



# Renaturation of SPARC Expressed in *Escherichia coli* Requires Isomerization of Disulfide Bonds for Recovery of Biological Activity

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SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin and BM-40) belongs to a group of secreted macromolecules that modulate cellular interactions with the extracellular matrix. During vertebrate embryogenesis, as well as in tissues undergoing remodeling and repair, the expression pattern of SPARC is consistent with a fundamental role for this protein in tissue morphogenesis and cellular differentiation. Human SPARC was cloned by the polymerase chain reaction from an endothelial cell cDNA library and was expressed in *Escherichia coli* as a biologically active protein. Two forms of recombinant SPARC (rSPARC) were recovered from BL21(DE3) cells after transformation with the plasmid pSPARCwt: a soluble, monomeric form that is biologically active (Bassuk *et al.*, 1996, *Archiv. Biochem. Biophys.* 325, 8–19), and an insoluble form sequestered in inclusion bodies. Aggregated rSPARC was unfolded by urea treatment, purified by nickel–chelate affinity chromatography, and renatured by gradual removal of the denaturant. Proper isomerization of the disulfide bonds was achieved in the presence of a glutathione redox couple. After final purification by high resolution gel filtration chromatography, a monomeric form of rSPARC displaying biological activity was obtained. The recombinant protein inhibited the spreading and synthesis of DNA by endothelial cells, two properties characteristic of the native protein. We conclude that the information for the correct folding of rSPARC resides in the primary structure of the protein, and suggest that post-translational modifications are required neither for folding nor for biological activity. Copyright © 1996 Published by Elsevier Science Ltd

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**Abbreviations:** AEBSF, aminoethylbenzenesulfonylfluoride; BAE, bovine aortic endothelial; BCA, 2,2'-bichinonic acid copper protein reagent; BSA, bovine serum albumin; CD, circular dichroism spectroscopy; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; IPTG, isopropyl- $\beta$ -D-galactopyranoside; Ni-NTA, nickel-nitrilotriacetate; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GSH, reduced glutathione; GSSG, oxidized glutathione.

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## INTRODUCTION

A recurring theme underlying cellular responses to injury and developmental signals is the necessity for a change in the morphology of adherent cells. The biological activities that modulate such changes in cell shape can be found in a group of extracellular proteins that include SPARC, tenascin, and thrombospondin (Sage and Bornstein, 1991). These proteins have been implicated in differentiation, stimulation or inhibition of migration and cell cycle, and the regulation of expression of certain genes. Such properties are thought to be linked to disruption of cell–matrix interactions, or a counter-

adhesive activity that leads to cell rounding and partial detachment from a substratum. The growth of blood vessels is a process determined by a dynamic interrelationship among endothelial cells, mitogens, and the extracellular matrix. We have proposed that SPARC alters the kinetics of this relationship and predisposes endothelial cells toward an activated state of remodeling and neovascularization. Early in the development of new blood vessels, for example, SPARC might be specifically cleaved (e.g. by plasmin) into peptides that elicit an angiogenic response (Lane *et al.*, 1994). At later stages of angiogenesis when endothelial cell proliferation ceases, intact SPARC is proposed to exert its known inhibitory effect on cell cycle progression (Lane and Sage, 1994; Sage and Vernon, 1994).

SPARC does not share appreciable identity in primary structure with tenascin or thrombospondin. Instead, SPARC belongs to a group of sequence-related proteins that can modulate the morphology and migration of endothelial cells (Bassuk *et al.*, 1993; Lane and Sage, 1994). This group is defined by a novel extracellular  $\text{Ca}^{2+}$ -binding (EC) module (Hohenester *et al.*, 1996) found in the following proteins: SPARC, rat brain SC1 (Johnston *et al.*, 1990), quail retina QR1 (Guermah *et al.*, 1991), human tonsil hevin (Girard and Springer, 1995), human testicular testican (Alliel *et al.*, 1993), and a mouse osteoblastic transforming growth factor  $\beta$ 1-regulated gene product (Shibanuma *et al.*, 1993). Within this group of proteins, a follistatin-like module is always followed by the EC module; the presence of these two modules might therefore define activities common to the group, with the specificity of each protein determined by a unique N-terminal domain.

A growth factor-free supply of biologically active SPARC is clearly necessary for structural and functional studies of this interesting protein. We have therefore developed a bacterial expression system that generated human endothelial SPARC as a biologically active recombinant protein (Bassuk *et al.*, 1996). Transcriptional control of SPARC RNA was dependent on an isopropylgalactopyranoside<sup>3</sup> (IPTG)-inducible T7 RNA polymerase promoter. Recombinant SPARC (rSPARC) was synthesized rapidly and was isolated as a soluble, monomeric form in a conformation similar to that of the active protein. Soluble rSPARC displayed an  $\alpha$ -helical conformation that was dependent on the addition of exogenous  $\text{Ca}^{2+}$ . Titration of the  $\text{Ca}^{2+}$ -saturated

protein with ethylenediaminetetraacetic acid (EDTA) reduced the  $\alpha$ -helical content (monitored experimentally by circular dichroism spectroscopy) and altered the environment around the tryptophan residue adjacent to the  $\text{Ca}^{2+}$ -binding EF-hand (monitored by ultraviolet fluorescence emission spectroscopy). These assays indicated that the conformation of soluble rSPARC was affected by removal of  $\text{Ca}^{2+}$  in a manner similar to that of murine SPARC. We concluded that both proteins were folded comparably. Soluble rSPARC also displayed the same biological activities as murine SPARC. When bovine aortic endothelial cells were placed into tissue culture wells containing rSPARC, the cells attached to the plastic surface, but their subsequent spreading was inhibited by rSPARC in a concentration-dependent manner. Moreover, binding experiments demonstrated a specific and concentration-dependent interaction with cultured endothelial cells.

Our yield of soluble rSPARC, however, represented a minimal fraction of the total rSPARC protein synthesized in *E. coli*. We report here that a second form of rSPARC is insoluble and is sequestered within inclusion bodies. Biochemical analysis of this rSPARC indicated that the protein sequence was complete but the disulfide bonding pattern was incorrect. Since the majority of rSPARC synthesized in *E. coli* was found within inclusion bodies, conversion of this insoluble form of the protein into a useful reagent was necessary.

A proven method for the recovery of active material from inclusion bodies includes the dissolution and denaturation of the protein in chaotropic reagents, and a subsequent refolding of the protein by gradient dialysis. We report here that denatured rSPARC can be refolded partially by the removal of chaotropic reagent by column chromatography. Steps in the folding process include the isomerization of disulfide bonds that are catalyzed by a glutathione redox couple. The final product, which is monomeric and biologically active, inhibited the incorporation of <sup>3</sup>H-thymidine into endothelial cell DNA, as previously reported for the native murine protein (Funk and Sage, 1991). We conclude that the reagent reported in this study is a useful alternative to vertebrate SPARC, and that its use will advance structural studies designed to delineate the function of this protein.

