The Copper Binding Domain of SPARC Mediates Cell Survival in Vitro via Interaction with Integrin β1 and Activation of Integrin-linked Kinase*

Secreted protein acidic and rich in cysteine (SPARC),2 a member of the matricellular family of proteins, is important for the development of the murine lens and the maintenance of its transparency. SPARC-null mice develop early onset cataracts due in part to aberrant assembly of the lens capsule and disruption of fiber cells (1–5). SPARC-null mice also show accelerated wound closure (6–8), enhanced tumor growth (9–11), and altered extracellular matrix (ECM) deposition in a variety of tissues. A secreted glycoprotein, SPARC binds to several integral components of the ECM and exhibits an anti-adhesive function that includes abrogation of focal adhesions and disruption of cell spreading and motility (14, 15).

SPARC has traditionally been described as a stress-response protein secreted at high levels by cultured cells (16–18). However, little is known about the role secreted SPARC plays during cellular stress. SPARC has been shown to act as a survival factor in stressed glioma cells (19, 20) and to potentiate the invasiveness of certain cancers. Recent work has shown that SPARC regulates cellular assembly of fibronectin by its stimulation of the activity of integrin-linked kinase (ILK) (20, 21). Whereas ILK, by virtue of its interaction with integrin cytoplasmic domains, has been located predominantly on the cytoplasmic side of the plasma membrane, secreted SPARC acts extracellularly at points of integrin-ECM interaction. The juxtaposition of ILK and SPARC across the cell membrane necessitates the involvement of transmembrane proteins for SPARC-mediated alteration of ILK activity. The integrin receptors are logical candidates, as they have been shown previously to interact with ILK and are major mediators of signal transduction between cells and the ECM.

ILK interacts with the cytoplasmic tails of the integrin β1/β3 subunits, which play a significant role in cell adhesion, motility, differentiation, and survival (for review, see Ref. 22) and with the intracellular complex associated with focal adhesions. ILK functions downstream and independently of phosphatidylinositol 3-kinase (PI3K) to phosphorylate several effector proteins including Akt, glycogen synthase kinase 3β, the forkhead transcription factor, and integrins β1/β3. Interaction between integrins and certain ECM ligands activates ILK via the integrin cytoplasmic tail. However, little is known about the role matricellular proteins play in the initiation or modulation of ILK signaling by virtue of their alteration of integrin-ECM interaction.

Although SPARC has been shown to interact with ECM proteins and to be taken up by cells in culture, a high affinity cell surface receptor for SPARC has not been found. Stabilin-1, a scavenger receptor on alternately activated macrophages, binds SPARC specifically (23, 24) and has been proposed to facilitate macrophage-mediated clearance of SPARC from damaged tissues (23). However, with its lineage-limited expression, stabi-
lin-1 seems unlikely to mediate signaling initiated by SPARC in epithelial, endothelial, and fibroblastic cells.

We hypothesized that, during the induction of stress in cultured cells, SPARC acts as a pro-survival factor at least in part by its augmentation of ILK activity through an interaction with specific integrins. By comparing lens epithelial cells (LECs) derived from WT and SPARC-null mice, we now show that SPARC decreases apoptosis in cultured cells subjected to different types of stress and that this activity resides in the Cu^{2+} binding domain of SPARC. A SPARC-dependent increase in ILK activity was required for the enhanced survival of WT LEC, whereas inhibition of ILK resulted in increased cellular apoptosis. SPARC showed a significantly increased interaction with integrin β1 in cells subjected to stress, and this interaction was decreased after the addition of blocking antibodies against either integrin β1 or to SPARC, concomitant with decreases in ILK activity and subsequent cell death.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—For immunoblotting, immunoprecipitation, and immunostaining procedures, the following antibodies were used: hamster anti-integrin β1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit (rb) anti-integrin β1 (Upstate Cell Signaling, Lake Placid, NY), hamster integrin β1-blocking antibody (BD Biosciences), mouse (ms) monoclonal (mAb) anti-integrin α6β1/4 (Chemicon, Temecula, CA), goat (gt) anti-mouse SPARC (R&D Systems, Minneapolis, MN), ms mAb and rb polyclonal anti-ILK (Upstate), rb anti-ILK IgG (25), rb anti-caspase-3 (Cell Signaling Technology, Beverly, MA), rb anti-phospho-glycogen synthase kinase 3β (Cell Signaling Technology), ms mAb anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion Inc., Austin, TX), ms mAb anti-phospho-myelin basic protein (MBP) (horseradish peroxidase (HRP)-conjugate) (Upstate), and rb anti-heavy chain-binding protein (BiP) (Cell Signaling Technology). For immunoprecipitation of ILK, SPARC-blocking assays, and epitope determination, our mAbs 236, 255, 293, and 303 against human (h) SPARC, rb serum against SPARC peptide 2.1, rb serum against SPARC peptide 3.2, and guinea pig serum against SPARC peptide 2.3 were used as previously described (25–28). Rabbit polyclonal antibodies from our laboratory against ILK and SPARC were affinity-purified against their respective immunogens (25–27).

