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ARTICLES

SPARC Regulates TGF-beta1-Dependent Signaling in Primary Glomerular Mesangial Cells

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Abstract Secreted protein acidic and rich in cysteine (SPARC), a member of the family of matricellular proteins, regulates the interaction of cells with pleiotropic factors and proteins of the extracellular matrix (ECM). Although it has been appreciated that transforming growth factor beta 1 (TGF-β1) induces SPARC and collagen type I, we have recently shown that SPARC regulates the expression of TGF-β1 and collagen type I in renal mesangial cells via a TGF-β1-dependent pathway, and have proposed a reciprocal, autocrine regulatory feedback loop between SPARC and TGF-β1. Herein, we sought to determine how SPARC regulates TGF-β1-dependent signal transduction. Our data indicate that SPARC modulates the TGF-β1-dependent phosphorylation of Smad-2 in primary mesangial cells derived from wild-type and SPARC-null mice. We also show that SPARC regulates the levels and activation of the stress-activated c-jun-N-terminal kinase (JNK) in mesangial cells by augmentation of the stimulatory effects of TGF-β1. Furthermore, we found that SPARC increases the levels and the activity of the transcription factor c-jun. These effects of SPARC on the TGF-β1 signaling pathway appear to be mediated through an interaction with the TGF-β1-receptor complex, but only in the presence of TGF-β1 bound to its cognate type II receptor. That SPARC is directly involved in the regulation of the TGF-β1 signaling cascade is consistent with the paradigm that matricellular proteins modulate interactions among cells, growth factors, and their respective receptors. J. Cell. Biochem. 91: 915–925, 2004.

Key words: SPARC; matricellular; mesangial cells; TGF-β; receptor interactions; transcription factors; Smad; c-jun; JNK

Secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein also known as BM-40 and osteonectin, modulates the interaction of cells with the extracellular matrix (ECM) through its regulation of cell adhesion and matrix assembly [Lane and Sage, 1994; Yan and Sage, 1999]. Specifically, SPARC has been shown to inhibit proliferation [Funk and Sage, 1991], disrupt focal adhesions, and prevent cell spreading in vitro [Motamed and Sage, 1998]. SPARC is known to bind to growth factors [Kupprion et al., 1998] as well as ECM proteins [Sasaki et al., 1998]. Furthermore, it affects the expression of a number of secreted proteins [Francki et al., 1999] including matrix metalloproteinases (MMP) [Tremble et al., 1993] in certain cell types and is thought to modulate interactions between cells and the surrounding ECM at least partially through this activity. The production of SPARC is increased in carcinomas [Porte et al., 1998], in

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atherosclerotic lesions [Raines et al., 1992], and in renal diseases such as passive Heymann nephritis [Floege et al., 1993] and mesangioproliferative glomerulonephritis [Pichler et al., 1996].

The multifunctional growth factor transforming growth factor beta 1 (TGF-β1) belongs to a family of related proteins and that function in physiological processes, which include growth, differentiation, proliferation, tissue remodeling, and wound healing [Pepper, 1997]. Although specific receptors have been found on nearly all mammalian cells, the effects of TGF-β1 differ according to cell type, growth conditions, and concentration of growth factor [Massagué, 1998]. TGF-β1 has been implicated in development and in the remodeling of tissues that takes place during adult life [Frank et al., 1996]. TGF-β1 mediates the formation of ECM via its stimulation of the synthesis of components such as collagen type I. Moreover, it inhibits the degradation of ECM by suppression of MMPs and induction of tissue inhibitors of these enzymes [Poncelet and Schnaper, 1998]. A number of reports have identified TGF-β1 as a critical factor in kidney diseases such as glomerulosclerosis [Gilbert et al., 1998] and glomerulonephritis [Yamamoto et al., 1996].

The objective of this study was to clarify the functions of SPARC in regard to the modulation of the TGF-β signaling cascade in glomerular mesangial cells that play a pivotal role in the exacerbation of glomerular diseases. We found that SPARC modulates TGF-β-dependent phosphorylation of Smad-2, but not of Smad-3, -4, -6, and -7 in primary mesangial cells derived from wild-type and SPARC-null mice. Furthermore, SPARC affects the activity of the stress-activated c-jun-N-terminal kinase (JNK) in mesangial cells by its augmentation of the effects of TGF-β1, and it increases both the expression and phosphorylation of the transcription factor c-jun, but not of c-fos, Creb-1, or SP-1. These effects of SPARC on the TGF-β signaling pathway appear to be mediated through an interaction with the TGF-β-receptor complex in the presence of TGF-β1 bound to its cognate type II receptor. Our results demonstrate a novel function of SPARC in the regulation of proteins of the TGF-β signaling pathway in mesangial cells in vitro and thus support the concept that matricellular proteins modulate interactions between cells and growth factors [Francki et al., 2003]. The data also indicate that SPARC might contribute to the progression of renal, and especially glomerular, disease.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of Murine Glomerular Mesangial Cells

129/SvJ × C57BL/6J wild-type and SPARC-null mice [Francki et al., 1999] were maintained in a pathogen-free facility. Prior to removal of the kidneys, mice were euthanized according to the recommendations of the American Veterinary Association and the US Department of Health and Human Services. The method for the preparation of primary mesangial cells is based on a partial collagenase digestion of isolated glomeruli [Francki et al., 1999]. Mesangial cells were characterized by immunocytochemistry for distinct antigens and were cultured under normal conditions as described in Francki et al. [1999]. All experiments were conducted with mesangial cells at low passage, ranging from three to nine.