## EXPERIMENTAL PROCEDURES

### Materials

Plasmid pSPARCwt, a pET22b (Novagen, Madison, WI, U.S.A.) derivative, encoding human umbilical vein endothelial cell SPARC under control of the T7 RNA polymerase promoter, has been described (Bassuk *et al.*, 1996). *E. coli* strain BL21(DE3) was from Novagen. Ni-NTA metal-chelate affinity resin was purchased from QIAGEN Inc. (Chatsworth, CA, U.S.A.). Disposable gel filtration columns (Econopak 10DG), Chelex-100, and precast SDS-PAGE gels were purchased from BioRad (Foster City, CA, U.S.A.). Ampicillin and carbenicillin were from Sigma (St Louis, MO, U.S.A.).  $^{35}\text{S}$ -dATP,  $^{125}\text{I}$ -Protein A, and  $^{125}\text{I}$ -NaI were obtained from Amersham Inc. (Arlington Heights, IL, U.S.A.). Aminoethylbenzenesulfonylfluoride (AEBSF) was purchased from Calbiochem (LaJolla, CA, U.S.A.).

### Growth of bacterial culture

Recombinant BL21(DE3) *E. coli* harboring the pSPARC-PET22b vector (Bassuk *et al.*, 1996), were grown at 37°C in 1 or 10 l pilot-scale fermentors in Luria broth that contained 50 µg/ml carbenicillin and 0.2% glucose. When the culture reached an  $\text{OD}_{600} = 0.5\text{--}0.7$ , IPTG was added to a final concentration of 1 mM to induce synthesis of rSPARC. At 3 hr post-induction, cells were harvested by sedimentation at 4000 g, resuspended in 0.1 vol  $\text{NaH}_2\text{PO}_4$  (pH 7.0) containing 10% glycerol (vol/vol), and lysed in a French Press at 12,000 psi (2 cycles). Soluble material was separated from insoluble material by centrifugation at 12,000 g for 20 min at 4°C. Soluble extracts and insoluble pellets were frozen at  $-80^\circ\text{C}$  until use.

### Isolation of rSPARC-containing inclusion bodies by sucrose gradient sedimentation

Material from the bacterial lysate that sedimented at 12,000 g was resuspended by homogenization in an 8.5% sucrose, 50 mM Tris-HCl (pH 8.0) buffer that contained 0.2 mM AEBSF and 10 mM Tris-HCl (pH 8.0). The following detergents were added to a final concentration of 0.5%: Triton X-100, Nonidet P-40, and sodium deoxycholate. The mixture (20 ml) was mixed for 1 hr at room temperature and was layered over a sucrose step gradient (67, 53, and 40%) poured into ultracentrifuge tubes. Centrifugation was performed for 16 hr at 4°C

in a Beckman SW41 rotor at 100,000 g. The inclusion bodies were recovered at the 53–67% interface and were removed with a Pasteur pipette. This material was washed three times with water, resedimented in a fresh sucrose gradient, washed again with water, and stored at  $-20^\circ\text{C}$ .

Washed inclusion bodies (0.2 µg) were thawed, dissolved in 8 M urea/0.1 M Tris-HCl (pH 8.0), and applied to a DEAE-Sepharose anion-exchange chromatography column. Bound protein was eluted with a linear gradient of 0–0.4 M NaCl and was analyzed by SDS-PAGE and immunoblotting.

### Purification of rSPARC by denaturing affinity chromatography

Insoluble and soluble proteins corresponding to 1–2 l of fermented culture were pooled and mixed with 8 M urea/0.1 M  $\text{NaH}_2\text{PO}_4$ /0.01 M Tris-HCl (pH 8.0)/0.05 M  $\text{NH}_4\text{OH}$ /1% octylthioglucoside. The pH of the mixture was adjusted to 11.0 with NaOH. After the solution was stirred for 2–4 hr at ambient temperature, the pH was adjusted to 7.5 with HCl, and dithiothreitol (DTT) and dithioerythritol (DTE) were each added to a final concentration of 0.05 M. After an additional 1 hr of stirring, the mixture was clarified by centrifugation at 20,000 g for 45 min at 25°C. Gel electrophoresis indicated the complete extraction of rSPARC from the residual, insoluble pellet. The supernate was dialyzed against buffer A (0.1 M  $\text{NaH}_2\text{PO}_4$ /0.01 M Tris-HCl/0.5 M NaCl/20% glycerol) adjusted to pH 7.8 and supplemented with 4.7 M urea according to the following protocol: 10 vol for 2–4 hr, 70 vol for 14–18 hr, and 10 vol for 1 hr. rSPARC was adsorbed to 8 ml of settled, equilibrated Ni-NTA agarose affinity resin by gentle stirring for 1 hr at ambient temperature. The inclusion of 5 mM imidazole during binding reduced the amount of nonspecific protein-resin interactions. The slurry was next poured into a 1.7 cm diameter chromatography column, and was washed with 4.7 M urea/buffer A/pH 7.8 at 0.5 ml  $\text{min}^{-1}$  until the  $A_{280} < 0.01$ . The combination of a carboxy-terminal His<sub>6</sub> tag and the endogenous 15 His residues of rSPARC permitted stringent washing of the column, due to a  $K_d \approx 10^{-15}\text{M}$  of His<sub>6</sub> with the Ni-NTA resin.

Contaminating proteins were removed with 10–20 column volumes of buffer A (pH 6.0) or until  $A_{280} < 0.01$ . rSPARC was eluted from the column with buffer A (pH 4.5). Eluates from the

wash at pH 4.5 were analyzed routinely by SDS-PAGE and found to be more than 95% free of contaminating proteins. Samples were stored at  $-20^{\circ}\text{C}$ .

Eluates at pH 4.5 were readjusted to pH 7.8 and were reappplied to the Ni-NTA column. The concentration of urea was reduced by a linear gradient from 4.5 M urea/buffer A (50 ml) to 1.0 M urea/buffer A (50 ml) at  $0.5\text{ ml min}^{-1}$  at ambient temperature. An additional 2–4 column volumes of 1 M urea/buffer A were passed through the column, prior to elution with 1 M urea/buffer A (pH 4.5). rSPARC was found to be stable in this solution and was kept frozen at  $-20^{\circ}\text{C}$ .