**Cell Culture**—C57Bl6/J × 129SVJ murine LEC were generated from lens epithelial explants as described previously (29). WT and SPARC-null mouse LEC were cultured in growth medium consisting of Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum (FBS), 10 units/ml penicillin G, 10 μg/ml streptomycin sulfate, and 0.25 μg/ml Fungizone® (Invitrogen). Cells were released with trypsin, seeded at 1.8 × 10^4 cells/cm² on tissue culture plastic, and incubated at 37 °C with 5% CO₂ for 24 h before stress induction. Tunicamycin-treated cells were incubated for 24 or 48 h in growth medium containing 5 μg/ml tunicamycin (Calbiochem). Serum-deprived cells were incubated for 48 h in growth medium lacking FBS but containing 0.1% BSA. Control cells were incubated in growth medium only and received fresh medium at the same time as their experimental counterparts.

**Immunoblotting**—Control, serum-deprived, and tunicamycin-treated cells were washed with PBS, lysed with ILK lysis buffer (1% Nonidet P-40, 50 mM HEPES, 150 mM NaCl, 5 mM Na₃VO₄, 5 mM NaF, 400 μg/ml DNAse, and Halt protease inhibitor mixture (Pierce)), and resolved by SDS-PAGE on 12% acrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA). Non-specific binding was blocked by incubation for 18–20 h at 4 °C in AquaBlock (East Coast Biologics, North Berwick, ME) diluted 1:1 with PBS containing 0.4% Tween 20. Membranes were incubated for 20 h at 4 °C in rb anti-BiP IgG (1:1000), gt anti-mouse SPARC IgG (1 μg/ml), ms anti-ILK IgG (1 μg/ml), rb anti-phospho-glycogen synthase kinase 3β IgG (1:1000), rb anti-caspase-3 IgG (1:1000), or ms anti-GAPDH IgG (1 μg/ml). Blots were washed 3 × 5 min in PBS containing 0.4% Tween 20 and incubated for 1 h in HRP-conjugated secondary antibody (donkey (dk) anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), dk anti-goat IgG (Jackson ImmunoResearch), or gt anti-mouse IgG (Pierce). Immobilon Western HRP substrate (Millipore) was used for detection of antibody-antigen complexes. Some blots were subsequently regenerated in Restore Western blot Stripping Buffer (Pierce) according to the manufacturer’s instructions and were blocked and re-probed as described above.

**Measurement of Reactive Oxygen Species**—Levels of cytosolic reactive oxygen species (ROS) in tunicamycin-treated WT and SPARC-null LEC were assessed as previously described (30) with slight modification. After a 24- or 48-h incubation with 5 μg/ml tunicamycin, 1 μg/ml 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Invitrogen) was incubated with the cells for 20–30 min followed by counting of fluorescent cells by flow cytometry (FACSCalibur, BD Biosciences).

**Quantification of Cell Death**—For analysis with acridine orange/ethidium bromide, procedures were followed as described previously (31) with some modification. Briefly, WT and SPARC-null LEC, grown for 24 h on glass coverslips in Dulbecco’s modified Eagle’s medium containing 10% FBS, were either deprived of serum (3 or 6 days) or exposed to tunicamycin (5 μg/ml for 24 or 48 h). Cells were incubated for 30 min at 37 °C in Dulbecco’s modified Eagle’s medium, FBS containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide. Cells were photographed with a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) and were scored as follows: full green nuclei, viable cells; condensed green nuclei, early mid apoptotic cells; condensed red nuclei, mid-late apoptotic cells; full red nuclei, necrotic cells. Routinely 3–4 panels containing 250–300 cells each were counted per condition. Additionally, the viability of trypsinized cells was determined by trypan blue exclusion.

