THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 30, pp. 22062–22071, July 27, 2007
© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

SPARC Regulates Processing of Procollagen I and Collagen Fibrillogenesis in Dermal Fibroblasts*

Received for publication, January 8, 2007, and in revised form, April 13, 2007 Published, JBC Papers in Press, May 23, 2007, DOI 10.1074/jbc.M700167200

Tyler J. Rentz[‡], Felicitta Poobalarahi[‡], Paul Bornstein[§], E. Helene Sage[¶], and Amy D. Bradshaw[‡1]

From the [‡]Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29412, the [§]Department of Biochemistry, University of Washington, Seattle, Washington 98195, and the [¶]Hope Heart Program, Benaroya Research Institute at Virginia Mason, Seattle, Washington 98101

A characterization of the factors that control collagen fibril formation is critical for an understanding of tissue organization and the mechanisms that lead to fibrosis. SPARC (secreted protein acidic and rich in cysteine) is a counter-adhesive protein that binds collagens. Herein we show that collagen fibrils in SPARC-null skin from mice 1 month of age were inefficient in fibril aggregation and accumulated in the diameter range of 60–70 nm, a proposed intermediate in collagen fibril growth. *In vitro*, procollagen I produced by SPARC-null dermal fibroblasts demonstrated an initial preferential association with cell layers, in comparison to that produced by wild-type fibroblasts. However, the collagen I produced by SPARC-null cells was not efficiently incorporated into detergent-insoluble fractions. Coincident with an initial increase in cell association, greater amounts of total collagen I were present as processed forms in SPARC-null *versus* wild-type cells. Addition of recombinant SPARC reversed collagen I association with cell layers and decreased the processing of procollagen I in SPARC-null cells. Although collagen fibers formed on the surface of SPARC-null fibroblasts earlier than those on wild-type cells, fibers on SPARC-null fibroblasts did not persist. We conclude that SPARC mediates the association of procollagen I with cells, as well as its processing and incorporation into the extracellular matrix.

Matricellular proteins are defined as proteins that are associated with the extracellular matrix (ECM)[2] but are not considered structural components of the ECM, in contrast to classical ECM proteins such as laminin and collagen I (1, 2). SPARC is a prototypic matricellular protein that exhibits counter-adhesive and anti-proliferative activity when added to cultured cells (3). SPARC has been shown to bind to a number of ECM proteins including collagens I, III, and IV (3).

Expression of SPARC is elevated during development and decreases upon differentiation in a majority of tissues (4). However, expression of SPARC persists in tissues in which ECM remodeling is ongoing, such as bone and gut epithelia (4). Increased levels of SPARC are detected in response to injury where ECM remodeling is initiated, with fibroses in liver, lungs, and kidney, and in the skin of individuals with scleroderma (5–8). Hence, SPARC expression patterns implicate this protein as an important mediator of collagen I deposition and/or remodeling.

The α1(I) and α2(I) subunits of procollagen I are synthesized with N- and C-propeptides that are enzymatically released by specific proteases to yield processed collagen I (9). Processing of procollagen I to collagen I is essential for correct assembly of collagen fibrils. Spatial and temporal regulation of procollagen processing has been proposed as a potential regulatory event in collagen fibril assembly (9). For example, antibodies against the N-propeptide of collagen I were immunolocalized exclusively to the surface of collagen fibrils that were 20–40 nm in diameter (10). Retention of the propeptides of procollagen I following incorporation into fibrils might present a steric hindrance to fibril fusion and thus limit fibril expansion. Alternatively, Watson *et al.* (11) reported that pN collagen I (procollagen I with N-propeptides retained) could be incorporated into collagen fibrils with the N-propeptide accommodated within the fibril. The authors suggested that the N-propeptide acts to enhance collagen fibril aggregation (11). In this case, premature processing of the N-propeptide would limit fibril expansion. The factors that influence the spatial and temporal regulation of procollagen I conversion to collagen I are predicted to have a profound influence on collagen deposition (9).

SPARC-null mice display a number of phenotypic abnormalities, the majority of which are manifested in aberrant ECM structure and assembly, consistent with a function of SPARC as a modulator of cell-ECM interactions (4). Adult SPARC-null mice exhibit decreased amounts of collagen in skin and bone (12, 13). In addition, fibrotic deposition of collagen is diminished in the absence of SPARC. For example, streptozoticin-induced renal fibrosis in SPARC-null mice was reduced in comparison to that of wild-type (14). Increased expression of SPARC is associated with collagen production, and the lack of SPARC results in decreased collagen accumulation. SPARC is therefore implicated as a key regulator of collagen incorporation in tissues.

To investigate further the mechanisms by which SPARC influences collagen deposition, we cultured SPARC-null and wild-type (WT) primary dermal fibroblasts and characterized collagen I maturation and fiber formation *in vitro*. Our studies

* This work was supported by National Institutes of Health Grants KO1 (to A. D. B.), GM-40711 (to E. H. S.), and AR 11248 (to P. B.) and a Veteran Affairs Career Development Award (to A. D. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Gazes Cardiac Research Institute, 114 Doughty St., Medical University of South Carolina, Charleston, SC 29425. Tel.: 843-792-4959; Fax: 843-876-5068; E-mail: bradshad@musc.edu.

[2] The abbreviations used are: ECM, extracellular matrix; WT, wild type; SPARC, secreted protein acidic and rich in cysteine; rSPARC, recombinant secreted protein acidic and rich in cysteine.

This is an Open Access article under the CC BY license.

indicate that, in the absence of SPARC, an initial increase in the levels of procollagen I in cell layers was coupled with an increase in the conversion of procollagen I to collagen I. The addition of recombinant SPARC (rSPARC) reduced the levels of procollagen I associated with cell layers and was accompanied by a decrease in conversion of procollagen I. Enhanced association of procollagen I with SPARC-null cells did not lead to a greater incorporation of collagen into detergent-insoluble fractions. In fact, SPARC-null fibroblasts demonstrated substantially less incorporation of collagen into detergent-insoluble fractions after extended culture. Likewise, collagen fibers formed sooner on the surface of SPARC-null cells but did not persist, in comparison to fibers on WT cells.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture reagents were from Invitrogen. rSPARC was generated by baculovirus infection of insect cells and purified as described in Ref. 15. Anti-murine collagen I antibodies were from MD Biosciences (Zurich, Switzerland). Antibodies generated against the C-propeptide of collagen α1(I) (LF41) were provided by Dr. L. Fisher (National Institutes of Health) ((16). Anti-actin antibodies were from Sigma.

Electron Microscopy—The SPARC-null mouse colony used in these studies has been described previously (17). Sections of skin were generated from 3 WT and 5 SPARC-null mice at 1 month of age and were prepared for electron microscopy (EM) as described in Ref. 12. Collagen fibril diameters were measured in scanned images generated from electron micrographs with NIH Image software. Collagen fibrils in at least 3 fields derived from sections of skin from each mouse were quantified; 2699 WT fibrils and 2658 SPARC-null fibrils were measured.

Primary Cell Culture—Primary dermal fibroblasts were isolated from age-matched WT and SPARC-null animals based upon the protocol described in Ref. 18 with the following exceptions: 1) the collagenase solution used in these experiments was 1/10 Blendzyme 3 (Roche Applied Science) in Dulbecco's modified Eagle's medium (Invitrogen), 2) cell preparations were rinsed 3 times in growth media (Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotic/antimycotic solution) prior to final plating, and 3) cells were filtered through a 100-μm cell strainer prior to final plating. The majority of animals used in these experiments were 2–3 months of age. Dermal fibroblasts were cultured in growth media supplemented with 50 μg/ml sodium ascorbate to induce collagen production and secretion as indicated. All experiments were performed with dermal fibroblasts between passages 1 and 4.

Analysis of ECM Production and Deposition—Primary dermal fibroblasts plated at equal concentrations were labeled with 25 μCi/ml [2,3,4,5-^{3}H]proline (PerkinElmer Life Sciences) in growth media for 18–24 h to assess collagen production in media and cell layers from WT and SPARC-null cells. Cell layers were collected by scraping in hot SDS-PAGE buffer with dithiothreitol (19) or in 1% deoxycholate with protease inhibitors (protease inhibitor mixture tablets, Roche). Deoxycholate-extracted cells were tumbled overnight at 4 °C to recover soluble proteins. Detergent-insoluble proteins were separated by centrifugation at 12,000 $\times$ *g* for 15 min at 4 °C. Cell fractions used for immunoblot and hydroxyproline analysis were also grown and collected in this manner.