#### *Isomerization of disulfide bonds by a glutathione redox potential*

Ten aliquots of  $40\text{ }\mu\text{g}$  of rSPARC ( $80\text{ }\mu\text{g/ml}$ ) were transferred to dialysis tubing (pore size = 12–14 kDa) and were dialyzed against 1 M urea/0.05 M Tris-HCl (pH 8.0) and subsequently against 1 M urea/0.05 M Tris-HCl (pH 8.0)/2 mM  $\text{CaCl}_2$ . Samples were next dialyzed against 1 M urea/0.05 M Tris-HCl (pH 8.0)/2 mM  $\text{CaCl}_2$ /2 mM reduced glutathione (GSH)/0.02 mM oxidized glutathione (GSSG) for the following durations: 0, 8, 16, 24, 32, 40, 48, 56, 64, and 72 hours. The reaction was quenched by the addition of SDS to 1% (vol/vol). Samples were dialyzed against 0.02 M Tris-HCl (pH 6.8), lyophilized, and resuspended in water; the concentration of protein was assayed with 2,2'-bichinonic acid copper protein reagent (Pierce, Rockford, IL, U.S.A.). Following SDS-PAGE of  $15.0\text{ }\mu\text{g}$  protein, the polyacrylamide gel was stained with Coomassie blue and photographed. The film negative was scanned with a Beckman DU70 spectrophotometer at the densitometric settings recommended by the manufacturer. The total area and the percentage area of rSPARC monomer were determined from scans at 560 nm. These data were graphed as the amount of rSPARC monomer formed as a function of the time of exposure to a 100:1 mixture of GSH:GSSG.

Alternatively, rSPARC was dialyzed against 0.1 M Tris-HCl (pH 8.0)/1 mM EDTA and was incubated with 5 mM GSH/1 mM GSSG (5:1) for 10 min. The reaction was quenched by the addition of 5 ml of 20% SDS heated to  $95^{\circ}\text{C}$ . The sample was lyophilized and was resuspended in 60 ml of 0.125 M Tris-HCl (pH 6.8)/10% glycerol/4% SDS/0.05% bromophenol blue. For preparative procedures, the

addition of SDS was omitted and the sample was concentrated as described below.

#### *Isolation of rSPARC monomer by gel filtration chromatography*

rSPARC samples were reduced in volume by the use of Centriplus-10 centrifugal concentrators (Amicon, Beverly, MA, U.S.A.) that had been previously submerged in 5% Tween-20 for 16 hr to block non-specific absorption of proteins. Concentrated samples were applied to a  $1.6 \times 60\text{ cm}$  chromatography column that contained Superdex-70 resin (Pharmacia, Piscataway, NJ, U.S.A.) and were fractionated as previously described (Bassuk *et al.*, 1996).

#### *Cell culture*

Bovine aortic endothelial (BAE) cells (< passage 13) were cultured on tissue culture plastic in Dulbecco's modified Eagle's medium (DMEM) + low glucose (Gibco, Grand Island, NY, U.S.A.), supplemented with 10% fetal bovine serum (FBS), 1% penicillin G, and 1% streptomycin sulfate, as previously described (Funk and Sage, 1991).

To evaluate potential toxic effects of exposure to rSPARC, we incubated BAE cells with  $60\text{ }\mu\text{g/ml}$  rSPARC for 16 hr as outlined above. Cells were washed twice with 1% FCS/DMEM, and were incubated in the same rSPARC-free medium for 96 hr. Cells were counted by hemocytometer at selected times after removal of rSPARC.

#### *Incorporation assay*

Contact-inhibited BAE cell cultures were made quiescent by an incubation of 3 days in serum-free DMEM. The growth-arrested cells were subsequently replated in 1% FCS/DMEM at subconfluent densities into 24-well tissue culture plates (Costar, Cambridge, MA, U.S.A.) as previously described (Sage *et al.*, 1995). Immediately after subculture, rSPARC or mouse SPARC synthetic peptide 4.2 ( $\text{NH}_2$ -TCDLDNDKYIALEEWAGCFG-COOH, residues 254–273) was added to respective wells at the specified final concentrations.

Control cells received volumes of phosphate-buffered saline (PBS) equivalent to those in which rSPARC was delivered. Following an incubation of 16 hr, cells were pulse-labeled with  $1\text{ }\mu\text{Ci/ml}$  of  $^3\text{H}$ -thymidine for 2 hr. Cells were subsequently washed with PBS, fixed with cold 10% trichloroacetic acid (30 min), washed with cold anhydrous ethanol, air dried, and

solubilized in 0.3 ml of 0.2 N NaOH. Quantification of radioactivity incorporated into cellular DNA was achieved by liquid scintillation counting.

#### *Inhibition of cell spreading by rSPARC*

Actively proliferating BAE cells (approximately 50–70% confluent) were gently dissociated from a 75 cm<sup>2</sup> flask by a brief exposure to trypsin/EDTA. Trypsin was inactivated by addition of 10% FBS/DMEM to the cells. Remaining traces of serum were removed by a wash with DMEM. Harvested cells were resuspended in 1% FBS/DMEM at  $2 \times 10^4$  cells/ml. BAE cells ( $10^4$ ) were plated into 24-well plastic dishes in the absence or presence of varying concentrations of rSPARC. After 4, 18, and 48 hr, cultures were photographed with an inverted phase-contrast microscope (Zeiss, Thornwood, NY, U.S.A.). The degree of cell spreading was quantified by the scoring of three photographic fields, each derived from a well of a triplicate set. A 'Rounding Index' (Lane and Sage, 1990) was determined according to the equation:  $RI = (1a + 2b + 3c)/(a + b + c)$ , where *a*, *b*, and *c* equal the total number of cells in the photographic field (Lane and Sage, 1990). An index of 1 represents completely spread cells, 2, partially spread cells, and 3, totally rounded cells.

## RESULTS

We had previously reported the isolation of a soluble, monomeric form of rSPARC with conformational and biological properties highly similar to those of the native protein (Bassuk *et al.*, 1996). However, less than 50% of the translated rSPARC was recovered in the soluble extracts, with the remaining sequestered within the insoluble fraction (Schneider *et al.*, 1996). We therefore developed a method to improve our yield of recovered protein that entailed (1) the denaturation and reduction of total bacterial proteins, and (2) the renaturation and refolding of rSPARC into a biologically active protein.

#### *Two forms of rSPARC are expressed in E. coli*

rSPARC was previously cloned from a human endothelial cDNA library into the bacterial expression vector pET22b and was termed pSPARCwt (Bassuk *et al.*, 1996). The expression of rSPARC was dependent on the addition of IPTG, which inactivates the *lac* repressor and induces the synthesis of chromo-

somally encoded T7 RNA polymerase under *lacUV5* control and subsequent transcription of rSPARC mRNA from its T7 promoter. Polyacrylamide gel electrophoresis and Western immunoblot analysis of exponentially growing recombinant BL21(DE3) *E. coli* after incubation with 1 mM IPTG revealed two forms of rSPARC. Fig. 1(A) demonstrates that the total rSPARC population in this system is defined by a soluble and an insoluble fraction (Fig. 1 (B) and (C)). Antibodies specific for the carboxy terminal 4.2 domain of rSPARC were used to detect the reduced protein after SDS-PAGE. Full-length rSPARC displayed a *M<sub>r</sub>* of approximately 40 kDa (Fig. 1(A)–(C)). Degradation fragments were also observed; these fragments were apparently cleaved at the N-terminus or central region of the protein, because the antibody used is specific for the C-terminus of SPARC (Lane and Sage, 1990).