Preparation of rhSPARC

Recombinant human (rh)SPARC was prepared in SF9 cells by the use of the baculoviral protein expression system and was collected in serum-free medium as described in Bradshaw et al. [2000]. The rhSPARC had activity similar to that of recombinant SPARC expressed in *E. coli* [Bassuk et al., 1996] and to SPARC synthesized by cultured mammalian cells [Funk and Sage, 1991], as measured by inhibition of proliferation and spreading [Bradshaw et al., 1999].

Preparation of Cellular Protein

Wild-type and SPARC-null mesangial cells were grown to 80% confluence in growth medium [Dulbecco's modified Eagle's medium (55%), F-12 nutrient mixture (20%) (Gibco, Grand Island, NY), fetal bovine serum (FBS) (20%) (Summit Biotechnologies, Stoughton, MA), trace elements (1%) (Biofluids, Inc., Rockville, MD), L-glutamine (2 mM), transferrin (5 μg/ml), insulin (125 U/ml), penicillin G (500 U/ml), streptomycin sulfate (500 U/ml), and amphotericin B (2 μg/ml) (Sigma, St. Louis, MO)], and subsequently cultured under growth-arresting conditions (24 h serum withdrawal) prior to treatment with the respective proteins. Total cellular protein was prepared by dissolution of the cells in 0.5% NP-40 lysis buffer as

described. Protein concentrations were determined by the BCA assay (Pierce Biotechnology, Inc., Rockford IL), according to the manufacturer's recommendation.

Western Blot Analysis

Primary mesangial cells from wild-type and SPARC-null mice were cultured to 80% confluence in growth medium and were deprived of serum for 24 h. Subsequently, the cells were incubated with TGF-β1 (10 ng/ml), rhSPARC (0.9 μM), as well as TGF-β1 and SPARC at these concentrations for 30 min. The cells were lysed as previously described. Equal amounts of protein per lane were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and were electrotransferred onto nitrocellulose membranes. The blots were subsequently blocked in 1% Tween-20 in phosphate buffered saline (PBS) and were incubated with the respective primary antibodies. Immunoreactivity was visualized by incubation of the blot with the respective secondary IgG coupled to horseradish peroxidase, followed by enhanced chemiluminescence. An antibody against β-tubulin was used to control for equal protein loading.

Antibodies

All primary antibodies used were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), (except the antibodies against phospho-Smad-2, which were kindly provided by Dr. C.H. Heldin, Ludwig Cancer Institute, Uppsala, Sweden) and are either specific for, or cross-react with, the mouse protein. Secondary antibodies were obtained from Santa Cruz Biotechnology, Inc., Bio-Rad Laboratories (Hercules, CA), and Kirkegaard & Perry Laboratories (Gaithersburg, MD).

SPARC Binding Experiments

To determine whether SPARC interacts with TGF-β1 and/or the TGF-β-receptor complex, we performed binding studies with recombinant human TGF-β1 as well as a TGF-β-receptor type II protein chimera (R&D Systems, Inc., Minneapolis, MN) that has the extracellular domain of the human TGF-β-receptor type II fused to the Fc region of human IgG1. Biotinylated rhSPARC [Francki et al., 2003] was incubated with TGF-β1 in standard binding buffer, and the complexes were immunoprecipitated with NeutraAvidin beads, transferred onto a PVDF membrane, and subsequently immunoblotted with antibodies specific for SPARC and TGF-β1. rhSPARC was incubated with the chimeric TGF-β-receptor type II in standard binding buffer in the presence or absence of TGF-β1. The complexes were immunoprecipitated with Protein-G beads, separated by SDS–PAGE, transferred onto a PVDF membrane, and subsequently immunoblotted with antibodies specific for SPARC, TGF-β1, or TGF-β-receptor type II.

Statistical Analysis

All immunoblots were scanned and converted to digital computer files with a UMAX S-6E scanner™ and Adobe Photoshop software™. Files were processed and analyzed by NIH Image software™ and are presented as composite figures. The data presented were derived from five independent preparations of mesangial cells isolated from pools of eight kidneys each. Results shown are from one experiment that was representative of four experiments conducted in each independent cell preparation. Variability among experiments was less than 10%.

RESULTS

SPARC Regulates the Activity of the Signal Transduction Adapter Protein Smad-2

We have shown previously that SPARC modulates collagen type I expression in mesangial cells via a TGF-β-dependent pathway [Francki et al., 1999]. To determine how SPARC exerts its effect on TGF-β1 signaling, we monitored one of the earliest activation events along the TGF-β-1-dependent signaling pathway, the phosphorylation of Smad-2 protein. In non-stimulated, growth-arrested mesangial cells from wild-type and SPARC-null mice, the levels of phosphorylated Smad-2 were significantly lower (approx. 70%) in SPARC-null cells in comparison to their wild-type counterparts, whereas the total cellular levels of Smad-2 remained unchanged between wild-type and SPARC-null cells (Fig. 1). To determine whether SPARC has multiple effects in regard to Smad signaling, we also investigated the total cellular protein levels of the receptor-activated Smad-3, the common mediator Smad-4, as well as the inhibitors Smad-6 and -7. There were no apparent differences in any of these proteins between wild-type and SPARC-null mesangial