Hydroxyproline analysis was carried out based on the method described in Ref. 20. Briefly, equal numbers of age-matched WT and SPARC-null dermal fibroblasts were cultured in growth media in Dulbecco's modified Eagle's medium without phenol red and supplemented with ascorbate (50 μg/ml) for the designated periods. Detergent-soluble and -insoluble cell layers were collected as stated above and placed in 6 N HCl for hydrolysis. Media fractions were freeze-dried, resuspended in 6 N HCl, and hydrolyzed at 120 °C. Cell extracts were hydrolyzed according to the same protocol. Samples were neutralized with 4 N NaOH. Samples and standards (L-hydroxyproline, Sigma) were incubated for 20 min at room temperature with chloramine T followed by addition of Ehrlich reagent (3.75 g of *p*-dimethylaminobenzaldehyde, 15 ml of 1-propanol, 6.5 ml of perchloric acid (60%) in 25 ml) for 20 min at 60 °C. Absorbance of standards and samples were read at $\lambda = 558$ nm. Values were calculated from a standard curve generated for each analysis. Amounts of total protein in detergent-soluble fractions were determined by bicinchoninic acid (BCA) assays according to the manufacturer's instructions (Pierce).

After separation by SDS-PAGE, metabolically labeled proteins were detected by fluorography on X-Omat film. Immunoblot analysis was performed by transfer of separated proteins to nitrocellulose and detection with anti-murine collagen I antibodies. Chemiluminescence was used to detect secondary antibodies conjugated to horseradish peroxidase. Quantification of protein bands was performed with NIH Image software.

Metabolically labeled conditioned media generated by equal numbers of WT and SPARC-null cells grown in ascorbate for 18–24 h were incubated with an anti-N-propeptide rabbit polyclonal antibody against recombinant mouse N-propeptide that was produced in insect cells, or polyclonal anti-murine SPARC antibodies (R&D Systems, Minneapolis, MN). Primary antibodies were precipitated with protein A/G-Sepharose beads (GE Healthcare). Immune complexes were boiled in SDS-PAGE buffer with dithiothreitol, separated by SDS-PAGE, and exposed to film.

Pulse-chase experiments were performed by labeling equal amounts of WT and SPARC-null fibroblasts in growth media. Ascorbate was added ~2 h prior to pulse labeling with 25 μCi/ml [^{3}H]proline for 12 min (pulse label). The pulse labeling media were removed, and the cell layer was rinsed in phosphate-buffered saline followed by incubation in chase media (growth media with ascorbate and 1 mg/ml unlabeled proline) for the indicated time periods. Cell layers were collected in SDS-PAGE buffer with dithiothreitol, and proteins were resolved on 7% SDS-polyacrylamide gels. Bands representing procollagen and collagen I were detected by fluorography and were quantified by the use of NIH Image software.

Immunohistochemistry—Age-matched WT and SPARC-null cells were plated in equal numbers on glass coverslips in growth media. Cells were treated with 50 μg/ml ascorbate and were grown for the indicated number of days. Cell layers were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.5) for 30–45 min. Coverslips were blocked in 1% normal goat serum in Tris-buffered saline (blocking solution) prior to addi-

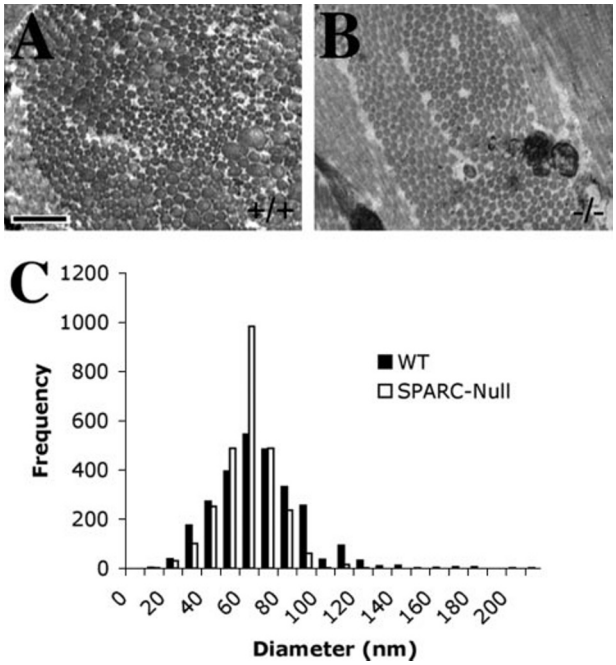

FIGURE 1. Collagen fibrils in the mid-dermis of SPARC-null (−/−) (*B*) mice exhibited a more uniform distribution of diameters in comparison to WT (+/+) (*A*) skin at 1 month of age. *C*, a higher frequency of collagen fibrils with diameters of 60–70 nm was observed in SPARC-null skin (*white bars*) *versus* WT skin (*black bars*). No fibrils >110 nm were observed in null skin, whereas WT fibrils reached 220 nm. *Bar* in *A*, 500 nm.

tion of anti-murine collagen I antibodies (1:200 dilution in blocking solution) or anti-cellular fibronectin (1:200 in blocking solution, Sigma). Primary antibodies were detected with goat anti-mouse fluorescein-conjugated secondary antibodies (Jackson Laboratories). Coverslips were mounted in Anti-Fade reagent (Molecular Probes, Eugene, OR) and were viewed on a Leica microscope equipped for epifluorescence.

RESULTS

EM of Collagen Fibrils from 1-Month-old Mice—Thin collagen fibrils characteristic of young dermis undergo lateral fusion to generate collagen fibrils of increasing diameter. We have reported that collagen fibrils in adult SPARC-null skin were smaller and more uniform in diameter than those of WT mice (12). We sought to determine whether the absence of SPARC was associated with phenotypic differences in collagen fibril morphology in the dermis during times characterized by active collagen fibril aggregation. As shown in Fig. 1, a more limited range of fibril diameters was observed in SPARC-null skin in comparison to WT at 1 month of age. Although the average diameter of WT fibrils was slightly larger than SPARC-null fibrils (60.2 *versus* 53.8 nm), the standard deviation of the WT fibril population was 24, whereas that of SPARC-null fibrils was 14.6, which is indicative of a more restricted range of fibril diameters in null dermis. Whereas collagen fibrils as large as 220 nm were observed in WT dermis, SPARC-null fibrils larger than 110 nm were not found. The frequency of SPARC-null collagen fibrils with diameters of ~60–70 nm was disproportionately represented in SPARC-null skin. Thirty-seven percent of measured SPARC-null collagen fibrils were ~60–70 nm in diameter, whereas 20% of WT fibrils were in this range. We

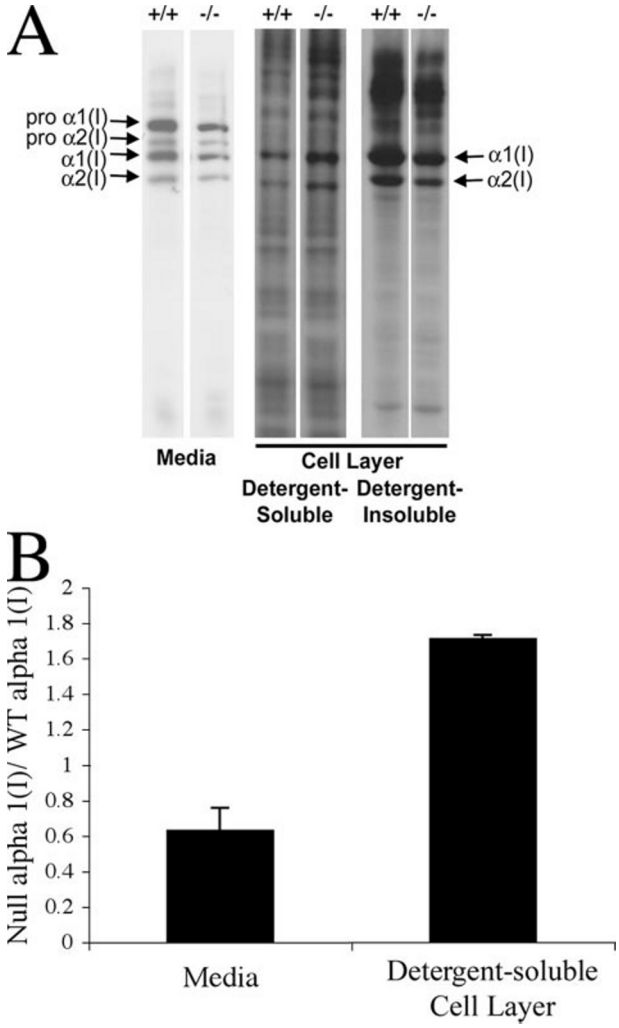

FIGURE 2. **Collagen I was preferentially associated with SPARC-null cell layers.** *A*, equal numbers of SPARC-null and WT primary dermal fibroblasts were metabolically labeled with [^{3}H]proline for 1 day. Equal amounts of labeled proteins determined by scintillation counting of conditioned media and cell layers were separated by SDS-PAGE and processed for fluorography. Decreased amounts of collagen α1(I) subunit were detected in the media of SPARC-null (−/−) cells in comparison to WT (+/+), whereas an increase in collagen I was observed in SPARC-null *versus* WT cell layer fractions. *B*, quantification of collagen α1(I) protein bands generated by fluorography from 7 separate cell isolations. The ratio of null α1(I)/WT α1(I) was <1 in conditioned media, but ratios >1 for null α1(I)/WT α1(I) were observed in cell layers.

conclude that in the absence of SPARC, collagen fibril accretion is compromised, as early as 1 month after birth.