**Immunoprecipitation**—Control, serum-deprived, and tunicamycin-treated cells were washed with PBS and were lysed with ILK lysis buffer as described above. As controls for immunoprecipitation in each trial, experimentally treated lysates were precipitated with beads only and a nonspecific antibody. Resulting immunoblots from these samples was negative unless otherwise indicated in each figure. For blocking experiments, 10 μg/ml nonspecific ms IgG, ms integrin β1-blocking antibody, or ms anti-SPARC mAb (236, 255, 293, or 303) was added.
to the media during induction of stress. Lysates were collected, and protein concentration was determined by BCA assay (21). Two μg of hamster anti-integrin β1 IgG, ms anti-integrin α6β1/4 IgG, or rb polyclonal antibody anti-ILK (25) was added to 150–200 μg of total cell lysates, and incubation occurred with gentle agitation for 20 h at 4 °C. Twenty μl of protein A/G⁺-agarose (Santa Cruz Biotechnology) was added to each sample, and incubation was continued for 1 h at 4 °C with agitation. Samples were purified by 2 sequential washes with ILK lysis buffer (modified to contain 750 mM NaCl) and boiled for 5 min.

Epitope Determination—mAbs raised against recombinant human SPARC were selected for their reactivity with both hSPARC and msSPARC. Epitopes of the four chosen mAbs were mapped by enzyme-linked immunosorbent assay against a library of SPARC peptides consisting of overlapping 10-mers homologous to hSPARC, with an overlap of 3 residues. The 91 overlapping SPARC peptides plus 5 non-SPARC 10-mers were synthesized and covalently bound to “pins” by Mimotopes International (Clayton, Victoria, Australia) according to their Multipin™ synthesis platform.

Enzyme-linked immunosorbent assays were performed according to the manufacturer's instructions. Briefly, the rack of pins (the peptide array) was blocked for 1 h in PBS containing 2% casein acid hydrolysate (Sigma-Aldrich) and 0.1% Tween 20, washed 3 × 10 min in PBS, incubated for 1 h in anti-SPARC mAb at 0.5 μg/ml in antibody diluent (PBS containing 0.1% BSA or casein acid hydrolysate, 0.1% Tween 20), washed 3 × 10 min in wash buffer (PBS, 0.1% Tween 20), incubated in peroxidase-labeled goat anti-ms IgG (secondary) (KPL, Gaithersburg, MD) at 0.2 μg/ml in antibody diluent, washed 3 × 10 min in wash buffer, and immersed in TMB substrate (OptEIA™, BD Biosciences). Assays testing the binding of the secondary alone were used to control for background reactivity. Color development was stopped at 1.5 min (mAb 255), 3 min (mAb 293), or 10 min (mAb 236, mAb 303, and secondary antibody alone); absorbance was recorded at 450 nm. The peptide array was regenerated by ultrasonication in 10× PBS containing 1% SDS and 0.1% β-mercaptoethanol.

Each antibody was assayed three times. The 96 absorbance readings from each assay were scaled from 0 to 1 for analysis. The average of the control assays (the secondary alone) was calculated and subtracted from each mAb anti-SPARC data point.

Dot Blot Analysis—Two μl of a 4 mm stock solution of SPARC peptides (2.1, 2.3, 3.2, and Z-2) (26, 27, 32) in PBS was spotted on a pre-wetted polyvinylidene difluoride membrane and allowed to dry. The membrane was subsequently blocked and probed as described above with 1 μg/ml non-specific ms IgG or anti-SPARC mAB (236, 255, 293, or 303). To validate the reactivity of spotted peptides, we independently probed the membrane with a 1:1000 dilution of antipeptide 2.1 (rb), anti-peptide 2.3 (guinea pig), or anti-peptide 3.2 (rb) (26, 27).

SPARC Peptide Competition—Control (48 h), serum-deprived (48 h), and tunicamycin-treated (24 h) cells were incubated with varying concentrations of SPARC peptides 2.3 or 1.1 (9, 27, 32). After the incubation, cells were washed with PBS and lysed with ILK lysis buffer, and lysates were immunoprecipitated as described above.

