are from one experiment that was representative of three separate experiments. Variability among experiments was less than 7% for each condition shown. **B**: Growth-arrested αR/FR cells were preincubated with or without SPARC (0.6 μM) for 2 h prior to stimulation with PDGF-AA (50–100 ng/ml) for 5–15 min. Cells stimulated with PBS (C) or SPARC (0.6 μM) alone for 15 min served as controls. Cell extracts were prepared at the indicated timepoints and immunoblotted with an anti-phospho-specific MAPK IgG (P-ERK1/2, **top panel**). For assessment of differences in protein loading, the membrane was stripped, blocked, and incubated with an anti-pan-ERK IgG (ERK1/2, **bottom panel**). Changes in activation of MAPK were determined by normalization of activated MAPK (P-ERK) to total MAPK levels. **C**: Growth-arrested PAEC/FGFR1 cells were preincubated for 2 h with PBS (**lane 1**), hrSPARC (1.2 μM) (lane 3), and SPARC peptide 4.2 (0.2 mM) (lane 4), or 30 min with the PI3-K pathway inhibitor LY294002 (10 μM) (lane 5), prior to stimulation with FGF-2 (40 ng/ml) for 10 min (**lanes 2–5**). Prepared cell extracts were resolved by electrophoresis on duplicate gels, and immunoblotted with either an anti-phospho-specific Akt IgG or an anti-pan-Akt IgG for the assessment of equal loading. Numbers under each lane denote percent change in levels of phospho-ERK, relative to PBS-treated controls (set at 100%). The results shown are from one experiment that was representative of at least three independent experiments. Variability among experiments was less than 10% for each protein.

with these growth factors. Moreover, these results indicate that the inhibition of FGF-2 activities by SPARC is achieved, at least in part, through modulation of signaling events downstream of FGFR1 activation.

Suppression of PKA Activity in Endothelial Cells Partially Reverses the Inhibitory Effect of SPARC on FGF-2-Mediated DNA Synthesis and MAPK Phosphorylation

Activation of PKA has been shown to suppress the mitogenic action of VEGF and FGF-2 in capillary endothelial cells through inhibition of the serine/threonine kinase activity of Raf-1 and a concomitant blockade of the MAPK signaling cascade [D'Angelo et al., 1997]. We asked whether treatment of HMVEC with specific inhibitors of PKA (H-89 and KT-5720) could reverse the suppressive effect of SPARC on DNA synthesis and MAPK activation stimulated by FGF-2. Simultaneous addition of SPARC (1.2 μM) and H-89 (10 nM) or KT-5720 (50 nM), but not the protein kinase C inhibitor calphostin C (50 nM, data not shown), to quiescent HMVEC reversed the inhibitory effect of SPARC on DNA synthesis stimulated by FGF-2 up to 51 and 38%, respectively (Fig. 6A, lanes h and i). In agreement with these findings, the inhibitory effect of SPARC on DNA synthesis was exacerbated (>20%) in the presence of 20 μM forskolin, a stimulator of adenylyl cyclase (Fig. 6A). Optimal concentrations of the PKA inhibitors used were determined empirically to have minimal inhibitory effects (<10%) on DNA synthesis stimulated by FGF-2 and to be able to block (by >90%) the anti-proliferative function of forskolin on HMVEC (data not shown).