Production and Deposition of Collagen I by SPARC-null Dermal Fibroblasts—Primary dermal fibroblasts from age-matched WT and SPARC-null mice were cultured to examine differences in collagen deposition that might implicate SPARC in collagen fibril formation. We have found that collagen secretion by primary murine dermal fibroblasts is greatly enhanced by the addition of ascorbate. As shown in Fig. 2, collagen produced by SPARC-null fibroblasts displayed an increased interaction with detergent-soluble cell layers in comparison to collagen produced by WT cells at ~18–24 h after induction of collagen secretion by ascorbate. Likewise, decreased amounts of collagen were present in media conditioned by SPARC-null compared with WT fibroblasts at this time. Fig. 2*A* shows a representative fluorograph generated from cells incubated with

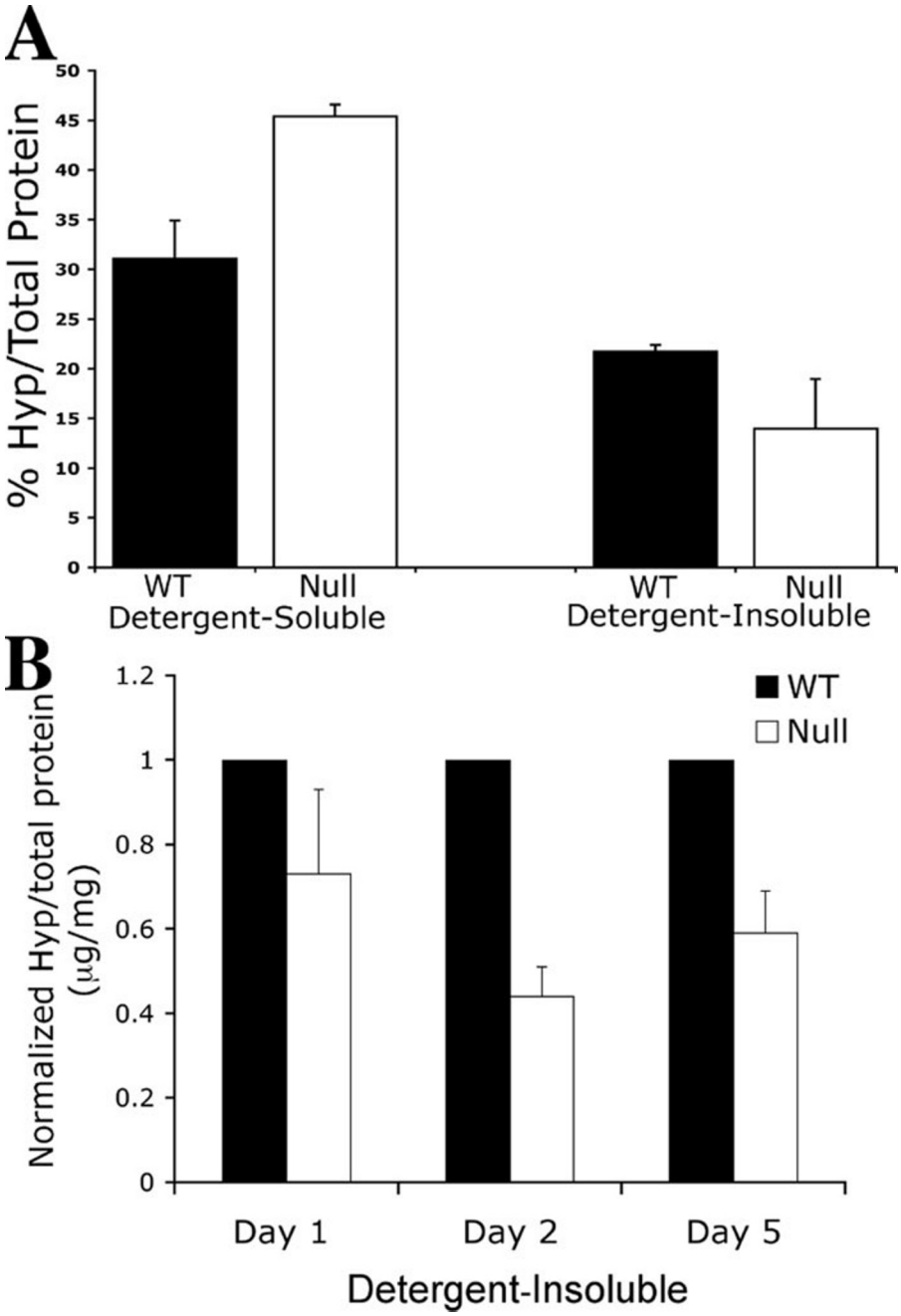

FIGURE 3. **Collagen initially associated with SPARC-null cells is not efficiently incorporated into detergent-insoluble cell layers.** *A*, hydroxyproline analysis of detergent-extracted cell layers from SPARC-null and WT cells 4 h after addition of ascorbate demonstrated an increase in the percent of total collagen I associated with detergent-soluble fractions in SPARC-null fibroblasts (*white bars*) *versus* WT cells (*black bars*). In contrast, detergent-insoluble cell layers from WT cells contained a greater percentage of total collagen in comparison to SPARC-null cells. *B*, hydroxyproline analysis of detergent-insoluble cell layers from SPARC-null (*white bars*) and WT fibroblasts (*black bars*) demonstrated decreased levels of collagen associated with SPARC-null cells at 1, 2, and 5 days after ascorbate addition. Amounts of hydroxyproline in detergent-insoluble fractions per total protein in detergent-soluble fractions in SPARC-null fibroblasts were normalized to WT values assessed in parallel cultures plated at equal density. *Bars* denote mean ± S.E.

[^{3}H]proline. Labeled collagen produced by WT (+/+) and null (−/−) cells was apparent in conditioned media, detergent-soluble, and detergent-insoluble cell layers. Quantification from 7 separate experiments performed with different cell preparations showed a consistent increase in collagen I associated with SPARC-null detergent-soluble cell layers that was accompanied by a decrease in the amounts of collagen I in the media, in contrast to the distribution of collagen observed in WT fibroblasts (Fig. 2*B*). As detergent-insoluble fractions did not completely dissolve in boiling Laemmli buffer, we relied on hydroxyproline analysis (see below) for quantification of collagen in detergent-insoluble cell extracts.

To characterize further the association of collagen I with separate cellular fractions, we performed hydroxyproline analysis of detergent-extracted WT and SPARC-null cells. As shown in Fig. 3*A*, the percent of hydroxyproline normalized to total protein (Hyp/total protein) associated with detergent-soluble layers was higher in the absence of SPARC after 4 h of collagen secretion. In contrast, the percent of Hyp/total protein in detergent-insoluble extracts was reduced in SPARC-null *versus* WT cells.

Deposition and accumulation of ECM occur over several days in cultured cells. We sought to determine whether initial increases in cell-associated collagen influenced deposition of collagen by SPARC-null cells over a 5-day time period. Hydroxyproline analysis carried out on cell layers from WT and SPARC-null fibroblasts cultured for 1, 2, and 5 days after ascorbate addition indicated that initial increases in cell-associated collagen did not result in increased collagen deposition. A significant decrease in collagen accumulation in detergent-insoluble cell layers in null *versus* WT cells was observed (Fig. 3*B*). The amounts of collagen were decreased in conditioned media from SPARC-null fibroblasts in comparison to WT levels at each day (data not shown).