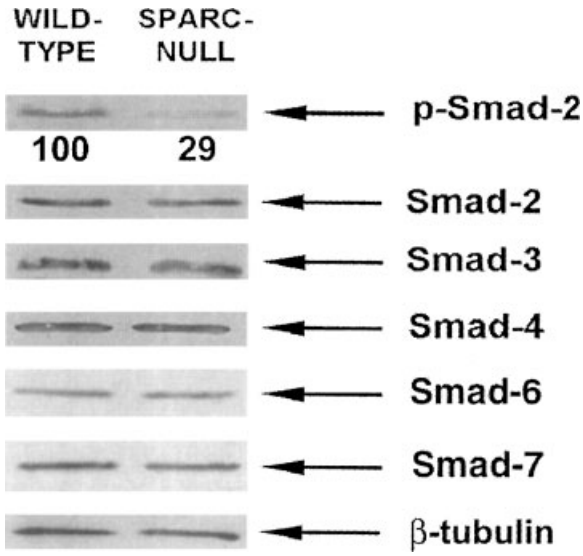

Fig. 1. Secreted protein acidic and rich in cysteine (SPARC)-null mesangial cells display diminished levels of phosphorylated Smad-2. Wild-type and SPARC-null cells were grown in standard growth medium until they were 80% confluent. The cells were washed with PBS and growth-arrested for 24 h under serum-free conditions. Total cellular protein lysates were prepared and equal amounts of protein were subjected to an immunoblot analysis with specific antibodies for Smad-2, phosphorylated Smad-2, -3, -4, -6, and -7. Numbers under each lane denote percent change in level of phosphorylated Smad-2, relative to that in wild-type cells (set at 100%).

cells (Fig. 1). Using RT-PCR analysis, we also evaluated the steady-state mRNA expression levels of the aforementioned Smads and did not find any significant differences between SPARC-null and wild-type mesangial cells (data not shown). As shown in our previous study [Francki et al., 1999], these results indicate that the lack of SPARC leads to a specific perturbation of the phosphorylation of Smad-2, which is a response of TGF-β1 signaling in mesangial cells.

We next asked whether the observed results were a direct effect of SPARC on the Smad signaling pathway or the consequence of the modulatory effect of SPARC on TGF-β1. To determine the effect of SPARC on the phosphorylation of Smad-2, we treated growth-arrested mesangial cells with rhTGF-β1 (10 ng/ml) and rhSPARC (0.9 μM), separately or in combination for 30 min. As expected, rhTGF-β1 stimulated the phosphorylation of Smad-2 in wild-type cells (approx. 4-fold) (Fig. 2, lanes 1 and 2). SPARC alone did not have an effect on Smad-2 phosphorylation in these cells (Fig. 2, lane 3); however, SPARC and TGF-β-1 showed a synergistic effect by their enhancing the phosphorylation approx. 8-fold (Fig. 2, lane 4). Interestingly, in SPARC-null cells, rhTGF-β1 (Fig. 2, lane 6), as well as rhSPARC (Fig. 2, lane 7), stimulated the phosphorylation of Smad-2 approx. 2- to 3-fold, but did not show a synergistic or additive effect when used in combination (Fig. 2, lane 8). Using RT-PCR analysis, we also evaluated the stimulated mRNA expression levels of the aforementioned Smads and again did not find any significant differences between SPARC-null and wild-type mesangial

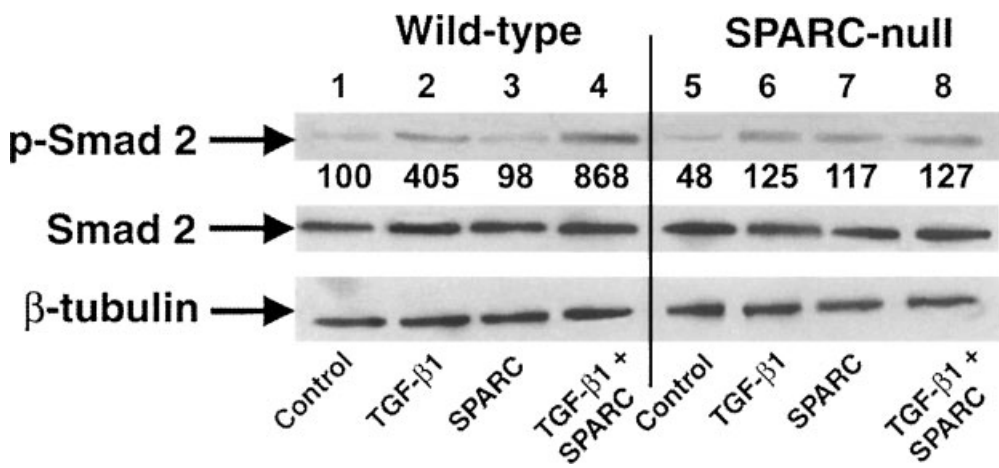

Fig. 2. SPARC and TGF-β1 regulate the phosphorylation of Smad-2. Wild-type and SPARC-null cells were treated as described in the legend to Figure 1. Subsequently, wild-type (lanes 1–4) and SPARC-null cells (lanes 5–8) were stimulated for 30 min with rhTGF-β1 at a concentration of 10 ng/ml in the presence or absence of SPARC (30 μg/ml, 0.9 μM). Total cellular protein lysates were prepared and equal amounts of protein were subjected to an immunoblot analysis with specific antibodies for phosphorylated and total Smad-2. β-tubulin was used as a control for equal protein loading. Numbers under each lane denote percent change in levels of respective proteins, relative to untreated wild-type cells (set at 100%).