Exogenous Murine SPARC Addition—Medium conditioned by WT LEC was determined to contain ~13.5 nm SPARC by comparative immunoblot analysis (data not shown). SPARC-null LEC were incubated with and without stress conditions in WT-conditioned media, serially diluted with SPARC-null-conditioned media to contain 0–13.5 nm murine SPARC. After incubation, cells were washed with PBS and lysed with ILK lysis buffer.
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RESULTS

SPARC Is Increased during Stress—The expression of SPARC increases as cells adapt to culture or are subjected to different forms of stress (16). To determine the role of SPARC in cell survival, we stressed LEC isolated from WT and SPARC-null mice by serum deprivation. In parallel, the inhibitor of dolichol-phosphate-mediated N-glycosylation, tunicamycin, was used to initiate an intracellular stress response, as previous work has shown that the administration of tunicamycin to LEC results in an endoplasmic reticulum-associated unfolded protein response (UPR) (30).

In culture, LEC produce and secrete SPARC into their media. When placed under stress conditions, cell-associated and secreted SPARC protein levels were increased relative to unstressed control cells (Fig. 1). ILK levels also were increased in response to stress, whereas integrin β1 appeared to decrease. Conditioned media were probed additionally for ILK, integrin β1, and GAPDH, with no reactivity (data not shown).

WT and SPARC-null LEC Undergo UPR—UPR is characterized by increased levels of ROS, the endoplasmic reticulum chaperone BiP, and caspase 3 and 12 activation as well as induction of apoptosis (for review, see Ref. 33). To characterize the UPR in the presence or absence of SPARC, we treated LEC with tunicamycin and assayed for levels of ROS or BiP. After the addition of redox-reactive dye, LEC were analyzed by flow cytometry. ROS levels in LEC increased over time after exposure to tunicamycin (Fig. 2, panels A and B). SPARC-null LEC consistently showed ROS levels higher than those of WT LEC before and during the addition of tunicamycin (p < 0.05). As an additional indicator of UPR induction, levels of BiP were determined by immunoblotting (Fig. 2C). Both SPARC-null and WT LEC displayed an ~4-fold induction of BiP after exposure to tunicamycin. Neither intracellular ROS nor BiP induction was observed as a consequence of serum deprivation (data not shown). Based on elevated ROS and BiP levels, both SPARC-null and WT LEC were shown to generate a UPR after exposure to tunicamycin.

SPARC Protects LEC against Apoptosis in Vitro—Fig. 3 shows the levels of SPARC in response to the two different types of stress. As expected, SPARC-null LEC exhibited no SPARC, whereas SPARC protein in WT LEC was increased after serum deprivation or exposure to tunicamycin (5- and 4-fold, respectively) (Fig. 3, panels A and D). LEC were also stained with trypan blue or a combination of ethidium bromide/acridine orange, the former to determine total cell death after the induction of stress (Fig. 3, panels B and E), whereas the latter was diagnostic for apoptosis by nuclear condensation and permeability (31, 34) (Fig. 3, panels C and F). Comparable staining with trypan blue and ethidium bromide/acridine orange indicated that stress-induced cell death in the two LEC populations occurs primarily through apoptotic pathways. In both serum-free and tunicamycin-supplemented culture media, SPARC-null LEC showed significantly increased rates of apoptosis in comparison with WT LEC.
SPARC-Integrin β1 Interaction Activates ILK

PI3K-dependent ILK Activity Is Affected by SPARC—As a survival factor, ILK has been shown to phosphorylate directly several downstream activator molecules and, indirectly, to affect the activity of several pro-/anti-apoptotic proteins. ILK interacts directly with Akt, glycogen synthase kinase 3β, and the forkhead transcription factor (22). Additional apoptosis-associated proteins affected by ILK include caspase-3, caspase-9, and Bcl-2 family members. As shown in Fig. 4, immunoblotting and in vitro kinase assays were performed to determine the relative level and activity of ILK and its PI3K dependence in wild-type and SPARC-null LEC. To determine ILK response during stress, we performed immunoblots with lysates from resting and stressed LEC (Fig. 4A). Both wild-type and SPARC-null LEC demonstrated increased ILK production during stress. For ILK activity in resting and stressed LEC, with and without exposure to the PI3K inhibitor LY294002, lysates were immunoprecipitated with anti-ILK antibodies and subjected to assays utilizing MBP as an ILK substrate. wild-type LEC showed increased, PI3K-dependent ILK activity when subjected to serum deprivation or tunicamycin (Fig. 4B). All results were normalized to the amount of ILK protein immunoprecipitated from each assay. ILK Activity Is Required for SPARC-mediated Survival—Previous studies indicated that SPARC has an anti-apoptotic function via its stimulation of Akt and ILK activity in gliomas (19, 20). Phosphorylated Akt is a downstream effector of the PI3K and ILK signaling pathways that promote survival. We reduced ILK activity by chemical inhibition in stressed LEC to ask whether the anti-apoptotic role of SPARC during stress is dependent on an ILK-mediated pathway.