#### *rSPARC is expressed as an insoluble form contained within inclusion bodies*

Analysis of recombinant cultures of exponentially growing BL21(DE3) *E. coli* after incubation with 1 mM IPTG for 3 hr revealed refractile inclusions within the cytoplasm of the bacteria (not shown). This morphology was not observed in plasmid-free BL21(DE3) cultures or

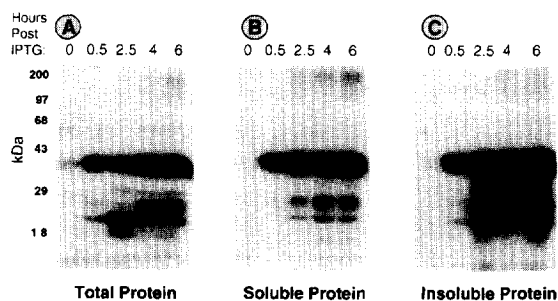


Fig. 1. Recombinant SPARC (rSPARC) is synthesized as two forms in bacteria. Cultures of recombinant BL21(DE3) *E. coli* were grown to  $OD_{600} = 0.6$  and rSPARC transcription was induced by the addition of IPTG to a final concentration of 1 mM at time 0. Aliquots of equal volumes were withdrawn from the growing cultures at 0, 0.5, 2.5, 4, and 6 hr. Cells were collected by sedimentation and lysed with Triton X-100 and lysozyme. Insoluble protein was removed by sedimentation. Protein samples were dissolved in sample buffer containing 2% SDS and 0.05 M dithiothreitol, fractionated by SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes. Shown are autoradiograms from immunoblots with immunoglobulins specific for the SPARC synthetic peptide 4.2 from domain IV. Detection of bound antigen-antibody complexes was achieved with <sup>125</sup>I-Protein A. Exposure time was 24 hr.

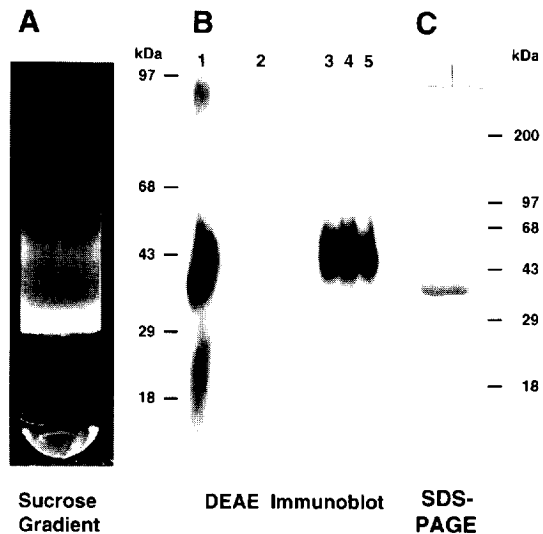


Fig. 2. Isolation of rSPARC-containing inclusion bodies by sedimentation through sucrose gradients. Banded material from the sucrose gradient in (A) was dissolved in 8 M urea and was fractionated by DEAE-Sepharose anion-exchange chromatography. (B) Autoradiogram from an immunoblot of the fractions from the ion-exchange column. rSPARC was detected with antibodies against the C-terminal  $\text{Ca}^{2+}$ -binding SPARC peptide 4.2 (Lane and Sage, 1990). Lane 1, initial isolate prior to chromatography; lane 2, the flow-through fraction; lanes 3-5, the 0.18-0.22 M NaCl eluate. (C) Coomassie blue-stained SDS gel of fractions pooled from the 0.18-0.22 M NaCl eluate.

in recombinant cultures that were not treated with IPTG (data not shown). Since we suspected that these refractile elements were likely to contain insoluble deposits of rSPARC that had been sequestered inside the cell, we isolated these inclusions by sucrose gradient sedimentation. Fig. 2(A) is a photograph of such inclusions after sedimentation to the 53-67% sucrose interface. Sedimentation in the absence of detergents resulted in an increase of contaminating proteins; rSPARC-containing inclusions therefore appeared to be associated with hydrophobic membrane components (data not shown). When this banded material was fractionated by denaturing anion-exchange chromatography, rSPARC eluted at a salt concentration of 0.18-0.22 M NaCl (Fig. 2(B), lanes 4-6). Material prepared in this manner was free of contaminating proteins (Fig. 2(C)). These data demonstrate that rSPARC-containing inclusion bodies can be treated as organelles and isolated by their sedimentation density in sucrose gradients. The yield of rSPARC was 50-100  $\mu\text{g}/\text{l}$  of fermentation culture. Particular reasons for these relatively low recoveries are (1) the inefficiency of dissolution of insoluble

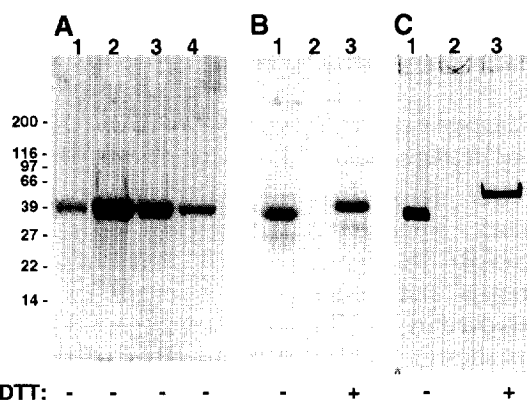
material in 8 M urea/0.1 M Tris-HCl (pH 8.0), and (2) the variability of the ion-exchange chromatography method due to a broad elution profile, and an ultimate dilution of the desired material.

Since the majority of rSPARC expressed in BL21(DE3) *E. coli* was insoluble, we decided to solubilize and renature the protein. Freshly isolated bodies could be dissolved partially in 8 M urea and fractionated by ion-exchange chromatography (Fig. 2(B)). The extraction of rSPARC into 8 M urea/6 M guanidine hydrochloride, or in 4 M guanidium isothiocyanate, was never complete (data not shown), and difficulties in the solubilization of rSPARC were encountered with prolonged storage. The extraction of rSPARC from total bacterial pastes was also found to be an inefficient process, owing to the incomplete solubilization of the protein and interference from nucleic acids. Increased recoveries of insoluble rSPARC were obtained after the mechanical lysis of the cells by French pressing, a process that also permitted the simultaneous shearing of nucleic acids. Only after we adjusted the pH of the extraction buffer to 11, and included octylthio-glucoside to a final concentration of 1-2%, were we able to extract 100% of rSPARC from the insoluble inclusion body fraction.