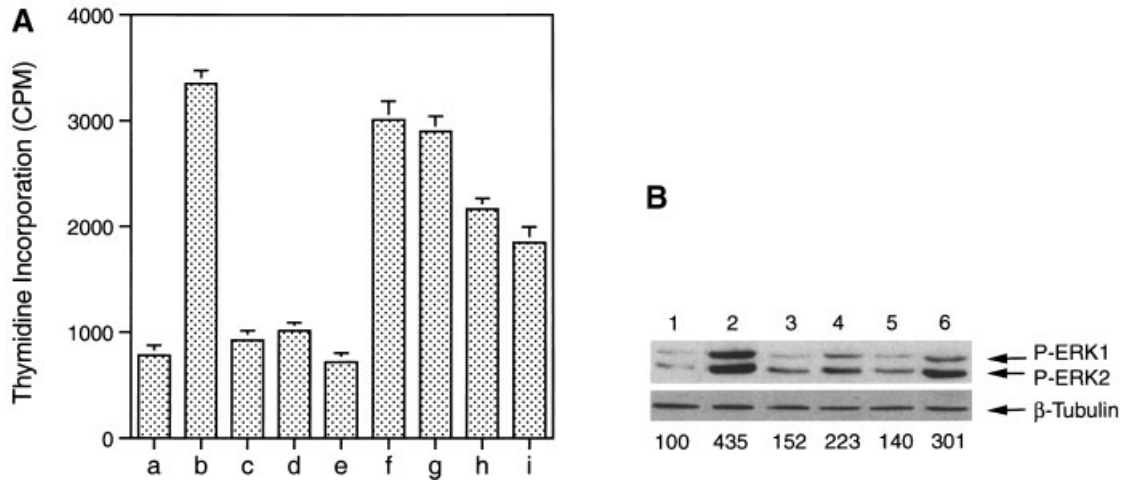

Fig. 6. Suppression of protein kinase A (PKA) activity partially reverses the inhibitory effect of SPARC on DNA synthesis and MAPK activation in HMVEC stimulated by FGF-2. **A**: HMVEC were grown to 80% confluence in fully supplemented medium. Cells were deprived of serum and growth supplements for 24 h, and were subsequently incubated with PBS (**lane a**), FGF-2 alone (20 ng/ml) (**lane b**), FGF-2 with either rhSPARC (1.2 μM) (**lane c**), forskolin (20 μM) (**lane d**), rhSPARC and forskolin (**lane e**), H-89 (10 nM), and forskloin (**lane f**), KT-5720 (50 nM), and forskolin (**lane g**), H-89 and rhSPARC (**lane h**), or KT-5720 and rhSPARC (**lane i**) for 15–17 h. Cells were pulse-labeled with [^{3}H]-thymidine for 4 h and were subsequently fixed for scintillation counting. Data shown are from one experiment, which was representative of three different experiments. Variability among experiments was less than 10% for each condition shown. **B**: Growth-arrested HMVEC as in (A) were stimulated with FGF-2 (40 ng/ml) alone for 10 min (**lane 2**) or preincubated for 2 h with rhSPARC (1.2 μM) (**lane 3**), rhSPARC and H-89 (10 nM) (**lane 6**), or forskolin (20 μM) for 30 min (**lane 5**) prior to stimulation with FGF-2 for 10 min. Cells treated with PBS (**lane 1**) or H-89 (**lane 4**) for 30 min served as controls. Prepared cell extracts were immunoblotted sequentially with an anti-phospho-specific MAPK IgG and an antibody against β-tubulin for the assessment of equal loading. Numbers under each lane denote percent change in levels of phospho-Akt, relative to PBS-treated controls (set at 100%). The results shown are from one experiment that was representative of three independent experiments. Variability among experiments was less than 8% for each protein.

Preincubation of HMVEC with SPARC (1.2 μM) and H-89 (5 μM) for 1–2 h prior to a 10 min stimulation with FGF-2 (40 ng/ml) reversed the inhibitory effect of SPARC on MAPK phosphorylation by as much as twofold (Fig. 6B, lane 6). Consistent with these results, preincubation of HMVEC with forskolin (50 μM) for 15 min prior to a 10 min stimulation with FGF-2, resulted in a significant suppression (greater than threefold) of MAPK phosphorylation (Fig. 6B, lane 5). In agreement with previously reported results [D'Angelo et al., 1997], addition of H-89 alone resulted in a significant activation of MAPK (greater than twofold) in the absence of FGF-2 (Fig. 6B, lane 4). Together, these results support the conclusion that SPARC suppresses DNA synthesis and MAPK activation stimulated by FGF-2 in HMVEC, at least in part, through activation of PKA.