cells (data not shown). Since we have shown that SPARC and TGF-β1 are involved in a positive regulatory feedback loop in glomerular mesangial cells [Francki et al., 1999], the data in Figure 2 indicate that SPARC enhances the effects of TGF-β1 vis-à-vis Smad-2 phosphorylation in wild-type mesangial cells, whereas it might have an additional, TGF-β1-independent effect in the corresponding cells that lack SPARC. Since it appears that the synergistic effects of TGF-β1 and SPARC require SPARC to be expressed endogenously, there might be a functional difference between this form of SPARC and the exogenous, rSPARC.

SPARC Modulates the Activity of the Transcription Factor c-Jun

Since the effect of SPARC on TGF-β1-dependent signaling events downstream of Smad activation has not been investigated, we asked whether SPARC-null cells might also modulate the activation of TGF-β1-dependent transcription factors, namely CREB-1, c-jun, and Sp-1. These transcription factors have been shown to interact with Smad complexes and thereby to enable the transcription of certain target genes [Liberati et al., 1999]. Therefore, we immunoblotted cell lysates from wild-type and SPARC-null mesangial cells with antibody specific for CREB-1, c-fos, c-jun, phosphorylated c-jun, and Sp-1. We observed substantially increased levels of total and phosphorylated c-jun (approx. 2-fold), whereas the levels of Sp-1, c-fos, and CREB-1 remained unchanged in mesangial cells from SPARC-null in comparison to wild-type mice (Fig. 3).

Potential differences were evaluated between wild-type and SPARC-null cells with respect to the time-dependent activation of c-jun after treatment for 30 min with rhTGF-β1 in the presence or absence of SPARC. We immunoblotted mesangial cell lysates with antibodies specific for c-jun and phospho-c-jun, as shown in Figure 4. In wild-type cells, rhTGF-β1 induced a rapid 3-fold phosphorylation of c-jun (Fig. 4, lane 2). In response to SPARC alone, only a slight increase (approx. 1.5- to 2-fold) in phosphorylation of c-jun was observed (Fig. 4, lane 3). The increase is less in comparison to that induced by rhTGF-β1. The combination of rhTGF-β1 and SPARC showed the same phosphorylation as was seen with rhTGF-β1 alone (Fig. 4, lane 4). The pattern of c-jun-phosphorylation changed in SPARC-null mesangial cells.

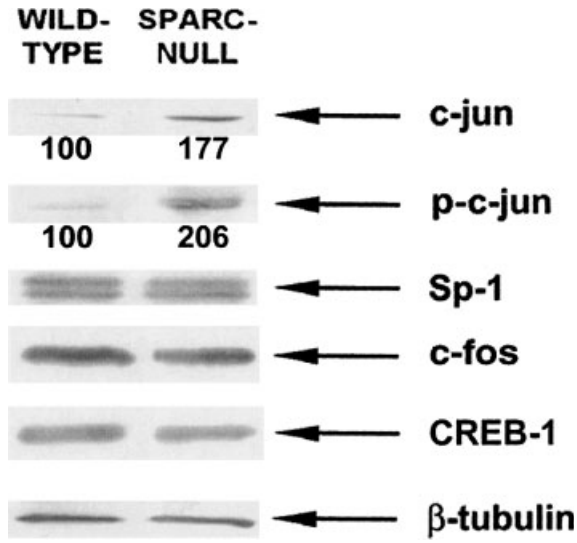

Fig. 3. SPARC-null mesangial cells display elevated levels of total and phosphorylated c-jun. Wild-type and SPARC-null cells were treated as described in the legend to Figure 1. Equal amounts of total cellular protein lysates were subjected to an immunoblot analysis with antibodies specific for CREB-1, c-fos, SP-1, c-jun, and phosphorylated c-jun. β-tubulin was used as a control for equal protein loading. Numbers denote percent change in levels of respective proteins, relative to wild-type cells (set at 100%).

Stimulation with rhTGF-β1 alone for 30 min showed a 4-fold increase in phosphorylation (Fig. 4, lane 6) compared to that of the wild-type mesangial cells (approx. 3-fold). The phosphorylation of c-jun in response to SPARC alone was also significantly higher (approx. 3- to 4-fold) when compared to wild-type cells (Fig. 4, lanes 3 and 7). The combination of rhTGF-β1 and SPARC showed a comparable level of phosphorylation (approx. 4-fold) relative to that induced by rhTGF-β1 alone (Fig. 4, lanes 6 and 8), again being substantially higher in comparison to their wild-type counterparts, which was approx. 2- to 3-fold (Fig. 4, compare lanes 2 and 6; lanes 4 and 8). With respect to the activation of c-jun, the SPARC-null mesangial cells appeared to be more responsive to TGF-β1, possibly due to the lower amounts of TGF-β1 and higher levels of c-jun produced by the cells. The TGF-β1-induced c-Jun phosphorylation is a direct response to TGF-β1 and is not modulated appreciably by SPARC in wild–type cells.