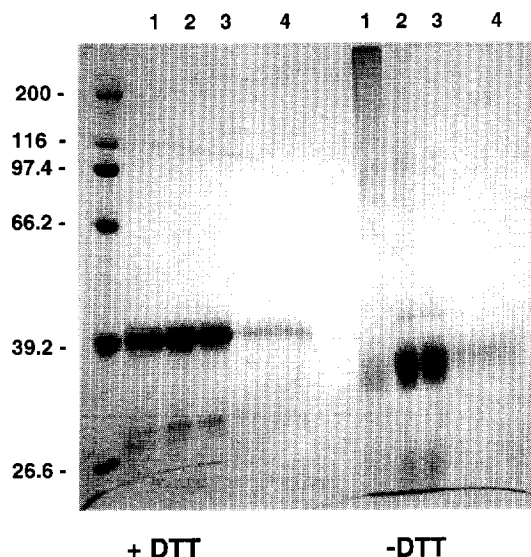
The presence of the carboxy terminal His<sub>6</sub> motif of rSPARC (Bassuk *et al.*, 1996) allowed us to develop rapid, one-step purification of the protein under denaturing conditions. Fig. 3 shows the results of one passage of total bacterial protein through a Ni-NTA metal-chelate affinity resin (Hochuli *et al.*, 1987). After an initial dissolution of bacterial proteins in 8 M urea, the concentration of urea was reduced to 4.7 M by dialysis, and from 4.7 to 1 M by a reverse gradient, during which time rSPARC remained immobilized on the Ni-NTA column. Partial refolding of the protein took place in association with the Ni-NTA matrix since no insolubility problems were encountered. The elution of rSPARC from the Ni-NTA column was achieved at pH 4.5, conditions under which the His residues of rSPARC were completely protonated and not available for interaction with  $\text{Ni}^{2+}$ . The configuration of rSPARC isolated in this manner was principally monomeric, since the apparent unreduced  $M_r$  after SDS-PAGE was 38 kDa (Fig. 3(A), lanes 1-4). However, upon removal of urea, rSPARC was found to aggregate (Fig. 4(B), lane 1), a condition enhanced by freeze/thaw cycling (not

shown). We therefore undertook a series of experiments to determine the nature of this aggregation.

The phenomenon was reversed by treatment of the sample with DTT prior to electrophoresis (Fig. 4(A), lane 1), a result that indicated that either incomplete or incorrect disulphide bonding was the cause of the aggregation. We reasoned that incubation of rSPARC with a mixture of reduced and oxidized glutathione (GSH and GSSG, respectively) could affect the isomerization of disulfide bonds in the protein and could produce a protein conformation that was stable and properly folded. Fig. 4 demonstrates that the cystine-dependent aggre-



**Fig. 3.** Purification and renaturation of rSPARC. (A) Denaturing Ni-NTA affinity chromatography. Total cellular protein was denatured and reduced as described in Experimental Procedures. Material insoluble in 8 M urea/0.1 M  $\text{NaH}_2\text{PO}_4$ /0.01 M Tris-HCl (pH 7.0)/0.05 M  $\text{NH}_4\text{OH}$ /1% octylthioglucoiside/0.05 M DTT/0.05 M DTE was removed by sedimentation at 20,000 *g* for 45 min at 25°C. Soluble, denatured proteins were dialyzed against 4.7 M urea/0.1 M  $\text{NaH}_2\text{PO}_4$  (pH 8.0)/0.01 M Tris-HCl/0.5 M NaCl/20% glycerol and were mixed with Ni-NTA resin for 1 hr at room temperature. The slurry was poured into a column, and chromatography was performed as described in Experimental Procedures. Shown is a Coomassie blue-stained, non-reducing, SDS-containing polyacrylamide (12%) electrophoretic gel. Lanes 1–4, fractions eluted at pH 4.5. (B) Glutathione-catalyzed disulfide bond isomerization. Purified proteins (pH 4.5 eluates) were reabsorbed to Ni-NTA resin at pH 7.8, the urea concentration was reduced by a linear gradient from 4.7 to 1 M at pH 6.0, and elution was performed at pH 4.5. Eluted protein was dialyzed against 0.1 M Tris-HCl (pH 8.0)/0.001 M EDTA. rSPARC (10  $\mu\text{g}$ ) was incubated for 10 min at ambient temperature with GSH:GSSG (5:1, 2:0.4 mM). The reaction was quenched with 5  $\mu\text{l}$  of hot 20% SDS. The sample was concentrated and was resolved by SDS-PAGE. Shown is a Coomassie blue-stained gel. Lanes 1 and 3, 10  $\mu\text{g}$  rSPARC. Lane 2 is blank. Lane 3 contained 0.05 M DTT in electrophoresis sample buffer. (C) Electrophoretic mobility shift of native SPARC after reduction. Lanes 1 and 3, 5  $\mu\text{g}$  murine SPARC isolated from parietal endoderm cells. Lane 2 contained 0.05 M DTT in sample buffer.



**Fig. 4.** Removal of urea from rSPARC and subsequent isomerization of disulfide bonds. SPARC was reappplied to a Ni-NTA affinity column in 6 M urea/0.1 M  $\text{NaH}_2\text{PO}_4$  (pH 8.0)/0.01 M Tris-HCl/0.5 M NaCl/20% glycerol, followed by 10 column vols of the same buffer at pH 6.0. The concentration of urea was reduced by a linear gradient of 50 ml of the above buffer to 50 ml of 1 M urea in the same buffer at a flow rate of 0.5 ml/min. rSPARC was eluted at pH 4.5 with 1 M urea in the same buffer. rSPARC was adjusted to concentrations of 1, 0.1, and 0.01  $\mu\text{g}/\text{ml}$  and was dialyzed against 100 mM Tris-HCl (pH 8.0)/150 mM NaCl/2 mM  $\text{CaCl}_2$ /2 mM GSH/0.02 mM GSSG. Samples were made up to 1% in SDS, concentrated, and resolved by SDS-PAGE. Shown is a Coomassie blue-stained gel. Lane 1, no GSH, no GSSG; lanes 2–4, GSH and GSSG present; lanes 1 and 2, rSPARC at 1 mg/ml; lane 3, rSPARC at 0.1 mg/ml, and lane 4, rSPARC at 0.01 mg/ml.

gation of rSPARC could be reversed by incubation with GSH:GSSG at 100:1 in the presence of 1 M urea. Since this reversal was accomplished at 1, 0.1, and 0.01 mg/ml, disulfide bond isomerization was independent of concentration over the range examined. The incubation with glutathione resulted in the formation of new disulfide bonds, because the protein displayed a lower electrophoretic mobility when DTT was included in the sample buffer (Fig. 3(B) and Fig. 4). This shift in mobility was also observed for native SPARC isolated from murine parietal endoderm cells (Fig. 3(C)). Fig. 5 shows a time-course of monomer formation that was dependent on the presence of a glutathione redox couple. The formation of monomeric rSPARC was observed to increase over the time period examined. The maximum amount of monomer formed under these conditions was approximately 40–50%, with the remaining protein as multimers.