We conclude from these results that in the absence of SPARC, procollagen I initially associated with cell surfaces to a greater extent than procollagen I produced concomitantly with SPARC. However, SPARC-null fibroblasts did not incorporate collagen as efficiently into an insoluble matrix.

Enhanced Procollagen Processing in the Absence of SPARC—As procollagen processing is a critical component of collagen I ECM assembly, we asked whether SPARC influenced procollagen I processing *in vitro*. Fig. 4 is a representative immunoblot from conditioned media and detergent-soluble cell layers col-

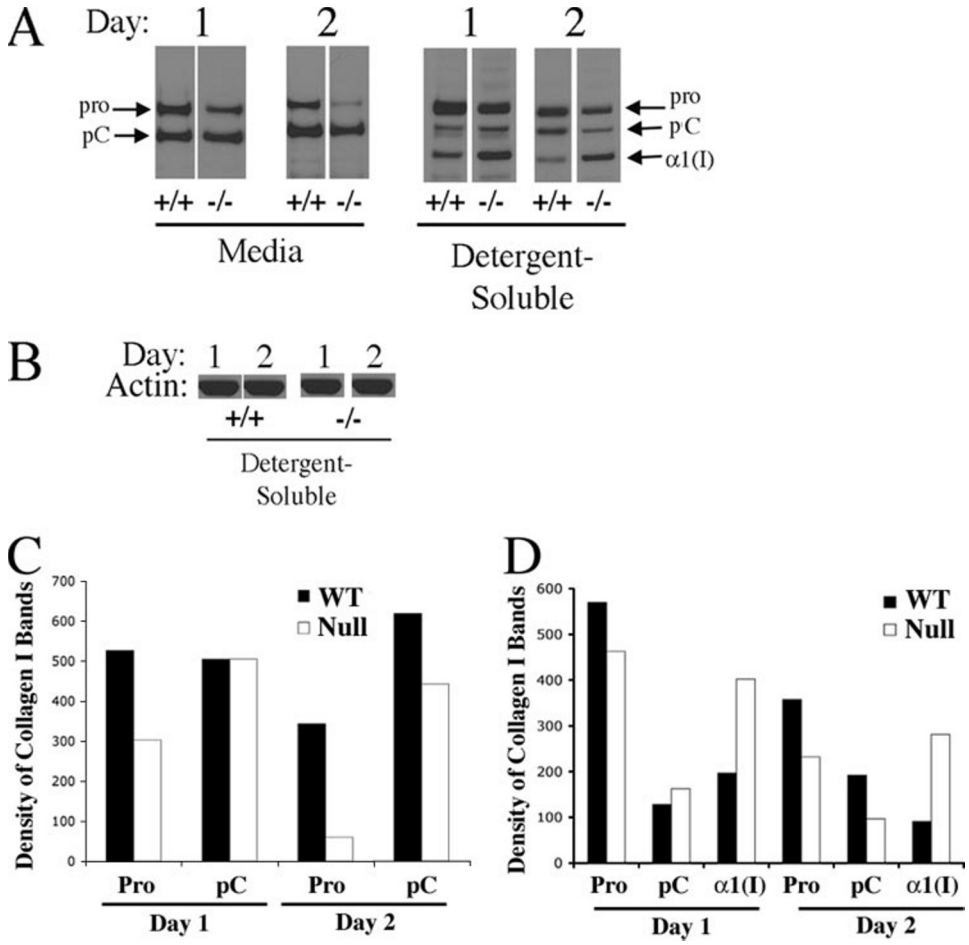

FIGURE 4. **Increases in processed collagen α1(I) were detected in SPARC-null cells and conditioned media.** *A*, immunoblot analysis using anti-collagen I antibodies showed elevated levels of processed collagen intermediate pC over procollagen I (*pro*: *arrows*) in media conditioned by SPARC-null *versus* WT cells. Detergent-soluble SPARC-null (−/−) cell layers exhibited increased amounts of fully processed α1(I) (α1(I): *arrows*) in comparison to WT (+/+) cells that contained higher levels of procollagen I (*pro*: *arrows*). Parallel cultures of null and WT cells from days 1 and 2 following addition of ascorbate are shown. *B*, actin levels in detergent-soluble fractions confirm equal cellular contribution from each genotype. *C*, quantification of band intensities from *A* exhibited a higher proportion of collagen I present as pC in SPARC-null media fractions (*white bars*) than in WT media (*black bars*). *D*, quantification of band intensities from *A* demonstrated that SPARC-null detergent-soluble fractions (*white bars*) contained higher amounts of processed collagen α1(I) at days 1 and 2 in comparison to WT fractions (*black bars*). *Pro*, procollagen α1(I); *pC*, pC-collagen α1(I); *α1(I)*, mature collagen α1(I).

lected 1 and 2 days after addition of ascorbate. The polyclonal antibody used in these experiments displayed a significant preference for the α1(I) subunit of collagen I, such that the procollagen α2(I) and its processed chains were not apparent. Three distinct bands that represent procollagen α1(I), pC α(I) (procollagen α1(I) with the N-propeptide removed), and collagen α1(I) were evident in detergent-soluble extracts. Procollagen α1(I) and pC α1(I) exhibit greater solubility in comparison with pN α1(I) and collagen α1(I) (21). Accordingly, conditioned media from SPARC-null and WT fibroblasts contained predominantly procollagen α1(I) and pC α1(I). However, a higher proportion of collagen I was present as pC α1(I) in SPARC-null *versus* WT conditioned media. Furthermore, an increase in the amounts of processed collagen α1(I) was also apparent in detergent-soluble cell layers from SPARC-null fibroblasts. Differences detectable at day 1 were more substantial at day 2 (Fig. 4*A*). The identity of pC collagen I was confirmed with antibodies against the C-propeptide of collagen I (LF41, data not shown) (16). The lack of detectable pN collagen I in detergent-soluble cell layers is most likely due to incorporation of pN collagen I into the detergent-insoluble fraction, which is difficult to solubilize and resolve by SDS-PAGE. Quantification of band intensity associated with collagen in the media (Fig. 4*C*) and in the detergent-soluble fraction (Fig. 4*D*) is shown.

An enhancement of procollagen I processing is expected to result in the production of greater amounts of cleaved propeptides in conditioned media. Immunoprecipitation was carried out with antibodies against the N-propeptide of collagen I to determine whether increased amounts of cleaved N-propeptide were observed in media conditioned by SPARC-null fibroblasts. A representative experiment is shown in Fig. 5*A*. Although a slight decrease in amounts of collagen I was observed in the starting material of SPARC-null *versus* WT conditioned media, increased amounts of N-propeptide were consistently found associated with SPARC-null conditioned media. As shown in Fig. 5*B*, quantification of three separate immunoprecipitation experiments with different primary cell isolates confirmed a reproducible increase in processed N-propeptide in media conditioned by SPARC-null *versus* WT fibroblasts.

We performed pulse-chase experiments to determine whether the absence of SPARC influenced procollagen I processing and/or whether longer periods of time were required to detect differences in procollagen I conversion in SPARC-null fibroblasts. A representative pulse-chase experiment is shown in Fig. 6*A*. Whereas mature collagen I was detected in SPARC-null cell layers at 36 min of chase, mature collagen I in WT cells was not detectable until 60 min of chase. An immunoblot analysis with anti-collagen I antibodies is shown in Fig. 6*A*, *lower panel*, to demonstrate that approximately equal amounts of total collagen I were present in SPARC-null and WT cells. The immunoblot also demonstrated the increase in processed collagen I associated with SPARC-null cells. Fig. 6*B* shows quantification from three separate experiments demonstrating that less time following pulse labeling was required to detect processed collagen I in SPARC-null *versus* WT cells. We conclude that SPARC diminishes procollagen I binding to cell surfaces and functions as a regulatory component in the conversion of procollagen I to collagen I.