DISCUSSION

The rate of neovascular growth is determined by a balance between endogenous positive and negative regulators of angiogenesis. FGF-2 and VEGF are the prominent stimulators of angiogenesis, whereas platelet factor-4 (PF-4), 16 kDa human prolactin, angiostatin, endostatin, tissue inhibitor of metalloproteinases, and thrombospondin (TSP)1 and 2, represent the better-studied endogenous negative regulators of angiogenesis [Pepper, 1996; Sage, 1997; Kontos and Annex, 1999; Ferrara, 2000; Folkman, 2002; Lawler, 2002]. To date, the mechanisms through which heparin-binding PF-4 and TSP1 inhibit FGF-2-stimulated angiogenesis have been delineated. PF-4 has been reported to antagonize proliferation of microvascular endothelial cells stimulated by FGF-2 through inhibition of FGF-2 dimerization, suppression of FGF-2 binding to high- and low-affinity receptors, and inhibition of FGF-2 internalization [Perollet et al., 1998]. TSP1 and its 140-kDa anti-angiogenic fragment have been shown to bind to FGF-2, moderately diminish ligand binding to high-affinity receptors without inhibition of their autophosphorylation, and strongly inhibit both ligand binding to low-affinity receptors and FGF-2 internalization [Taraboletti et al., 1997].

SPARC, like TSP1 and 2, is a member of the matricellular family of proteins known to affect various aspects of endothelial cell behavior that are relevant to angiogenesis [for review see Brekken and Sage, 2000; Murphy-Ullrich, 2001; Bornstein and Sage, 2002]. SPARC has been shown to antagonize the activity of two major angiogenic growth factors (VEGF and FGF-2) through apparently different mechanisms. SPARC inhibited VEGF-stimulated mitogenesis of microvascular EC through direct binding to the growth factor, suppression of its high-affinity receptor phosphorylation, and inhibition of ERK activation [Kupprion et al., 1998]. Conversely, inhibition of FGF-2-stimulated proliferation and migration of BAEC by SPARC was shown to be independent of a direct interaction between FGF-2 and SPARC or an interference with ligand binding to high-affinity FGF receptors. Moreover, inhibition of FGF-2-stimulated EC proliferation, but not migration, by SPARC was shown to require the presence of serum [Hasselaar and Sage, 1992].

In this study, we investigated the mechanism through which SPARC inhibits the proliferative effect of FGF-2 in endothelial cells. The results of our in vitro co-precipitation studies WERE in agreement with the contention that SPARC antagonizes FGF-2 activity in the absence of a direct interaction with the ligand or its high-affinity receptor. Contrary to the reported serum requirement for inhibition of FGF-2-stimulated mitogenesis by SPARC in BAEC, significant suppression of DNA synthesis in HMVEC was observed under serum-free conditions. This apparent discrepancy in serum requirement for SPARC activity could be attributed to vessel origin and/or species differences of endothelial cells used in the two studies. Moreover, simultaneous addition of FGF-2 and SPARC, a peptide from the carboxy-terminal Ca^{2+}-binding region of the protein (peptide 4.2), inhibited DNA synthesis in HMVEC, whereas a 2 h pretreatment of HMVEC with SPARC appeared to be required for significant suppression of ligand-induced FGFR1 phosphorylation and MAPK activation. Since it is plausible that SPARC pretreatment could potentially release matrix-bound FGF-2, initiate a burst of MAPK activation, and result in FGFR1 desensitization, we analyzed the state of MAPK activity for the duration of the exposure of HMVEC to SPARC. The observed minimal changes in MAPK activity during this timecourse, however, did not support this possibility.