SPARC Regulates the Activity of the Stress-Activated Kinase JNK in Mesangial Cells

Since c-jun is activated directly by the stress activated kinase JNK, we measured the levels

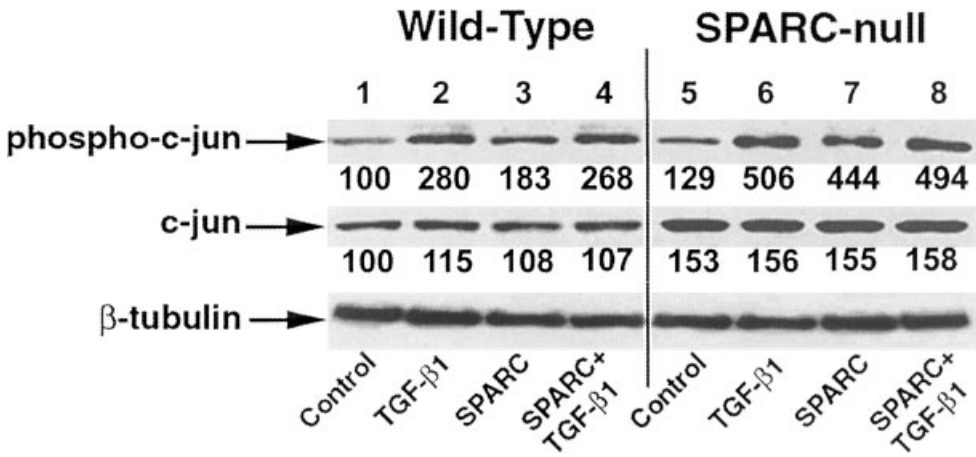

Fig. 4. SPARC and TGF-β1 regulate the activation of c-jun in mesangial cells. Wild-type and SPARC-null cells were treated as described in the legend to Figure 1. Subsequently, wild-type (lanes 1–4) and SPARC-null cells (lanes 5–8) were stimulated for 30 min with rhTGF-β1 at a concentration of 10 ng/ml in the presence or absence of SPARC (30 μg/ml, 0.9 μM). Total cellular protein lysates were prepared, and equal amounts of protein were subjected to an immunoblot analysis with antibodies specific for phosphorylated and total c-jun. β-tubulin was used as a control for equal protein loading. Numbers under each lane denote percent change in levels of respective proteins, relative to untreated wild-type cells (set at 100%).

and the activity of JNK in SPARC-null mesangial cells and their wild-type counterparts in response to rhTGF-β1 and SPARC. Mesangial cells were incubated for 30 min with rhTGF-β1 and SPARC, and equal amounts of total cellular lysates were exposed on blots to antibodies specific for JNK. We found that SPARC-null mesangial cells expressed elevated levels of JNK (approx. 2- to 3-fold) in comparison to wild-type cells, and that SPARC enhanced the TGF-β1-driven activation of JNK (which was approx. 3- to 4- fold higher relative to untreated cells) (Fig. 5), essentially the stimulation pattern observed for c-jun (Fig. 4). In wild–type cells, SPARC exhibited an enhancing effect on the induction of JNK activation by TGF-β1, and such an effect was not observed for c-jun. Thus, SPARC also modulates TGF-β1-induced JNK activation, but not c-jun phosphorylation, in wild–type cells. This represents a potential branch point for TGF-β1 signaling pathways in mesangial cells.

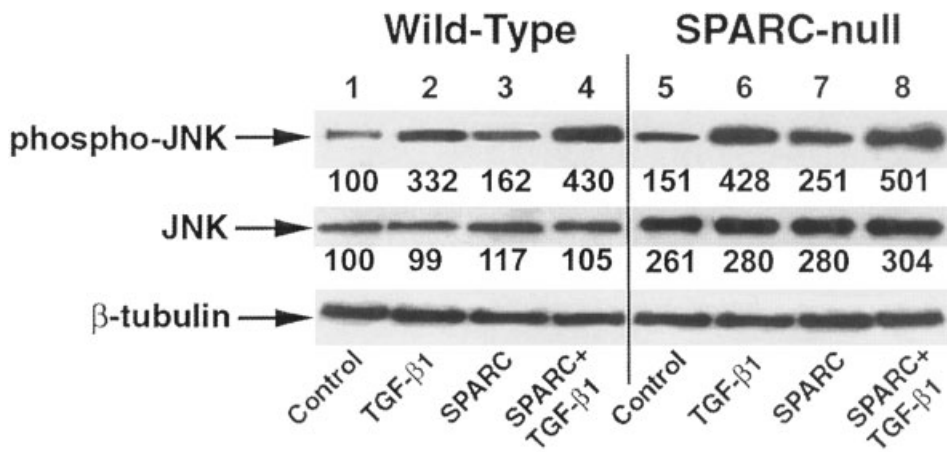

Fig. 5. SPARC and TGF-β1 regulate the levels and the activation of JNK in mesangial cells. Wild-type and SPARC-null cells were treated as described in the legend to Figure 1. Subsequently, wild-type (lanes 1–4) and SPARC-null cells (lanes 5–8) were stimulated for 30 min with rhTGF-β1 at a concentration of 10 ng/ml in the presence or absence of SPARC (30 μg/ml, 0.9 μM). Total cellular protein lysates were prepared and equal amounts of protein were subjected to an immunoblot analysis with antibodies specific for phosphorylated JNK and total JNK. β-tubulin was used as a control for equal protein loading. Numbers under each lane denote percent change in levels of respective proteins, relative to untreated wild-type cells (set at 100%).