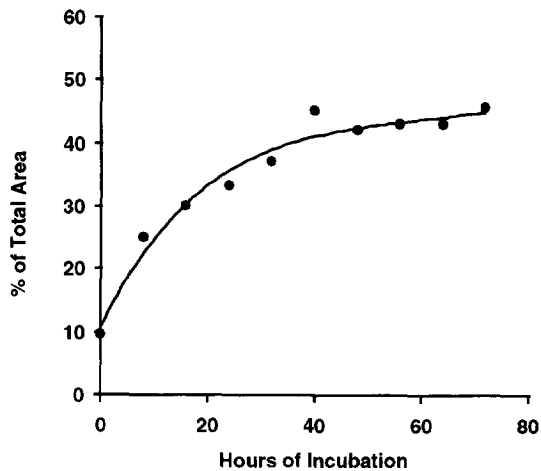


Fig. 5. Time course of glutathione-dependent rSPARC monomer formation. rSPARC was isolated by denaturing metal-chelate affinity chromatography, and the urea concentration was reduced to 1 M as described in Experimental Procedures. Ten aliquots of 40  $\mu$ g of rSPARC (80  $\mu$ g/ml) were dialyzed against 1 M urea/0.05 M Tris-HCl (pH 8.0)/2 mM CaCl<sub>2</sub>/2 mM GSH/0.02 mM GSSG for the following times: 0, 8, 16, 24, 32, 40, 48, 56, 64, and 72 hr. The reaction was quenched by the addition of SDS to 1%. After concentration, 15  $\mu$ g of protein was fractionated by SDS-PAGE, stained with Coomassie blue, and photographed. The film negative was densitometrically scanned at 560 nm, and the percentage area that corresponded to monomeric rSPARC was calculated and graphed.

Alternatively, isomerization was performed in solutions that lacked urea but contained higher concentrations of glutathione. Shown in Fig. 3(B) is the protein after an incubation with a 5:1 ratio of GSH:GSSG.

Table 1 summarizes the yields of rSPARC after purification under denaturing conditions. After a final gel filtration chromatography step on Superdex-70 resin, we calculated a yield of 2–4 mg of biologically active monomeric protein per 1.3 l of fermentation culture. This range of

recovery is approximately 2–3-fold higher than that obtained from soluble lysates (Bassuk *et al.*, 1996), and compares favorably with that predicted by Schneider *et al.* (1996), who used video-densitometric scanning methods for quantification of recoveries in their study of optimal culture conditions for rSPARC in BL21(DE3) *E. coli*.

#### Circular dichroism spectroscopy

The  $\alpha$ -helical content of renatured rSPARC was found to increase by the addition of Ca<sup>2+</sup> as determined by circular dichroism spectroscopy. This effect was reversed by the addition of EDTA. These observations are in agreement with the behavior of the soluble form of rSPARC (Bassuk *et al.*, 1996) and with the native murine protein (Engel *et al.*, 1987).

#### Renatured and refolded rSPARC is monomeric and displays biological activity

Our preparations of rSPARC after disulfide bond isomerization frequently contained rSPARC configurations that tended to form trimers and tetramers. Such oligomers were removed by gel filtration chromatography (data not shown). Final preparations of rSPARC used in subsequent assays were judged to be > 90% free from contaminating trimers and tetramers. We have previously reported that soluble preparations of rSPARC displayed biological activities similar to those of the native murine protein (Bassuk *et al.*, 1996). Soluble rSPARC inhibited the spreading of BAE cells and bound specifically to BAE cell monolayers. These activities were not due to contaminating endotoxin, as the determined amount of this lipopolysaccharide present in our preparations was well below the threshold that elicits a

Table 1. Recovery of rSPARC from *E. coli*<sup>a</sup>

Fractions	Total proteins (%)	Total proteins (mg)
Total rSPARC	20–30 <sup>b</sup>	—
Soluble lysate	12–20 <sup>b</sup>	825 <sup>c</sup>
Ni-NTA <sup>d</sup>	—	3.5 <sup>c</sup>
Superdex-70	—	1–2 <sup>c</sup>
Inclusion body	6–12 <sup>b</sup>	—
Sucrose gradient <sup>e</sup>	—	12–15 <sup>c</sup>
Ni-NTA 1 <sup>c</sup>	—	12–15 <sup>c</sup>
Ni-NTA 2 <sup>f</sup>	—	6–8
Superdex-70	—	2–4 <sup>c</sup>

<sup>a</sup>Data compiled from six 1.3 l fermentation experiments performed under identical conditions. <sup>b</sup>Calculated by integration of video-densitometric scans of Coomassie-blue stained gels (Schneider *et al.*, 1996). <sup>c</sup>Protein content determined with bicinchoninic acid copper protein reagent (Bassuk *et al.*, 1996). <sup>d</sup>From a pH 5.3 eluate. <sup>e</sup>From a pH 4.5 eluate in denaturing conditions. <sup>f</sup>From a pH 4.5 eluate after gradient removal of urea.

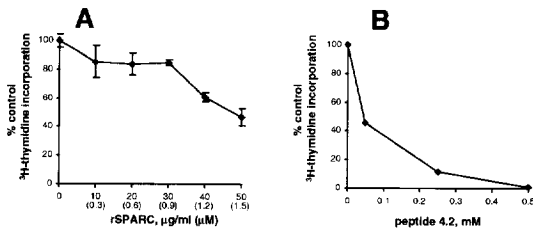


Fig. 6. Inhibition of endothelial cell DNA synthesis by rSPARC. rSPARC was isolated by denaturing metal-chelate affinity chromatography and was renatured as described in Experimental Procedures. Indicated amounts of rSPARC (A) or SPARC synthetic peptide 4.2 (B) were incubated for 16 hr with freshly plated, growth-arrested BAE cells. Cells were labeled with  $1 \mu\text{Ci/ml}$  of  $^3\text{H}$ -thymidine for 2 hr, washed, and dissolved in  $0.2 \text{ N NaOH}$ . The radioactivity in a fraction of acid-insoluble cellular DNA was determined by scintillation counting.

significant change in the gene expression of BAE cells and causes cellular detachment (Bassuk *et al.*, 1996).