To prove that inhibition of mitogenic activity of FGF-2 by SPARC does not involve other FGF receptors, we utilized several endothelial and non-endothelial cell systems, for example, MM14 myoblasts, in which FGFR1 is expressed almost exclusively. In the presence of FGF-2 and serum, myoblasts proliferate and their terminal differentiation is suppressed. Upon removal of FGF-2, myoblasts exit the cell cycle and initiate their differentiation process [Olwin and Hauschka, 1988]. In the presence of FGF-2, SPARC, or peptide 4.2 significantly inhibited proliferation of MM14 myoblasts and promoted their differentiation to myocytes. We then verified the anti-proliferative effect of SPARC on PAEC lines stably expressing either full-length or chimeric FGFR1 as their predominant FGF receptor. These findings, in agreement with the results of our binding studies, strongly support the claim that SPARC antagonizes the DNA synthesis and MAPK activation mediated by FGFR1 in the absence of a direct interaction with either FGF-2 or the extracellular growth factor-binding domain of the receptor. Inhibition of mitogenic activity of an angiogenic growth factor by an endogenous inhibitor of angiogenesis in the absence of direct binding has previously been reported by Gengrinovitch et al. [1995]: PF-4 efficiently negated the mitogenic activity of the non-heparin-binding $VEGF_{121}$ isoform in human umbilical vein endothelial cells in the absence of a direct interaction with the ligand or its high-affinity receptors. Finally, to test whether SPARC inhibits the activation of signaling pathways other than MAPK downstream from FGFR1 phosphorylation, we used PAEC/FGFR1 cells in which FGF-2 has been shown to stimulate PI 3-K activation to significant levels [Cross et al., 2000]. Phosphorylation of Y766 in FGFR1 has been shown to be dispensable for the FGF-2-mediated proliferation of this cell line, but necessary for activation of PI 3-K and cytoskeletal reorganization [Cross et al., 2000]. Pretreatment of PAEC/FGFR1 with SPARC or peptide 4.2 did not result in significant changes in the levels of FGF-2-stimulated phosphorylation of Akt, a downstream target of PI3-K activity, but significantly inhibited their DNA synthesis and MAPK activation. Such independent inhibition of FGFR1-mediated ERK phosphorylation but not Akt phosphorylation in

endothelial cells has recently been reported for PF-4 [Sulpice et al., 2002]. Collectively, these results are consistent with the hypothesis that SPARC, like PF-4, disrupts FGF-2 signaling via an intracellular mechanism of inhibition that occurs downstream of FGFR1 phosphorylation. In support of this hypothesis, SPARC has been found associated with the nuclear matrix [Gooden et al., 1999], suggestive that it might also modulate intracellular and nuclear processes.

Recent studies suggest that PKA participates in the regulation of growth factor signaling both in vivo and in vitro [D'Angelo et al., 1999; Pursiheimo et al., 2002a,b]. Inhibition of FGF-2-stimulated angiogenesis in chicken chorioallantoic membranes by $\alpha5\beta1$ antagonists is claimed to be mediated by activation of PKA [Kim et al., 2000]. Moreover, it was recently reported that parathyroid hormone inhibits FGF-2-stimulated angiogenesis in vivo by activation of PKA [Bakre et al., 2002]. Elevation of PKA activity has also been shown to inhibit the mitogenic action of FGF-2 in microvascular endothelial cells through a blockade of the activation of Raf-1 [D'Angelo et al., 1997]. Phosphorylation of Raf-1 on serine 43 by activated PKA is believed to inhibit its kinase activity and block the MAPK signaling cascade [Wu et al., 1993; Hafner et al., 1994]. In this report, we have provided evidence that the inhibition of FGFR1-mediated MAPK activation and DNA synthesis, but not Akt phosphorylation, by SPARC and SPARC peptide 4.2 in endothelial cells is mediated in part by the activation of PKA. This partial reversal of the anti-proliferative effect of SPARC by specific inhibitors of PKA indicates the existence of other as yet unidentified pathways.