SPARC Interacts With the TGF-β-Receptor Complex

To determine how SPARC modulates TGF-beta 1 response, we tested whether SPARC interacts with TGF-β1 or with the TGF-β-receptor complex. We performed binding studies with biotinylated rhSPARC and rhTGF-β1 (Fig. 6). Lanes 1 through 5 depict the NeutrAvidin- (Fig. 6A, IP) and protein G-precipitated (Fig. 6B, IP) proteins, Lanes 6 through 10 show the non-precipitated proteins (Fig. 6A,B, IDE). There was no binding between SPARC and TGF-β1 alone (Fig. 6A, lane 4), or SPARC and the chimeric TGF-β-receptor type II alone (Fig. 6B, lane 5) at equimolar ratios. Furthermore, we performed these binding studies at various molar ratios of SPARC, TGF-β1, and the chimeric TGF-β-receptor type II, but we were not able to detect any interaction when only two proteins were present (data not shown). Interestingly, there was binding of SPARC to the chimeric TGF-β-receptor type II only in the presence of TGF-β1 (Fig. 6B, lane 4). These data indicate that SPARC might bind to a structural epitope that encompasses TGF-β1 and the TGF-β-receptor type II, or that SPARC interacts with the TGF-β-receptor type II only upon a conformational change of the TGF-β-receptor type II that is induced by TGF-β1. We suggest that, as depicted in the model in Figure 7, the presence of SPARC alters and/or enhances the activity of the TGF-β-receptor complex and the subsequent signaling cascade, in comparison to the responses elicited by TGF-β1 alone.

DISCUSSION

The importance of SPARC during the progression of renal, and especially glomerular, diseases has gained credence from numerous studies published over the last several years [Floege et al., 1993; Pichler et al., 1996]. Although it has been appreciated that TGF-β1 induces SPARC and collagen type I [Reed et al.,

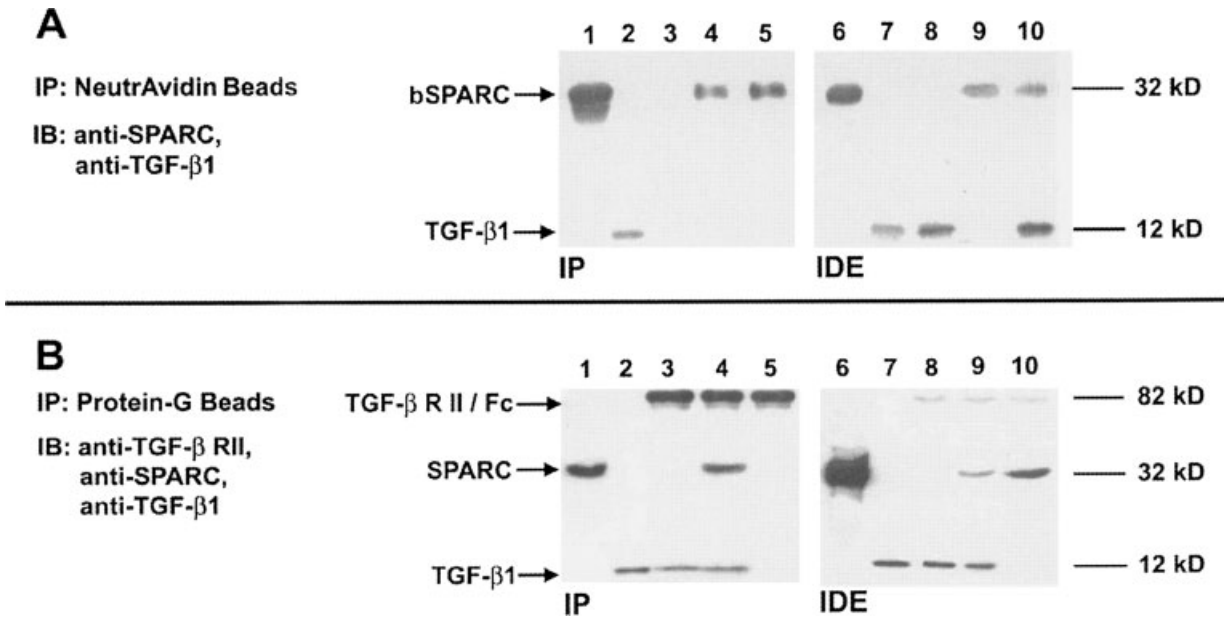

Fig. 6. SPARC binds to the TGF-β receptor type II. **A**: Biotinylated rhSPARC (bSPARC) was incubated at an equimolar ratio with TGF-β1 in standard binding buffer, and the complexes were immunoprecipitated with NeutrAvidin beads, transferred onto a PVDF membrane, and subsequently immunoblotted with antibodies specific for SPARC and TGF-β1. Lanes: (1) rhSPARC control; (2) rhTGF-β1 control; (3) rhTGF-β1 IP; (4) rhSPARC IP; (5) rhTGF-β1 + SPARC IP; (6) rhSPARC control; (7) rhTGF-β1 control; (8) rhTGF-β1 IDE; (9) rhSPARC IDE; (10) rhTGF-β1 + SPARC IDE. **B**: rhSPARC was incubated with the chimeric TGF-β-receptor type II in standard binding buffer in the presence or absence of TGF-β1 at equimolar ratios. The complexes were immunoprecipitated with Protein-G beads, resolved by SDS–PAGE, transferred onto a PVDF membrane, and subsequently immunoblotted with antibodies specific for SPARC, TGF-β1 and TGF-β-receptor type II. Lanes: (1) rhSPARC control; (2) rhTGF-β1 control; (3) rhTGF-β1 + TGF-β-receptor type II IP; (4) rhSPARC + rhTGF-β1 + TGF-β-receptor type II IP; (5) rhSPARC + TGF-β-receptor type II IP; (6) rhSPARC control; (7) rhTGF-β1 control; (8) rhTGF-β1 + TGF-β-receptor type II IDE; (9) rhSPARC + rhTGF-β1 + TGF-β-receptor type II IDE; (10) rhSPARC + TGF-β-receptor type II IDE. IP, immunoprecipitate; IDE, immunodepleted extract; IB, immunoblot.