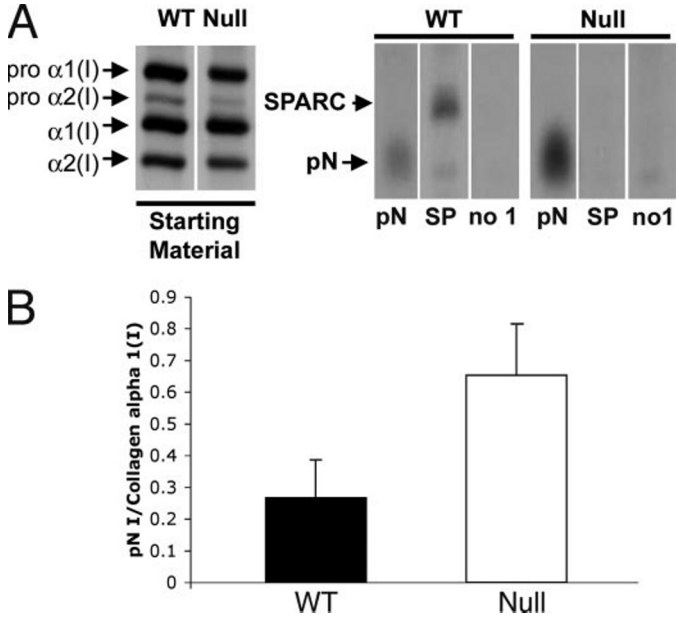

FIGURE 5. **Increased levels of cleaved N-propeptide of collagen α1(I) were detected in SPARC-null conditioned media.** *A*, a representative experiment in which media conditioned for ~24 h by metabolically labeled WT and SPARC-null cells was immunoprecipitated with anti-SPARC (*SP*) or anti-N-propeptide (*pN*) antibodies or no primary antibody (*no 1*). *B*, quantification of the density of immunoprecipitated N-propeptide (as detected by fluorography) relative to collagen α1(I) from three separate experiments is shown. A reproducible elevation in levels of N-propeptide of collagen α1(I) was found associated with null- (*white bars*) *versus* WT- (*black bars*) conditioned media. *Bars* denote mean ± S.E.

rSPARC Reverses Increases in Cell-associated Collagen I and in Collagen I Processing in SPARC-null Cells—We asked whether restoration of SPARC activity to SPARC-null fibroblasts reversed the differences in procollagen conversion in SPARC-null cells. Addition of recombinant SPARC (rSPARC) to SPARC-null fibroblasts led to an appreciable decrease in procollagen I associated with null cell layers (Fig. 7*A*, compare *lane 3* (−*rSPARC*) to *lane 4* (+*rSPARC*)). Coincident with a decrease in overall amounts of cell-associated collagen I, a significant inhibition of procollagen processing was evident upon addition of rSPARC.

Quantification from three separate experiments is shown in Fig. 7, *B* and *C*. We consistently recorded a decrease in collagen I in detergent-soluble SPARC-null cell layers treated with rSPARC, in comparison to that of control null cells treated with equal amounts of nonspecific protein (ovalbumin, Fig. 7*B*). In addition, the proportion of collagen present as processed collagen α1(I) in detergent-soluble fractions was significantly decreased by addition of rSPARC (Fig. 7*C*). The decrease in amounts of procollagen I converted into collagen I was accompanied by a decrease in the levels of collagen I associated with SPARC-null cell layers.

Fiber Formation on SPARC-null Versus WT Fibroblasts—Immunofluorescence studies to detect collagen fibers on the surface of WT and SPARC-null dermal fibroblasts were carried out to discern whether the differences seen in collagen I processing influenced collagen fiber formation *in vitro*. At 1 (Fig. 8, *C* and *D*) and 2 h (*E* and *F*) after addition of ascorbate, more collagen immunoreactivity was associated with SPARC-null cells (*D* and *F*) in comparison to WT cells (*C* and *E*). These results are consistent with the initial increases in cell-associated collagen as described in Fig. 2.

After addition of ascorbate for 1 day, collagen fibers were apparent on WT and SPARC-null fibroblasts (Fig. 9, *A* and *B*). However, 3 days after treatment with ascorbate, collagen fibers on the surface of SPARC-null fibroblasts (Fig. 9*D*) were diminished, whereas those associated with WT cells persisted (Fig. 9*C*). Collagen fibers on WT fibroblasts at day 3 appeared to have increased staining over those present at day 1. We conclude that collagen fibers on WT cells continued to develop with time as expected, whereas collagen fibers formed in the absence of SPARC did not persist.

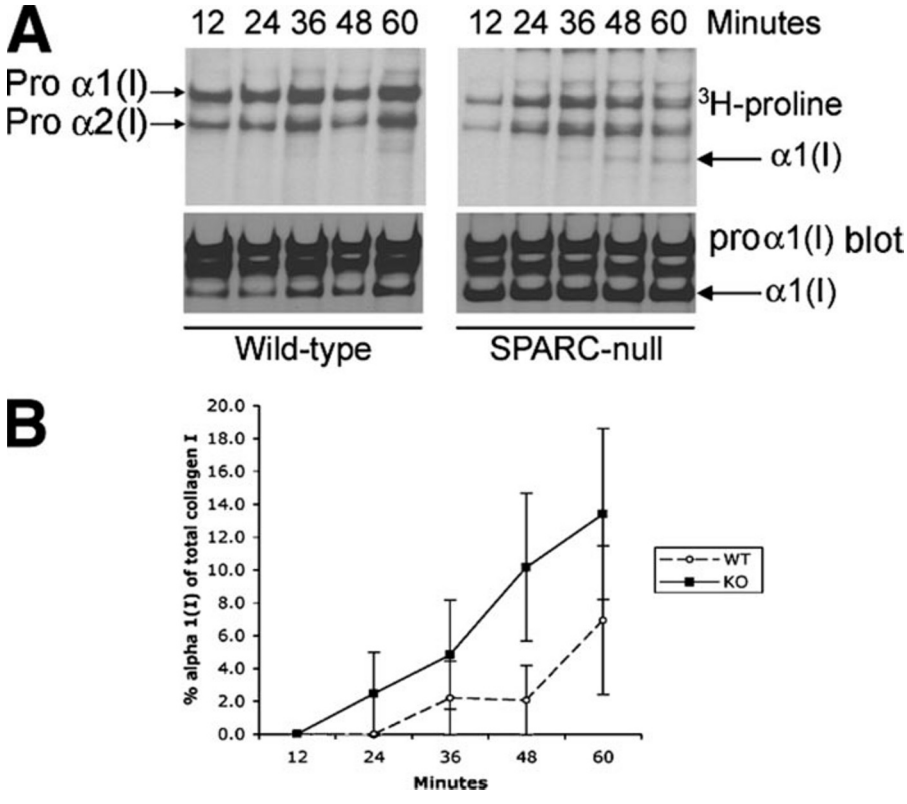

FIGURE 6. **Processed α1(I) was found in SPARC-null cells at earlier time points after pulse labeling than in WT cells plated at equal density.** *A*, equal numbers of WT and SPARC-null cells were pulsed for 12 min with [^{3}H]proline and subsequently chased with unlabeled proline, and cell layers were collected at 12-min intervals. An immunoblot showing total collagen α1(I) is shown in the *lower panel*. *B*, quantification of fluorography from three separate pulse-chase experiments showed that processed α1(I) was reproducibly detected in greater amounts in SPARC-null cell layers (*dashed line*) as early as 24 min after pulse labeling, whereas lesser amounts of processed α1(I) were associated with WT cells (*solid line*).