To determine whether the increased ILK activity observed in wild-type LEC accounted for enhanced survival, we stressed wild-type and SPARC-null LEC as before but with the addition of the small molecule, ILK-specific inhibitor KP-392 (35). Total cell death was calculated by inclusion of trypan blue dye. The addition of the ILK inhibitor to serum-deprived LEC (Fig. 5A) or tunicamycin-treated (Fig. 5B) LEC elevated total death in wild-type LEC to levels comparable in SPARC-null cells. KP-392 added to SPARC-null LEC did not increase total cell death after induction of stress (data not shown).

Extracellular SPARC-Integrin β1 Interaction Is Enhanced during Stress—Although previous work has shown that SPARC affects ILK activity (20, 21), the mechanism for an interaction between a secreted (SPARC) and intracellular (ILK) protein has not been resolved. SPARC has also been shown to diminish focal adhesion complexes in vitro (14). Integrins are a primary component of focal adhesions, which enable cross-talk between the ECM and intracellular signaling molecules, e.g., ILK. It was, therefore, of interest to
explore the role of integrins as mediators between SPARC and ILK during stress induction in LEC.

Lysates from LEC that were serum-deprived or exposed to tunicamycin (Fig. 6A) were immunoprecipitated with anti-integrin β1 antibodies and were subsequently probed for SPARC. Increased levels of SPARC-integrin β1 complex were observed in WT LEC after stress induction. Additionally, a SPARC-integrin β1 complex was revealed after SPARC-null LEC were stressed in WT LEC-conditioned media containing secreted, murine SPARC (Fig. 6A).

To verify the apparent interaction between SPARC and integrin β1, we used several different antibodies in co-immunoprecipitation assays. Anti-α6β1/4 integrin antibodies did not immunoprecipitate SPARC from stressed LEC. However, multiple anti-integrin β1 antibodies, with different specificities, revealed increased SPARC-integrin β1 interaction after induction of stress (Fig. 6B).

Because both SPARC and integrin β1 are processed through the classical secretory pathway, interaction between the two proteins might be an intracellular process before secretion of SPARC. To address this issue, we periodically washed and subsequently incubated stressed cells with either conditioned medium or fresh medium without secreted SPARC. When cells were given SPARC-null conditioned or fresh medium, the SPARC-integrin β1 interaction was eliminated (Fig. 6C) relative to the slight decrease observed in cells receiving WT-conditioned medium.

For confirmation of the formation of a complex containing SPARC, integrin β1, and ILK, cells were labeled and cross-linked with UV-sensitive amino acid derivatives. Western analysis showed the formation of a large protein complex that included ILK and integrin β1 under all conditions as well as SPARC under conditions of stress (Fig. 6D).

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**ILK-mediated Survival Is Dependent on SPARC-Integrin β1 Interaction**—SPARC and integrin-blocking antibodies were added to LEC in an attempt to inhibit the interaction between SPARC and integrin β1 (Fig. 7 and Fig. 8). Nonspecific, isotype control antibodies showed no effect on SPARC-integrin β1 interaction, but the addition of integrin β1-blocking antibodies eliminated SPARC from the immunoprecipitated complexes (Fig. 7A). In vitro kinase reactions were used to determine
whether inhibition of the SPARC-integrin β1 interaction influenced ILK activity during stress (Fig. 7B). Integrin β1-blocking antibodies, also shown to block SPARC-integrin β1 complex formation, lowered ILK activity in serum-deprived or tunicamycin-treated WT LEC.

Anti-SPARC mAbs varied in their capacity to block the interaction of SPARC with integrin β1 (Fig. 8A). Total cell death, as measured by trypan blue staining, of tunicamycin-treated WT LEC was increased to levels observed in SPARC-null LEC by the addition of SPARC-blocking mAb 255 (Fig. 8B). In contrast, nonspecific antibodies (IgG) or a non-blocking anti-SPARC mAb (303) exhibited minimal effects on the death of WT LEC cultured in the presence of tunicamycin (Fig. 8B).