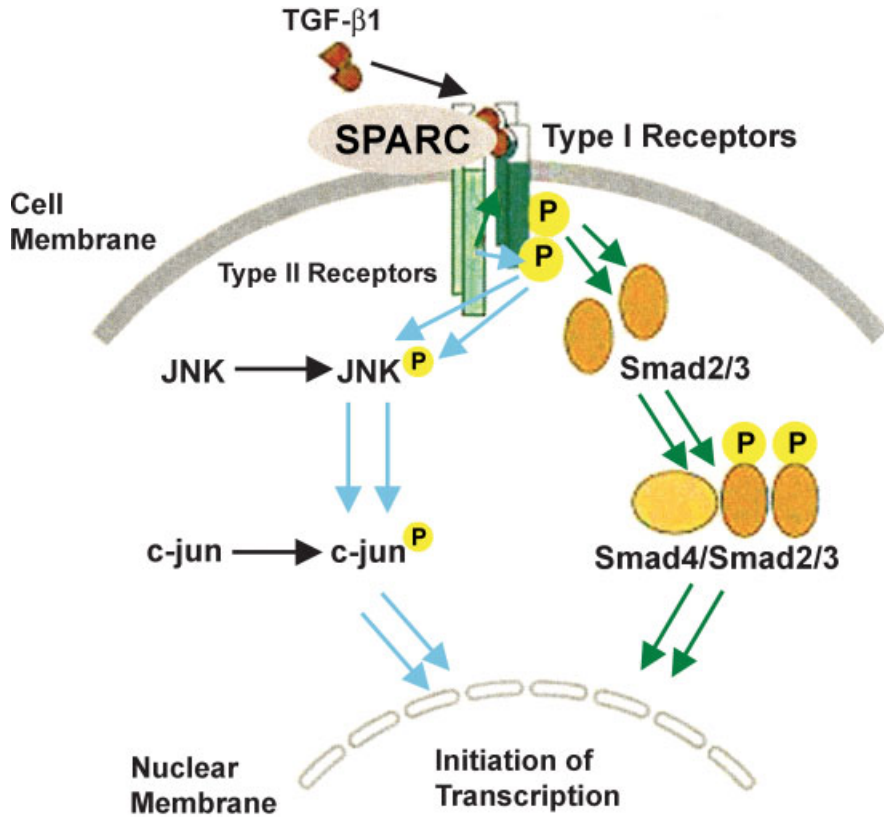

Fig. 7. Modulation of the TGF-β1-dependent signal transduction cascade by SPARC. SPARC interacts with the receptor-complex in a ligand-dependent manner and might thereby alter the structure of the receptors, the subsequent intrinsic activation steps, and/or the kinase activity of the type I receptor. Thus, SPARC might induce formation of a different receptor complex and thereby enhance subsequent TGF-β1-dependent signaling (e.g., activation of Smad 2/3, JNK). SPARC, in comparison to TGF-β1 alone, might also change the conformation of the TGF-β-receptor complex to alter the binding efficiency of the type I receptor kinase for certain signal transduction molecules and thereby induce specific downstream signaling events (blue and green arrows).

1994], we have recently shown that SPARC regulates the expression of TGF-β1 and collagen type I in mesangial cells, and have proposed a reciprocal, autocrine regulatory feedback loop between SPARC and TGF-β1 [Francki et al., 1999]. Herein, we present results that SPARC does not bind to TGF-β directly but interacts with the TGF-β-receptor complex only in the presence of TGF-β.

Why is it important to determine whether SPARC regulates the activation of TGF-β-receptors? TGF-β1 is one of the most prominent and important factors expressed by mesangial cells during the remodeling process [Floege et al., 1991; Poncelet and Schnaper, 1998; Francki and Sage, 2001]. The expression of TGF-β-receptors, as well as the binding of TGF-β1, are highest in mesangial cells near confluence [Riser et al., 1999], and the expression of the TGF-β-receptors is enhanced after stimulation with TGF-β1 [Siegert et al., 1999]. Interestingly, SPARC is also produced at high levels in glomerulonephritis [Francki and Sage, 2001] and under certain conditions in vitro [Lane and Sage, 1994; Francki et al., 1999]. SPARC regulates proliferation by controlling the activity of several growth factors [Raines et al., 1992; Kupprion et al., 1998]. Thus, SPARC could function as a positive mediator of TGF-β1 activity in mesangial cells. The interaction of these proteins might be critical in the maintenance of a proper response to injury in the kidney, as TGF-β1 controls the expression of other growth factors and ECM proteins [Francki and Sage, 2001].