DISCUSSION

In this study, we have found that collagen fibrils generated in the

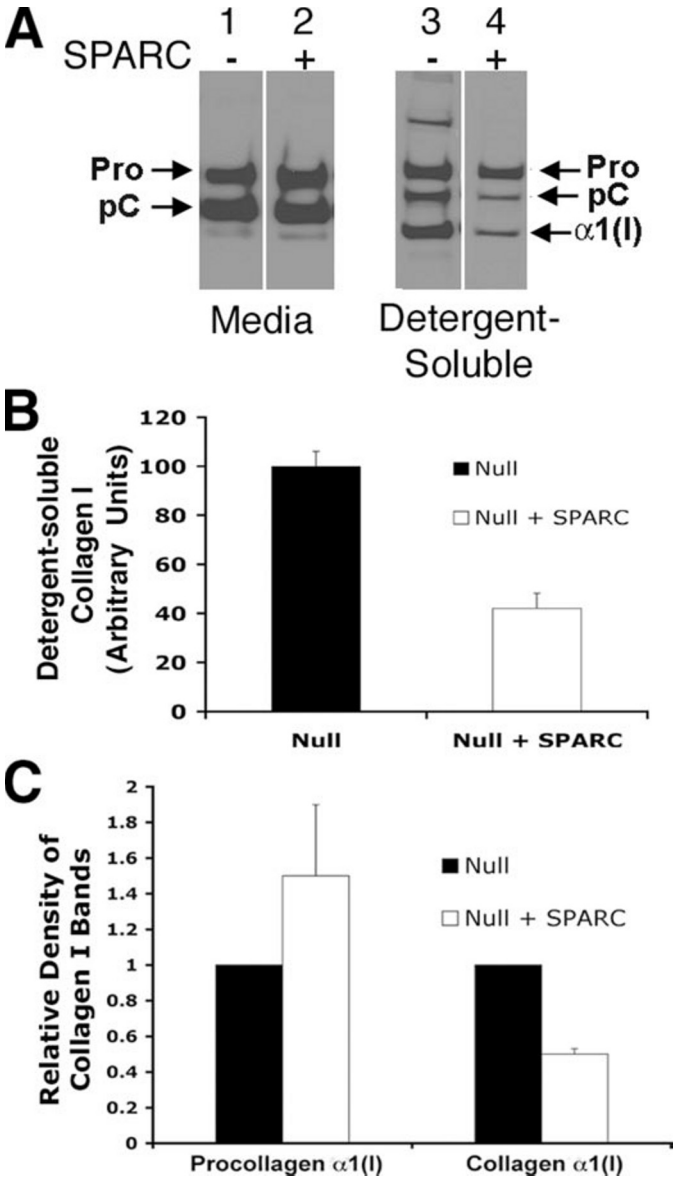

FIGURE 7. **rSPARC rescues the increases in the cellular association and processing of procollagen in SPARC-null cells.** *A*, addition of rSPARC to SPARC-null cultures reversed the increase in processed collagen α1(I) (*arrows*). Equal numbers of SPARC-null cells were cultured with rSPARC (+) or with ovalbumin (−), and media and cell layers were collected 1 day after addition of ascorbate. Levels of procollagen (*pro*: *arrows*) and processed collagen α1(I) (*pC*, *α1(I)*) were detected by immunoblot analysis of conditioned media (*lanes 1* and *2*) and cell layers (*lanes 3* and *4*). *B* and *C*, quantification of immunoblot analyses from three separate experiments demonstrated a consistent reduction in cell-associated collagen I coincident with addition of rSPARC and a reproducible decrease in procollagen I processing upon addition of rSPARC to SPARC-null cells. *Bars* are mean ± S.E.; *white bars* indicate addition of rSPARC. $p = 0.02$ in *B*.

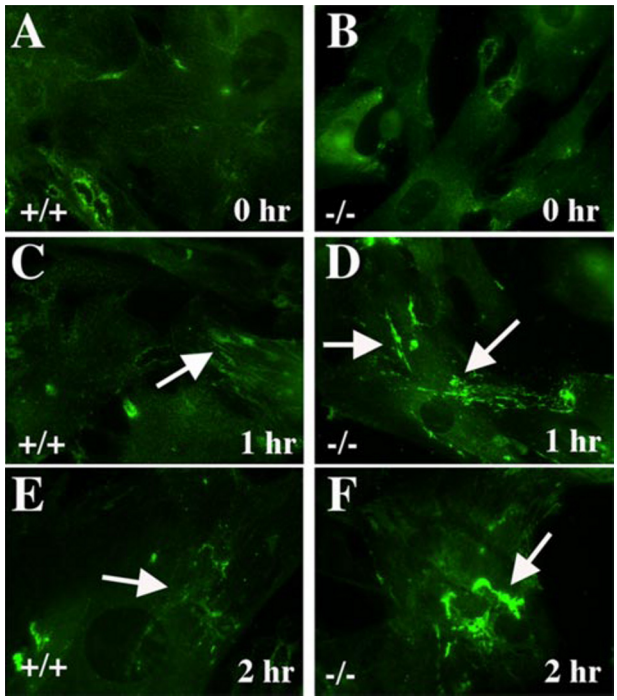

FIGURE 8. **Collagen fibers on SPARC-null cells are increased at early times after addition of ascorbate.** Collagen fibers visible on SPARC-null cells (−/−, *D*) 1 h after addition of ascorbate exhibited greater intensity than those on WT cells (+/+, *C*) at 1 h. Similar differences were observed at 2 h (*F* and *E*). Significant collagen I immunoreactivity was not detected on either cell type prior to addition of ascorbate (*A* and *B*). *Arrows* indicate collagen fibers. These fields are representative of at least 10 fields generated from three separate cell preparations.

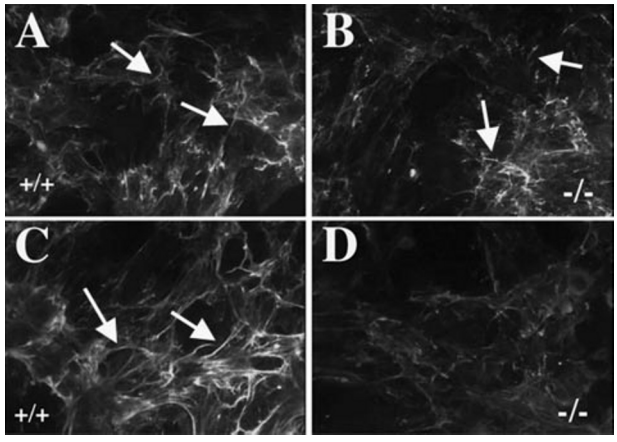

FIGURE 9. **Collagen I fibers formed on SPARC-null cell surfaces are not stable.** By 1 day (*A* and *B*) after addition of ascorbate, SPARC-null cells (−/−, *B*) displayed a distribution of collagen fibers similar to that of WT (+/+, *A*). However, at day 3 (*C* and *D*), collagen fibers on WT cells (*C*) appeared more numerous or robust, whereas collagen fibers associated with SPARC-null cells (*D*) were reduced in number and staining intensity. *Arrows* indicate collagen fibers. These fields are representative of at least 10 fields generated from three separate cell preparations.

absence of SPARC in mice at 1 month of age appeared to be stalled at a stage of fibril aggregation and accumulated as proposed intermediates in fibril fusion (22). Previous studies demonstrated a substantial decrease in amounts of collagen in SPARC-null dermis (12). This observation suggested to us that SPARC was essential for collagen fibril growth and deposition in skin. To elucidate the cellular basis of the SPARC-null collagen phenotype, we investigated collagen I production in SPARC-null dermal fibroblasts. We have shown, by a number of different techniques, that SPARC-null dermal fibroblasts exhibited an increased association of collagen I with cell layers and enhanced processing of procollagen α1(I) to collagen α1(I). The processed collagen I was not efficiently incorporated into SPARC-null detergent-insoluble cell layers, and collagen fibers that formed on SPARC-null cells did not persist to the same extent as those on WT cells. These results indicate that regulation of collagen association with cell surfaces by SPARC is crit-

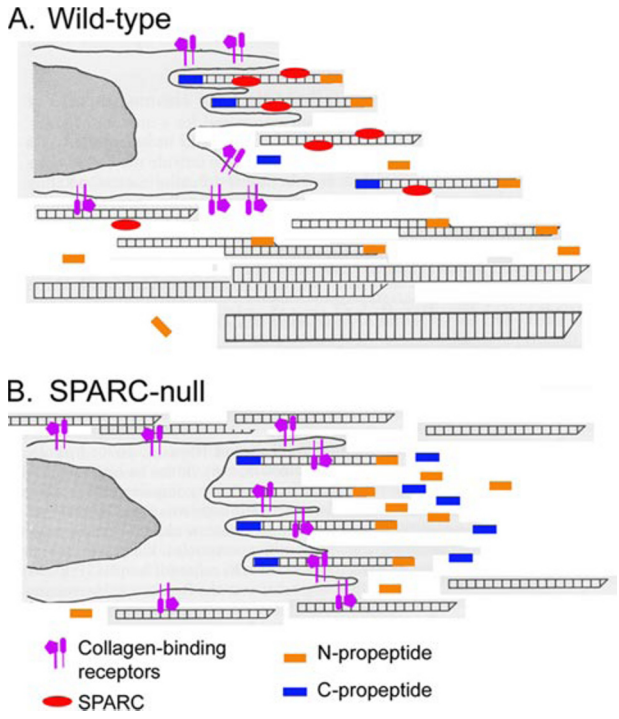

FIGURE 10. **SPARC activity in procollagen processing.** In *A*, procollagen fibrils are bound by SPARC, which diminishes collagen engagement by cell-surface receptors. In the absence of SPARC (*B*), procollagen interacts with receptors to a greater degree and is tethered to cell surfaces. Propeptide cleavage is enhanced in the pericellular milieu. SPARC-null fibrils fail to aggregate as efficiently as fibrils on WT cells.

ical for appropriate procollagen processing and formation of stable collagen fibers. A schematic of our current hypothesis of SPARC activity in procollagen I maturation is shown in Fig. 10.