We assayed our final preparations of monomeric rSPARC for additional activities previously described for the native murine protein. rSPARC was shown to inhibit the incorporation of  $^3\text{H}$ -thymidine by BAE cells *in vitro* (Fig. 6(A)). At  $1.5 \mu\text{M}$  ( $50 \mu\text{g/ml}$ ), we observed a 50% reduction in the incorporation of the radioactive deoxyribonucleotide. For comparison, the reported  $\text{ED}_{50}$  for native murine SPARC in an analogous assay is  $0.6 \mu\text{M}$  ( $20 \mu\text{g/ml}$ ) (Funk and Sage, 1991). For further comparison, soluble rSPARC was reported to inhibit the uptake of  $^3\text{H}$ -thymidine by cultured rat glomerular mesangial cells (Pichler *et al.*, 1996). In this study, soluble rSPARC ( $1.5 \text{ mM}$ ,  $50 \mu\text{g/ml}$ ) inhibited 55–60% of incorporation of

$^3\text{H}$ -thymidine by cells stimulated with either 1% serum or  $10 \text{ ng/ml}$  platelet-derived growth factor. We also evaluated a preparation of SPARC synthetic peptide 4.2 (Fig. 6(B)). The  $\text{ED}_{50}$  of this peptide was approximately  $50 \mu\text{M}$ . This result, which is consistent with that reported previously (Sage, 1992), provided an internal reference for the inhibition of DNA synthesis by this strain of BAE cells.

Purified rSPARC blocked the spreading of BAE cells in a concentration-dependent manner (Table 2). Cell rounding indices of  $2.12 \pm 0.07$  and  $2.26 \pm 0.11$  were observed with  $0.6 \mu\text{M}$  ( $20 \mu\text{g/ml}$ ) added protein over a 4 and 18 hr time period, respectively. These values are in agreement with the index of  $2.15 \pm 0.11$  reported for  $0.6 \mu\text{M}$  ( $20 \mu\text{g/ml}$ ) native murine SPARC on BAE cells (Sage, 1992), and with the index of  $2.10 \pm 0.1$  reported for  $0.6 \mu\text{M}$  ( $20 \mu\text{g/ml}$ ) soluble rSPARC on the same cells (Bassuk *et al.*, 1996). Buffer controls, obtained from a protein-free fraction derived from the eluate of a Superdex gel filtration column, produced indices of  $1.6 \pm 0.05$  and  $1.49 \pm 0.27$  at 4 and 18 hr, respectively.

To assess the toxicity of rSPARC, we examined the proliferation of BAE cells after a 16 hr exposure to rSPARC. As shown in Table 3, there was a 2.7-fold increase in cell number 96 hr after the removal of exogenous rSPARC from the culture medium. We therefore concluded that the effect of rSPARC on DNA synthesis was reversible and that rSPARC was not toxic to endothelial cells, which are otherwise highly sensitive to endotoxin. The data shown in Table 3 are in agreement with the previously reported experiments on the

Table 2. Inhibition of BAE cell spreading by rSPARC<sup>a</sup>

Time (hr)	Ni-NTA column buffer	PBS	rSPARC $\mu\text{g/ml}$ ( $\mu\text{M}$ )	Rounding index
4	+	–	–	$1.65 \pm 0.05$
	–	+	–	$1.60 \pm 0.14$
	+	–	20 (0.6)	$2.12 \pm 0.07$
	–	–	35 (1.05)	$2.29 \pm 0.08$
	–	–	50 (1.5)	$2.47 \pm 0.10$
18	–	–	–	$1.49 \pm 0.27$
	–	+	–	$1.47 \pm 0.22$
	–	–	20 (0.6)	$2.26 \pm 0.11$
	–	–	35 (1.05)	$2.27 \pm 0.11$
	–	–	50 (1.5)	$2.27 \pm 0.08$
48	–	–	50 (1.5)	2.30
17	–	–	20 (0.6)	$2.15 \pm 0.11^b$
			Murine SPARC	

<sup>a</sup> BAE cells ( $10^4$ ) were plated in the presence of 20–50  $\mu\text{g/ml}$  rSPARC/1% FBS/DMEM. After 4, 18, and 48 hr, the degree of spreading in each group was converted into a Rounding Index (Lane and Sage, 1990), in which an index of 1 represents a culture with only spread cells. A culture with increasing numbers of unspread and round cells would approach the maximum index of 3. Values were calculated from multiple photographic fields of three independent cultures and are presented as the mean  $\pm$  SD. <sup>b</sup> (Sage, 1992).

Table 3. Recovery of BAE cells after incubation with rSPARC

Time	Total cells/ml
Prior to addition of rSPARC <sup>a</sup>	$3.0 \times 10^5$
15 hr after medium change	$2.5 \times 10^5$
48 hr after medium change	$4.0 \times 10^5$
96 hr after medium change	$7.5 \times 10^5$

<sup>a</sup> Growth-arrested BAE cells were plated in presence of 60  $\mu\text{g/ml}$  (1.8  $\mu\text{M}$ ) rSPARC/1% FBS/DMEM for 16 hr. The medium was replaced with 1% FBS/DMEM, and the total number of cells/ml was determined with a hemocytometer.

reversibility of the anti-proliferative effect of native SPARC (Sage *et al.*, 1989).

### DISCUSSION

Our laboratory has established a bacterial expression system that generates quantities of human endothelial cell recombinant SPARC (rSPARC) that are biologically active. The protein-encoding region was cloned into a pET vector under the control of an inducible T7 RNA polymerase in a BL21(DE3) lysogen of *E. coli* (Bassuk *et al.*, 1996). A soluble, monomeric form of the protein was purified, which bound to BAE cells and inhibited their spreading *in vitro* (Bassuk *et al.*, 1996). Soluble rSPARC also inhibited DNA synthesis by cultured rat glomerular mesangial cells and has been implicated in the resolution of Thy-1 experimental glomerulonephritis (Pichler *et al.*, 1996). The conformation of this soluble, recombinant protein was dependent on bound  $\text{Ca}^{2+}$ . rSPARC isolated from soluble extracts of recombinant bacterial cultures therefore manifests an intrinsic ability to fold into its proper conformation, despite its 14 Cys residues and the reducing environment of the bacterial cytoplasm. Support for this contention comes from a recent report in which domains III and IV of SPARC were shown to comprise a self-folding and crystallizable extracellular  $\text{Ca}^{2+}$ -binding (EC) module (Maurer *et al.*, 1995; Hohenester *et al.*, 1996).

A second conformer of rSPARC found in recombinant BL21(DE3) *E. coli* is an insoluble form sequestered in inclusion bodies. Since we have not observed a consistent pattern of degradation of rSPARC associated with these isolated bodies, we conclude that the bacterium is overwhelmed by an abundance of rSPARC folding intermediates, a fraction of which aggregates in the cytoplasm (Schneider *et al.*, 1996). Efforts to increase the expression of

soluble rSPARC by co-transformations of recombinant BL21(DE3) with plasmids harboring constructs encoding the bacterial chaperones DnaK–DnaJ or GroEL–GroES were unsuccessful (Schneider *et al.*, 1996). Therefore, models describing modes of action of these chaperones may be oversimplified, and other molecular chaperones could be implicated in the rSPARC folding pathway. The rSPARC that is correctly folded must therefore be present in low concentrations to minimize protein–protein interactions and to promote the formation of stable end-products from intermediate forms in the folding pathway. This hypothesis is supported by data from this report showing that total cellular rSPARC can be denatured and refolded into a correct conformation at significantly higher yields than those obtained with regard to the soluble form.