One possibility is that SPARC could potentially interfere with FGF-2 signaling by its competing for the binding of FGF-2 to its HSPG co-receptors. It is believed that HSPGs modulate the binding of FGF-2 to FGFRs through formation of high-affinity FGF-2/HSPG/FGFR ternary complexes and/or by the induction of FGF-2 oligomerization, which would facilitate FGFR dimerization and signal transduction [reviewed in 1997; Klint and Claesson-Welsh, 1999; Szebenyi and Fallon, 1999; Nugent and Iozzo, 2000]. Our results with HSPG-deficient myeloid cells that express FGFR1 demonstrated that, in the presence of heparin, SPARC significantly inhibited FGF-2-mediated mitogenesis of these cells but had no effect on their proliferation after they were stimulated with IL-3. At this point, we can not definitively exclude interference with HSPG function as a possible mechanism of inhibition of FGF-2-stimulated proliferation by SPARC in HMVEC. However, our studies involving inhibition of HSPG sulfation by chlorate treatment and enzymatic degradation of cell-surface HSPGs by heparinases and heparatinases indicate that SPARC mediates its anti-proliferative effect on FGF-2-stimulated proliferation of HMVEC through HSPG-independent mechanisms (Motamed, data not shown). Unlike TSP1 and PF-4, SPARC does not have a known heparin-binding domain and does not bind to heparin affinity columns [Sage et al., 1984]. However, the presence of cryptic heparin-binding sites within SPARC and/or its preferential binding to selected heparan sulfate moieties can not be excluded. Circumstantial evidence for such an interaction, in which the heparin-binding region of vitronectin was shown to bind directly to the extracellular high-affinity Ca^{2+}-binding domain of SPARC, was reported by Rosenblatt et al. [1997].

Diminution of the activation of MAPK in HMVEC by the angiogenesis inhibitor angiostatin has been reported to involve the action of a tyrosine phosphatase [Redlitz et al., 1999]. Suppression of FGF-2-stimulated MAPK activation by SPARC could potentially be mediated through the action of MAPK phosphatases (MKP). Full enzymatic activity of MAPK requires dual phosphorylation on a Thr183-X-Tyr185 motif by MAPK kinase, whereas dephosphorylation at either site results in its inactivation [reviewed in Seger and Krebs, 1995; Chang and Karin, 2001; Chong et al., 2003]. Suppression of MAPK phosphorylation by PKA has been shown to involve the induction of dual-specificity tyrosine/threonine MKPs 1 and 2 [Brondello et al., 1997]. Since inactivation of PKA could only partially reverse the anti-proliferative effect of SPARC, it could be hypothesized that complete inhibition of MAPK activation by SPARC involves activation of a tyrosine and/or a serine/threonine phosphatase. We presently cannot exclude this possibility because preincubation of HMVEC with either the tyrosine phosphatase inhibitor sodium orthovanadate, or the serine/threonine phosphatase inhibitor okadaic acid, with or without SPARC, resulted in significant levels of sus-

tained basal MAPK activity that interfered with the measurement of FGF-2-stimulated MAPK activation (Motamed, unpublished results). A better understanding of the role of phosphatases in the context of SPARC function awaits the use of different phosphatase-deficient cells coupled with phosphatase assays in vitro.

In addition to regulation of the activity of growth factors by confining their availability, matricellular proteins appear to have devised alternative modes of action to provide a tight modulation of the growth of vascular cells. In this study, we have shown that SPARC inhibits the proliferative effect of FGF-2 without diminishing the interaction between the ligand and its high-affinity FGFR1. Moreover, the inhibition of FGF-2 function was shown to involve a mechanism of intracellular inhibition and to be mediated in part by activation of PKA and the concomitant suppression of the Ras/MAPK signaling cascade.

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