Our data are consistent with a function for SPARC in the diminishment of procollagen I engagement of cell surface receptors. Recently, the binding sites of SPARC on collagen I were determined by atomic force microscopy and were found to overlap or to be in close proximity to those previously identified as collagen-binding integrin sites (23, 24). $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the best characterized collagen receptors expressed by dermal fibroblasts (25). We predict that collagen I bound by SPARC presents a poor substrate for engagement by $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins by virtue of steric hindrance. We suggest that during active deposition of collagen I by fibroblasts, engagement of integrin receptors is counterproductive because $\alpha_1\beta_1$ signals inhibition of collagen I expression, and $\alpha_2\beta_1$ increases collagenase production in dermal fibroblasts (26, 27). SPARC might therefore function to decrease integrin engagement and signaling that interfere with collagen deposition. Alternatively, SPARC bound to distinct sites on procollagen I might direct integrin binding to specific sites that enhance matrix assembly. Nonetheless, we hypothesize that, in the absence of SPARC, collagen is overly engaged by integrin receptors and is rapidly turned over at the cell surface (Fig. 10). As $\alpha_2\beta_1$ promotes phagocytosis of collagen I, a plausible explanation for the reduction in collagen I incorporation into the ECM, which we have observed *in vitro* and *in vivo* in the absence of SPARC, is enhanced procollagen conversion mediated by increased integrin engagement favoring collagen I uptake rather than incorporation into ECM (28).

We do not observe significant differences in procollagen processing in conditioned media generated from metabolic labeling experiments, a finding that we attribute to the timing of secretion of the labeled procollagens. Upon addition of ascorbate, accumulated procollagen within intracellular organelles will be secreted prior to newly synthesized, labeled procollagen. We postulate that cell binding sites for procollagen will bind unlabeled procollagen released prior to labeled procollagen. Hence, we predict that differences in procollagen conversion are more readily detected by immunoblot analysis, which represents the entire pool of secreted procollagen, rather than by fluorography of labeled procollagen, which detects only a portion of the secreted pool.

The C-propeptide of collagen I directs in intracellular trimer formation in the endoplasmic reticulum (9). Removal of the C-propeptide by bone morphogenic protein-1 (or related tolloid family members) is thought to be a critical step in collagen I deposition, as its removal decreases the solubility of procollagen I to a level similar to that of processed collagen I (21, 29). Although association of pC collagen I with cell layers is not observed in some cell types, Lamande and Bateman (30) reported that mutations that nullified glycosylation of the C-propeptide in primary murine fibroblasts resulted in significant increases in pC collagen I in the media of cells expressing the mutant form of collagen I *versus* WT collagen I with fully glycosylated C-propeptide. Hence, glycosylation of the C-propeptide of collagen I increased interactions with cell surfaces. We found higher levels of pC relative to pN collagen I in detergent-soluble cell layers produced by primary dermal fibroblasts. We predict that the majority of pN collagen I resides in the detergent-insoluble fraction.

Whereas pC collagen I is not thought to be included in collagen fibrils, pN collagen I incorporation in growing fibrils has been observed in developing skin and tendon by EM immunolocalization (10, 31). Incorporation of pN collagen I might limit collagen fibril diameter, such that regulation of N-propeptide removal by ADAMTS-2 (or related family members ADAMTS-3 and -13) might control further fibril aggregation and growth (32, 33). In the event that N-propeptide functions as a facilitator of collagen fibril accretion, removal of N-propeptide prior to fibril incorporation would be expected to result in smaller collagen fibrils (11). Interestingly, SPARC-null dermis exhibited small collagen fibril diameters at both 1 month and in adults. Hence premature cleavage of N-propeptide resulting in inefficient collagen fibril fusion would be consistent with a function of SPARC in modulation of procollagen I processing at the cell surface. We consider the most likely explanation for increased processing in SPARC-null cells to be an increase in receptor engagement of procollagen I that retains procollagen in the pericellular milieu, in which N- and C-propeptide proteinases accumulate. Greater exposure of procollagen I tethered by receptors in the pericellular space enhances processing and perhaps subsequent phagocytosis.

Recently, SPARC has been reported to affect integrin-linked kinase activity in lung fibroblasts (34). SPARC-null fibroblasts exhibited reduced integrin-linked kinase activity that resulted in decreased fibronectin assembly and a reduction in fibronectin-induced stress fiber formation. As Barker *et al.* (34) did not

induce collagen production by addition of ascorbate, the effects of SPARC on collagen production in these cells were not assessed. We have also observed a slight reduction in fibronectin fibrils on the surface of SPARC-null dermal fibroblasts in the absence of ascorbate (data not shown). However, differences in fibronectin fibrils were not detectable between SPARC-null and WT fibroblasts in the presence of ascorbate (data not shown). As collagen and fibronectin fibril assembly have been shown to be coincident *in vitro*, collagen I production by SPARC-null fibroblasts at early times after ascorbate addition might mask alterations in fibronectin assembly (35, 36). Possibly, a diminished capacity to assemble fibronectin fibrils over time contributes to the decreased stability of collagen fibers on SPARC-null cell surfaces observed at times >3 days (Fig. 9).

SPARC-null mice develop cataracts that appear to be based, at least in part, on aberrant ECM assembly by lens epithelial cells (37). SPARC might function to reduce lens epithelial cell binding to collagen IV, another SPARC-binding collagen. Immunohistochemistry of lens basement membrane from SPARC-null mice demonstrated disorganized collagen IV associated with β_1 integrin-positive cellular protrusions into the matrix (38). Failure of lens epithelial cells to release collagen IV from β_1 integrin engagement might contribute to the disorganized basal lamina in SPARC-null lens.

Recently, Canty and Kadler (39) reported that some procollagen I conversion to collagen I takes place in intracellular compartments within embryonic tendon. We cannot rule out that some processing of procollagen I in our dermal fibroblast cultures might take place within cells. As SPARC shares characteristics with heat shock proteins, the possibility that SPARC might function in the capacity of a chaperone is conceivable. In this case, procollagen I produced in the absence of SPARC might exhibit properties distinct from those of WT procollagen I that influence cell surface association. Notably, in assays of protein folding, addition of SPARC resulted in disulfide rearrangement of a model substrate that was consistent with chaperone-like activity (40). However, SPARC has a secretory signal peptide, contains no recognizable endoplasmic reticulum or Golgi apparatus retention sequences, and is readily detected in conditioned media from a number of different cell types (3). In the event that SPARC does perform a chaperone-like function for procollagen I (and/or other ECM proteins), SPARC is likely to be secreted with procollagen I.

A growing number of extracellular proteins are known to participate in collagen fibril formation. Decorin, lumican, fibromodulin, biglycan, and mimecan regulate collagen fibril diameters, presumably through binding of these proteoglycans to collagen fibrils in the extracellular space, where they serve either to inhibit or promote fibril aggregation (41–44). In addition, disruption of the genes encoding the matricellular proteins tenascin X, thrombospondin 2, osteopontin, dermatopontin, and hevin have revealed a function for these proteins in collagen fibril formation (45–49). In the majority of cases, decreased expression of the aforementioned collagen fibril-effector proteins resulted in increased collagen fibril diameters with irregular profiles, in contrast to SPARC-null collagen fibrils that are small and regular in diameter. SPARC is the first reported collagen fibril effector protein to influence procollagen I processing events.

SPARC expression is often associated with fibrosis. Recently, Zhou *et al.* (50) reported that reduction of SPARC expression in scleroderma fibroblasts by RNA interference was sufficient to reduce collagen expression by these cells that otherwise exhibit increased expression of SPARC and collagen I, in comparison to normal fibroblasts. SPARC therefore is an attractive target for the development of strategies to counteract fibrotic deposition of collagen. We postulate that disruptions in procollagen I processing influenced by SPARC result in decreased stability of collagen I fibrils. SPARC is therefore identified as an essential modulator of collagen I-cell interaction that regulates the deposition of stable collagen I fibrils in the extracellular space.

Acknowledgments—We express appreciation to Juliet Carbon, Carrie Murri, and Gail Workman for expert technical assistance.