TGF-β1 mediates its activity by binding to TGF-β-receptor II, a constitutively active serine/threonine kinase [Massagué and Weis-Garcia, 1996]. Upon binding, this receptor recruits and phosphorylates TGF-β-receptor I

and thereby activates a multitude of downstream signaling events [Heldin et al., 1997]. These events are dependent on cell type, concentration of ligand, number of receptors, and the amount of intracellular adapter proteins. Thus, it becomes important to explore the function of modulating proteins like SPARC on the TGF-β1-signaling pathway to explain the pleiotropic effects of TGF-β1.

We suggest that SPARC interacts with the TGF-β-receptor complex in a ligand-dependent manner and thereby alters the structure of the receptors, their subsequent intrinsic activation steps, as well as the kinase activity of the type I receptor. Thus, in contrast to TGF-β1 alone, SPARC might induce a different receptor complex formation and thereby enhance subsequent TGF-β1-dependent signaling (e.g., activation of Smad-2). SPARC, in comparison to TGF-β1 alone, might also change the conformation of the TGF-β-receptor complex to alter the binding efficiency of the type I receptor kinase for certain signal transduction molecules and thereby induce specific downstream signaling events. Interestingly, in corroboration with our findings, it was recently described that SPARC inhibits proliferation of the Mv1Lu epithelial cell line by altering the TGF-β signaling system through a TGF-β-receptor- and Smad2/3-dependent pathway [Schiemann et al., 2003]. However, it will be imperative to ascertain that the binding of SPARC to the TGF-β1 ligand-receptor complex also occurs in vivo, studies that are currently underway. The fact that SPARC regulates the activity and the levels of certain members of the Smad-protein family, the transcription factor c-jun, and its activating kinase JNK in mesangial cells, is indicative of interactions between SPARC and proteins of the TGF-β-pathway. In addition, we report that SPARC is involved in the regulation of stress-activated pathways, namely the activation of JNK in mesangial cells. Figure 7 is a cartoon depicting these several possibilities, based on the data in this study.

How does SPARC modulate the TGF-β1-dependent signal transduction cascade? SPARC is a multifunctional matricellular protein that exerts its effects through several pathways. SPARC has been shown to interact with the ECM, growth factors, and growth factor receptors. Previous studies in our laboratory also showed that SPARC exerts its proximal, counteradhesive effects and its distal, antiproliferative effects through two independent signal transduction pathways [Motamed and Sage, 1998]. Recent studies have demonstrated that SPARC regulates the phosphorylation of fibroblast growth factor receptor type I without interacting with fibroblast growth factor-2 [Motamed et al., 2003]. In our previous studies, we showed that SPARC induces the expression of collagen type I, an effect we could mitigate with TGF-β-blocking antibodies [Francki et al., 1999]. TGF-β1 is known to induce collagen type I expression [Poncelet and Schnaper, 1998], and it is well-established that the effects of TGF-β1 are transduced by members of the Smad family [Chen et al., 1999]. Active Smads interact with several transcription factors including ATF-2 [Sano et al., 1999] and c-jun [Heldin et al., 1997], form transcription complexes, and initiate gene transcription. Inhibitory Smads compete with the regulatory Smads for binding to the TGF-β-receptor type I [Afrakhte et al., 1998]. The activity of Smads in the cell is controlled by a second regulatory feedback mechanism: TGF-β1 induces the expression of proteins like Smurf2, which then bind to the Smads and target them for ubiquitination and subsequent proteasomal degradation [Bonni et al., 2001]. We hypothesize that SPARC has a bimodal effect on the regulation of the expression and activation of these signal transducing proteins (Smads) and transcription factors (c-jun). SPARC might modulate an early response, such as the activation of Smads and transcription factors, by modulating ligand/receptor interaction on the surface of the cell, and subsequently a later response by its altering the kinetics of de novo synthesis of these proteins as well as that of TGF-β1 itself. These late effects of SPARC could also be TGF-β-independent, possibly exerted through a cognate receptor for SPARC, and are currently under investigation.

It has also been suggested that TGF-β1 acts through receptor-dependent, but Smad-independent effects. Recent reports suggest that TGF-β1 activates the MAP kinase signaling cascade [Blanchette et al., 2001], the protein kinase C signaling pathway [Yakymovych et al., 2001], and kinases of the stress-activated pathway, for example JNK [Minet et al., 2001]. Therefore, TGF-β-receptor type I can activate distinct downstream effectors, possibly through a number of different binding sites in its cytoplasmic domain. Our data imply such a mechanism in regard to the effect of SPARC on the

activation of JNK. We suggest that SPARC acts by altering the conformation of the TGF-β-receptor complex and thus modulates the interaction of other intracellular signaling proteins (e.g., JNK), with the intrinsic TGF-β-receptor type I kinase, through a Smad-independent pathway. However, the precise mechanism of these signaling events triggered by SPARC in mesangial cells needs to be examined further.

Our data support a functional link between SPARC and the TGF-β1-dependent signaling pathway. Moreover, results from this study help us to define the specific effects of SPARC on TGF-β1-dependent signaling, as well as the general modulatory functions of matricellular proteins on the interactions between cells and growth factors. To our knowledge, this is the first evidence that SPARC is directly involved in the regulation of expression and/or activation of TGF-β1 and its signaling cascade in primary glomerular mesangial cells, which is in agreement with the paradigm that matricellular proteins modulate interactions between growth factors and cells.

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