The Follistatin Domain of SPARC Interacts with Integrin β1—The differences between the four anti-SPARC mAbs in their capacity to block the SPARC-integrin β1 interaction prompted an investigation into the epitopes of each antibody. Potential epitopes were first identified by testing the mAbs in a series of enzyme-linked immunosorbent assays against a Multipin™ peptide array representing the full-length amino acid sequence of hSPARC. The peptide array contained 91 overlapping 10-mers homologous to hSPARC, each offset from its neighbor by 3 residues. Each mAb was assayed against the entire peptide array three separate times. Background activity of the HRP-conjugated secondary antibody alone was subtracted from each reading (Fig. 9). Potential epitopes were selected according to 1) high net reactivity with adjacent overlapping peptides, and 2) an absorbance pattern indicative of a peak (candidate peaks are circumscribed by red boxes). Potential epitope sequences are shown in red in the insets (Fig. 9). Two of the mAbs, 236 and 293, yielded multiple peaks within the N-terminal half of SPARC, indicating the possibility that the mAbs bind to peptide sequences separated by nonbinding sequences within the primary structure of SPARC but brought into spatial proximity by its tertiary structure. This supposition was borne out by reacting mAbs with soluble 20- and 50-mers into spatial proximity by its tertiary structure. This supposition was borne out by reacting mAbs with soluble 20- and 50-mers approximating domains of SPARC (26, 32) (Fig. 10). Several of the peptides detected predicted by the data from the Multipin™ assay and did not detect peptides for which there were no corresponding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10). mAbs 236 and 293 detected responding peaks (Figs. 9 and 10).
A Multipin™ peptide array representing the full sequence of hSPARC as contiguous and overlapping 10-mers (see “Experimental Procedures”) was used to identify epitopes recognized by 4 anti-SPARC mAbs. Each graph (A–D) shows the reactivity of the mAb against overlapping, sequential peptides in three separate experiments. The inset panel displays predicted epitope position (red) in a molecular model of SPARC derived from A chain coordinates of Protein Bank Accession 1BMO (40). The blue bar along the x axis denotes the sequence represented in the molecular model. A, anti-SPARC mAb 236; B, anti-SPARC mAb 255; C, anti-SPARC mAb 293; D, anti-SPARC mAb 303. Potential epitopes were selected on the basis of high reactivities with contiguous peptides, and predicted epitopes are designated by red rectangles.

The most striking developmental characteristic of SPARC-null mice is early onset cataractogenesis. Catracts are generally associated with alterations in LEC behavior, lens capsule formation, deposition of integral ECM components, and general stress responses in lens fiber cell layers (36). Several common lens pathologies result from the sensitivity of this tissue to external stressors such as hypoxia, fluctuations in ionic balance, hyperglycemia, and ultraviolet irradiation. The discovery of SPARC in our laboratory was an outcome of its production by cells subjected to stress, e.g., “culture shock” (16). Although SPARC was presented as a pro-survival factor in a recent study (20), its role in cell survival after stress induction had not been elucidated. A potential clue to a mechanism by which SPARC might regulate cell survival is its capacity to increase the activity of ILK during fibronectin assembly (21). Despite an apparent colocalization of SPARC and ILK on the surface of cells, the mechanism by which ILK activity is enhanced by SPARC is not known. We hypothesized that SPARC integrates a pro-survival function in inhibitory mAb 303 was found to react only with a sequence that was more C-terminal (i.e., in the EC domain). Furthermore, the three inhibitory mAbs (236, 255, and 293) reacted with peptides encompassing the SPARC copper binding sequence KKGHK, as shown both by the Multipin™ assay and by the dot blot of peptide 2.3 (Fig. 10, panels A and B, respectively).

**DISCUSSION**

The most striking developmental characteristic of SPARC-null mice is early onset cataractogenesis. Cataracts are generally associated with alterations in LEC behavior, lens capsule formation, deposition of integral ECM components, and general stress responses in lens fiber cell layers (36). Several common lens pathologies result from the sensitivity of this tissue to external stressors such as hypoxia, fluctuations in ionic balance, hyperglycemia, and ultraviolet irradiation. The discovery of SPARC in our laboratory was an outcome of its production by cells subjected to stress, e.g., “culture shock” (16). Although SPARC was presented as a pro-survival factor in a recent study (20), its role in cell survival after stress induction had not been elucidated. A potential clue to a mechanism by which SPARC might regulate cell survival is its capacity to increase the activity of ILK during fibronectin assembly (21). Despite an apparent colocalization of SPARC and ILK on the surface of cells, the mechanism by which ILK activity is enhanced by SPARC is not known. We hypothesized that SPARC integrates a pro-survival function in...
SPARC-Integrin β1 Interaction Activates ILK