We found that high concentrations of chaotropic agents, an alkaline pH, and a non-ionic detergent were essential for the dissolution of bacterial rSPARC. We therefore denatured and subsequently reduced total bacterial protein prior to nickel–chelate affinity chromatography. The gradual removal of urea while rSPARC was immobilized on the affinity resin was a critical procedure that served to stabilize the protein during refolding and to prevent its precipitation. SDS–PAGE of purified, unreduced rSPARC in the presence of 1 M urea exhibited an entirely monomeric protein. However, upon removal of urea, we frequently found that a portion of the monomers tended to aggregate, a phenomenon that we attributed to incorrect or incomplete disulfide-bond formation. SPARC has 14 Cys residues and there are no free SH groups in the secreted native protein. We exposed rSPARC to a mixture of reduced and oxidized glutathione in the presence or absence of 1 M urea. Incubation of rSPARC in varying ratios of GSH:GSSG resulted in an increased yield of the monomer. This observation is consonant with many reports of the intrinsic ability of proteins to refold themselves in the absence of external catalytic activity (Anfinsen, 1973). The redox potential imparted by the glutathione system represents a useful experimental system in which to probe the folding of rSPARC during post-translational modifications in the endoplasmic reticulum of eukaryotic cells. This organelle is well-known to contain glutathione as well as enzymes that might act as catalysts or chaperones for secreted proteins (Bassuk and

Berg, 1989). We observed that rSPARC refolded at protein concentrations in excess of 1 mg/ml, a result supporting our hypothesis that the information required for the folding of rSPARC into a biologically active protein is contained within its primary structure.

rSPARC has been expressed in yeast (Yost *et al.*, 1994), kidney 293 cells (Pottgiesser *et al.*, 1994; Xie and Long, 1995), and *E. coli* (Bassuk *et al.*, 1996). The polypeptide appears to be correctly folded in each system, since the isolated protein is a monomer as revealed by non-reducing SDS-PAGE. The bacterial and yeast proteins were biologically active, and both the bacterial and mammalian proteins displayed a  $\text{Ca}^{2+}$ -dependent conformation. rSPARC can therefore be expressed in heterologous systems, and its biological activity has been linked to conformational states that are dependent on bound  $\text{Ca}^{2+}$  (Motamed *et al.*, 1995). Comparable conformations of rSPARC and native SPARC have been established by studies using intrinsic fluorescence emission and circular dichroism spectroscopies (Pottgiesser *et al.*, 1994; Bassuk *et al.*, 1996).

Functionality of carbohydrate is another issue relevant to rSPARC proteins. Three different N-linked glycosylation sites have been reported for the seven SPARC and three SPARC-like proteins. Due to its bacterial origin, rSPARC lacks carbohydrate but nonetheless exhibits biological activity in assays of cellular morphology, proliferation, and binding interaction *in vitro*. A mutant mouse rSPARC, expressed in yeast and lacking the known N-linked carbohydrate attachment site, was found to be anti-adhesive (Yost *et al.*, 1994) and reduced focal contacts in bovine aortic endothelial cells (Murphy-Ullrich *et al.*, 1995). It appears, for these activities at least, that the carbohydrate at this site is not an essential feature. But is carbohydrate required for maximal activity? When we compared the  $\text{ED}_{50}$  of rSPARC to that reported for native murine SPARC, we noticed that twice as much rSPARC was needed to inhibit 50% of the incorporation of [ $^3\text{H}$ ]-thymidine by BAE cells. Although we cannot rule out the possibility that inactive forms of rSPARC in our preparations could account for the diminished specific activity of rSPARC in these assays, the greater specific activity of the glycosylated murine protein is more likely due to a decreased affinity for types III and/or V collagen, components of the BAE extracellular matrix. Xie and Long

(1995) reported that the removal of carbohydrate from SPARC resulted in a two-fold increase in binding capacity for type V. A reduced affinity of glycosylated SPARC for type V or other collagens could enhance its interaction with BAE cells, with subsequent decreases in [ $^3\text{H}$ ]-thymidine incorporation. For rSPARC, the lower carbohydrate content would result in more of the protein bound to the extracellular matrix, and less available to interact with endothelial cells. This reduced ability of rSPARC to inhibit [ $^3\text{H}$ ]-thymidine incorporation could account directly for the lower specific activity of rSPARC. The synergistic effect of domain II (containing the carbohydrate-attachment site) and domain IV (which binds to BAE cells) in the modulation of endothelial cell behavior (Sage *et al.*, 1995) indicates that there is a structural cooperativity between the follistatin-like (Patthy, 1991) and EC modules (Hohenester *et al.*, 1996).

Alternatively, the existence of carbohydrate on SPARC could promote the folding of the nascent polypeptide within the endoplasmic reticulum. The chaperones Bip and calnexin, the latter known to bind selectively to carbohydrates within glycoproteins, could bind SPARC to facilitate its proper folding and disulfide bond formation (Kim and Arvan, 1995; Helenius, 1994). The oligosaccharide might then play an important role in the efficient secretion process associated with the post-translational processing of SPARC. Since millimolar concentrations of  $\text{Ca}^{2+}$  are stored within the endoplasmic reticulum, the SPARC leaving this compartment is expected to contain bound  $\text{Ca}^{2+}$  and would therefore be secreted with a maximal content of  $\alpha$ -helix. Our studies describing the folding properties of rSPARC *in vitro* support our hypothesis that a self-folding SPARC might require ancillary chaperones to effect its secretion. The driving force behind this self-folding property is the EC module that is likely to form a larger structural or functional unit with the follistatin-like module. The conserved spacing of multiple Cys residues and the presence of a carboxy-terminal  $\text{Ca}^{2+}$ -binding EF-hand are likely to provide the conformational properties that mediate one or more of the functions proposed for these proteins (Bassuk *et al.*, 1993). The specificity for each protein would then be determined by a unique N-terminal domain. For SPARC, elements in domain I are likely to contribute to the control of endothelial cell shape, as both the glycosy-

lated murine and the non-glycosylated bacterial protein demonstrated equivalent activities at the same dose in cell-spreading assays. A fundamental role for SPARC in the genesis or repair of tissues and organs can now be reconciled in part to specific structural domains. It will be interesting to define the metal-binding capabilities of SPARC in the context of additional functions.

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