REFERENCES

1. Bornstein, P. (1995) *J. Cell Biol.* **130,** 503–506
2. Bornstein, P., and Sage, E. H. (2002) *Curr. Opin. Cell Biol.* **14,** 608–616
3. Brekken, R. A., and Sage, E. H. (2001) *Matrix Biol.* **19,** 816–827
4. Bradshaw, A. D., and Sage, E. H. (2001) *J. Clin. Investig.* **107,** 1049–1054
5. Frizell, E., Liu, S. L., Abraham, A., Ozaki, I., Eghbali, M., Sage, E. H., and Zern, M. A. (1995) *Hepatology* **21,** 847–854
6. Kuhn, C., and Mason, R. J. (1995) *Am. J. Pathol.* **147,** 1759–1769
7. Pichler, R. H., Hugo, C., Shankland, S. J., Reed, M. J., Bassuk, J. A., Andoh, T. F., Lombardi, D. M., Schwartz, S. M., Bennett, W. M., Alpers, C. E., Sage, E. H., Johnson, R. J., and Couser, W. G. (1996) *Kidney. Int.* **50,** 1978–1989
8. Vuorio, T., Kahari, V. M., Black, C., and Vuorio, E. (1991) *J. Rheumatol.* **18,** 247–251
9. Prockop, D. J., and Kivirikko, K. I. (1995) *Annu. Rev. Biochem.* **64,** 403–434
10. Fleischmajer, R., Timpl, R., Tuderman, L., Raisher, L., Wiestner, M., Perlish, J. S., and Graves, P. N. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7360–7364
11. Watson, R. B., Holmes, D. F., Graham, H. K., Nusgens, B. V., and Kadler, K. E. (1998) *J. Mol. Biol.* **278,** 195–204
12. Bradshaw, A. D., Puolakkainen, P., Dasgupta, J., Davidson, J. M., Wight, T. N., and Sage, E. H. (2003) *J. Investig. Dermatol.* **120,** 949–955
13. Delany, A. M., Kalajzic, I., Bradshaw, A. D., Sage, E. H., and Canalis, E. (2003) *Endocrinology* **144,** 2588–2596
14. Taneda, S., Pippin, J. W., Sage, E. H., Hudkins, K. L., Takeuchi, Y., Couser, W. G., and Alpers, C. E. (2003) *J. Am. Soc. Nephrol.* **14,** 968–980
15. Bradshaw, A. D., Bassuk, J. A., Francki, A., and Sage, E. H. (2000) *Mol. Cell. Biol. Res. Commun.* **3,** 345–351
16. Fisher, L. W., Stubbs, J. T., 3rd, and Young, M. F. (1995) *Acta Orthop. Scand. Suppl.* **266,** 61–65
17. Norose, K., Clark, J. I., Syed, N. A., Basu, A., Heber-Katz, E., Sage, E. H., and Howe, C. C. (1998) *Investig. Ophthalmol. Vis. Sci.* **39,** 2674–2680
18. Francki, A., Bradshaw, A. D., Bassuk, J. A., Howe, C. C., Couser, W. G., and Sage, E. H. (1999) *J. Biol. Chem.* **274,** 32145–32152
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685
20. Woessner, J. F., Jr. (1961) *Arch. Biochem. Biophys.* **93,** 440–447
21. Prockop, D. J., and Hulmes, D. J. S. (1994) *Assembly of Collagen Fibrils de novo from Soluble Precursors*: *Polymerization and Copolymerization of Procollagen*, *pN-collagen*, *and Mutated Collagens*, Academic Press, San Diego, CA
22. Birk, D. E., Zycband, E. I., Woodruff, S., Winkelmann, D. A., and Trelstad, R. L. (1997) *Dev. Dyn.* **208,** 291–298
23. Wang, H., Fertala, A., Ratner, B. D., Sage, E. H., and Jiang, S. (2005) *Anal. Chem.* **77,** 6765–6771
24. Xu, Y., Gurusiddappa, S., Rich, R. L., Owens, R. T., Keene, D. R., Mayne, R., Höök, A., and Höök, M. (2000) *J. Biol. Chem.* **275,** 38981–38989
25. Heino, J. (2000) *Matrix Biol.* **19,** 319–323

26. Gardner, H., Broberg, A., Pozzi, A., Laato, M., and Heino, J. (1999) *J. Cell Sci.* **112,** 263–272
27. Ravanti, L., Heino, J., Lopez-Otin, C., and Kahari, V. M. (1999) *J. Biol. Chem.* **274,** 2446–2455
28. Lee, W., Sodek, J., and McCulloch, C. A. (1996) *J. Cell Physiol.* **168,** 695–704
29. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) *Science* **271,** 360–362
30. Lamande, S. R., and Bateman, J. F. (1995) *J. Biol. Chem.* **270,** 17858–17865
31. Fleischmajer, R., Perlish, J. S., Timpl, R., and Olsen, B. R. (1988) *J. Histochem. Cytochem.* **36,** 1425–1432
32. Colige, A., Sieron, A. L., Li, S. W., Schwarze, U., Petty, E., Wertelecki, W., Wilcox, W., Krakow, D., Cohn, D. H., Reardon, W., Byers, P. H., Lapiere, C. M., Prockop, D. J., and Nusgens, B. V. (1999) *Am. J. Hum. Genet.* **65,** 308–317
33. Colige, A., Vandenberghe, I., Thiry, M., Lambert, C. A., Van Beeumen, J., Li, S. W., Prockop, D. J., Lapiere, C. M., and Nusgens, B. V. (2002) *J. Biol. Chem.* **277,** 5756–5766
34. Barker, T. H., Baneyx, G., Cardo-Vila, M., Workman, G. A., Weaver, M., Menon, P. M., Dedhar, S., Rempel, S. A., Arap, W., Pasqualini, R., Vogel, V., and Sage, E. H. (2005) *J. Biol. Chem.* **280,** 36483–36493
35. Li, S., Van Den Diepstraten, C., D'Souza, S. J., Chan, B. M., and Pickering, J. G. (2003) *Am. J. Pathol.* **163,** 1045–1056
36. Velling, T., Risteli, J., Wennerberg, K., Mosher, D. F., and Johansson, S. (2002) *J. Biol. Chem.* **277,** 37377–37381
37. Yan, Q., Clark, J. I., Wight, T. N., and Sage, E. H. (2002) *J. Cell Sci.* **115,** 2747–2756
38. Yan, Q., Blake, D., Clark, J. I., and Sage, E. H. (2003) *J. Histochem. Cytochem.* **51,** 503–511
39. Canty, E. G., and Kadler, K. E. (2005) *J. Cell Sci.* **118,** 1341–1353
40. Emerson, R. O., Sage, E. H., Ghosh, J. G., and Clark, J. I. (2006) *J. Cell Biochem.* **98,** 701–705
41. Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E., and Iozzo, R. V. (1997) *J. Cell Biol.* **136,** 729–743
42. Ezura, Y., Chakravarti, S., Oldberg, A., Chervoneva, I., and Birk, D. E. (2000) *J. Cell Biol.* **151,** 779–788
43. Corsi, A., Xu, T., Chen, X. D., Boyde, A., Liang, J., Mankani, M., Sommer, B., Iozzo, R. V., Eichstetter, I., Robey, P. G., Bianco, P., and Young, M. F. (2002) *J. Bone Miner. Res.* **17,** 1180–1189
44. Tasheva, E. S., Koester, A., Paulsen, A. Q., Garrett, A. S., Boyle, D. L., Davidson, H. J., Song, M., Fox, N., and Conrad, G. W. (2002) *Mol. Vis.* **8,** 407–415
45. Mao, J. R., Taylor, G., Dean, W. B., Wagner, D. R., Afzal, V., Lotz, J. C., Rubin, E. M., and Bristow, J. (2002) *Nat. Genet.* **30,** 421–425
46. Kyriakides, T. R., Zhu, Y. H., Smith, L. T., Bain, S. D., Yang, Z., Lin, M. T., Danielson, K. G., Iozzo, R. V., LaMarca, M., McKinney, C. E., Ginns, E. I., and Bornstein, P. (1998) *J. Cell Biol.* **140,** 419–430
47. Liaw, L., Birk, D. E., Ballas, C. B., Whitsitt, J. S., Davidson, J. M., and Hogan, B. L. (1998) *J. Clin. Investig.* **101,** 1468–1478
48. Takeda, U., Utani, A., Wu, J., Adachi, E., Koseki, H., Taniguchi, M., Matsumoto, T., Ohashi, T., Sato, M., and Shinkai, H. (2002) *J. Investig. Dermatol.* **119,** 678–683
49. Sullivan, M. M., Barker, T. H., Funk, S. E., Karchin, A., Seo, N. S., Hook, M., Sanders, J., Starcher, B., Wight, T. N., Puolakkainen, P., and Sage, E. H. (2006) *J. Biol. Chem.* **281,** 27621–27632
50. Zhou, X., Tan, F. K., Guo, X., and Arnett, F. C. (2006) *Arthritis. Rheum.* **54,** 2626–2631