A

N-Terminus

C-Terminus

Follistatin domain

EC domain

B

2.1

2.3

3.2

Z-2

Lysate

Ms IgG

mAb 236

mAb 255

mAb 293

mAb 303

anti-SPARC-peptide antiserum

FIGURE 10. Confirmation of epitopes predicted by Multipin™ peptide array. SPARC peptides (20- and 30-mers) were dotted onto polyvinylidene difluoride membranes and probed with anti-SPARC mAbs 236, 255, 293, and 303. A, schematic diagram of SPARC showing relative positions of the portion of SPARC for which crystallographic data exist (red bar, follistatin domain; blue bar, extracellular calcium binding (EC) domain). SPARC peptides (gray bars), and mAb epitopes predicted by Multipin™ peptide array (colored bars). B, dot blot shows immunodetection by anti-SPARC mAbs of peptides containing the predicted epitopes (236, red; 255, green; 293, yellow; 303, blue). Reactivity of dotted peptides was confirmed by the use of guinea pig and rabbit antisera against the respective peptides (anti-SPARC peptide antisera). Composite blots for peptides 2.1, 2.3, 3.2, and Z-2 are from single membranes and are representative of three separate experiments.

stressed cells at least in part by its augmentation of downstream ILK activity through interaction with ILK-associated integrins β1/β3. Our results here indicate that cell death resulting from stress induction is apoptotic, as defined by nuclear morphology. Because we have not pursued additional apoptotic indicators or additional downstream ILK signaling, it is formally possible that cell death after stress is non-apoptotic. In either circumstance the role of SPARC is one of pro-survival during cellular response to stress.

As had been demonstrated previously, induction of stress in cultured cells increased expression of both SPARC and ILK, including increased secretion of SPARC into the culture medium (Fig. 1). In contrast to SPARC and ILK proteins, integrin β1 appeared to decrease with stress. However, subsequent immunoprecipitations with anti-integrin antibodies showed integrin β1 protein levels equivalent to those in resting cells, results allowing reasonable estimates of SPARC interaction (Figs. 6 – 8).

Before secretion, SPARC is prominent in the endoplasmic reticulum and has been proposed as a molecular chaperone that guides the folding of basement membrane proteins (37). Emerson et al. (38) have recently demonstrated that SPARC acts as a chaperone on model protein substrates. It was, therefore, of interest to generate endoplasmic reticular stress and the subsequent UPR in WT and SPARC-null cells. Survival after the UPR is dependent on endoplasmic reticulum-specific chaperone activity and is causally related to cataract formation in the lens (30). As indicated by increased ROS and BiP production, both WT and SPARC-null LEC undergo an UPR after overnight exposure to the inhibitor of N-glycosylation, tunicamycin (Fig. 2). There is a slight increase in ROS production and a reduced induction of BiP in SPARC-null LEC, the data indicating a possible role for SPARC during the UPR and correlating with elevated apoptosis in SPARC-null LEC.

For comparison with the intracellular effects of exposure to tunicamycin, cells were cultured in the absence of FBS for extended periods of time. Deprivation of serum or exposure to tunicamycin led to apoptotic cell death, as verified by trypan blue inclusion and observation of nuclear morphology/integrity (Fig. 3). After induction of stress, WT LEC exhibited increased production of SPARC and a decreased apoptotic rate relative to SPARC-null LEC under similar conditions. Although not explored here, it is possible that alterations in protein glycosylation on SPARC or survival-related proteins could result in increased SPARC-mediated survival. Previous work in our laboratory has shown that alterations in glycosylation do not change SPARC function, but alterations to other proteins remain a factor for consideration.

ILK signaling in the presence of SPARC was characterized by immunoblotting and immunocytochemistry. Immunoblots or staining of WT and SPARC-null LEC lysates after serum deprivation or exposure to tunicamycin (Fig. 4A) revealed enhanced ILK signal in both LEC lines. Whereas stress
The cytoplasmic tails of integrins have direct interaction at the cell surface, but they do indicate a functional relationship. The increased protein levels of ILK in both WT and SPARC-null LEC, ILK activity was seen only in WT LEC (Fig. 4B). Further analysis revealed that inhibition of PI3K eliminated all the SPARC-mediated ILK activity in cultured LEC (Fig. 4B). Additionally, ILK-specific inhibition eliminated the survival advantage of WT LEC relative to their SPARC-null counterparts, whereas inhibition of ILK did not affect the survival of SPARC-null LEC (Fig. 5). These results indicate a specific, PI3K-dependent role for ILK activity in the anti-apoptotic function of SPARC.

SPARC affects adhesion in vitro in part by its disruption of focal adhesions and the binding of integrins to ECM components (14). The cytoplasmic tails of integrins β1/β3 bind to ILK and in turn modulate its activity (22). We, therefore, asked whether integrins could bridge signaling between extracellular SPARC and intracellular ILK. After stress induction in the absence of serum or in the presence of tunicamycin, there was a significant increase in SPARC protein that was co-immunoprecipitated with integrin β1 in WT LEC (Fig. 6A). Immunoprecipitation of integrin β1 from SPARC-null cells exposed to WT-conditioned medium also isolated interacting SPARC protein under stress conditions. The SPARC-integrin β1 interaction appears to be specific, as several anti-integrin β1 antibodies recognizing different epitopes produced similar results (Fig. 6B). Elimination of SPARC-integrin β1 interaction via periodic removal of conditioned media demonstrated that secreted SPARC is required for the observed complex formation. This finding validates an extracellular connection between the SPARC and integrin β1 (Fig. 6C). Finally, protein cross-linking revealed a large, cell-associated complex containing ILK and integrin β1, and induction of stress resulted in the inclusion of SPARC in this complex (Fig. 6D).

We propose that the SPARC protein co-immunoprecipitated with anti-ILK antibodies during stress and/or during fibronectin assembly is in a complex with ILK and integrin β1. These findings do not eliminate the possibility that ILK and SPARC have direct interaction at the cell surface, but they do indicate a
role for integrins in SPARC-mediated signaling, as a functional interface between ILK and SPARC.

The use of anti-SPARC mAbs or a function-blocking antibody eliminated SPARC from the immunoprecipitated complex before and especially after stress induction (Fig. 8A; Fig. 7A). Blocking of a SPARC-integrin β1 interaction impaired both downstream ILK activation (Fig. 7B) and survival of WT LEC under stress (Fig. 8B). These results confirm that stress-induced SPARC-ILK signaling is mediated at least in part by integrin β1 in vitro. The antibodies we used to immunoprecipitate and block integrin β1 impair the adhesive function of this integrin. Because active integrins are required for functionality on the cell surface, we conclude that SPARC forms a complex primarily with the active form of integrin β1 on the cell surface. These findings do not preclude the possibility that SPARC and integrin β1 do not directly interact. It is possible that additional cellular factors are required to mediate or enhance the SPARC-integrin β1 interaction demonstrated here.

To explain the varied success of the anti-SPARC mAbs to inhibit complex formation between integrin β1 and SPARC, we determined specific epitopes for each of the four mAb. Those mAb that inhibited the binding of SPARC to integrin β1 (236, 255, and 293; Fig. 8A) were shown to recognize epitopes in the follistatin domain of SPARC (Figs. 9 and 10). The capacity of each mAb to block SPARC appeared coincident with its reactivity with SPARC peptide 2.3 (27, 32). Competition assays showed that SPARC peptide 2.3 was capable of specific, concentration-dependent inhibition of SPARC-integrin β1 interaction (Fig. 11). Further analysis indicated peptide 2.3 was capable of inhibiting (i) the integrin β1 binding, (ii) the integrin-ILK complex formation, and (iii) the ILK signaling conferred by extracellular SPARC on stressed LEC (Figs. 12 and 13).

Peptide 2.3 represents a copper binding sequence that stimulates angiogenesis via its enhancement of the endothelial cell cycle (27, 39). KKGHK can be proteolytically released (with flanking amino acids) from SPARC after degradation by extracellular proteases associated with wound repair and tissue remodeling in vivo (27, 32). It is plausible that during injury, development, tissue remodeling, and/or stress in vivo, the localized increase in SPARC protein results in an interaction with integrin β1 in which intact SPARC or SPARC proteolytic fragments participate. Subsequently, the pro-survival and angiogenic signaling required for the resolution of injury would occur. In conclusion, SPARC has an ILK-dependent, anti-apoptotic function in cultured LEC that is dependent on its copper binding domain mediating interaction with integrin β1.

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Note added in Proof—The final version of this paper differs from the Papers in Press version in that the graphics for some of the figures initially did not comply with the JBC requirements. Specifically, individual portions of composite gels were not clearly delineated. The data in the paper have not been changed.

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