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Identification of a Sequence in the Matricellular Protein SPARC That Interacts With the Scavenger Receptor Stabilin-1

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ABSTRACT

SPARC (osteonectin/BM-40), a secreted matricellular protein that promotes cellular deadhesion and motility in wound healing, carcinogenesis, and inflammation, binds to the scavenger receptor stabilin-1 in alternatively activated macrophages and undergoes endocytosis and clearance from the extracellular space. Both SPARC and stabilin-1 are expressed by endothelial cells during inflammation, but their interaction in this context is unknown. We have identified a binding site on SPARC for stabilin-1 by a solid-state peptide array coupled with a modified enzyme-linked immunosorbent assay. A monoclonal antibody that recognizes the identified binding site was also characterized that could be an inhibitor for the SPARC-stabilin-1 interaction in macrophages or endothelial cells. J. Cell. Biochem. 112: 1003–1008, 2011.
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KEY WORDS: VASCULAR PERMEABILITY; ALTERNATIVELY ACTIVATED MACROPHAGES; PEPTIDE ARRAY; EPITOPE; OSTEONECTIN; EXTRACELLULAR MATRIX

Secreted protein acidic and rich in cysteine (SPARC), a nonstructural glycoprotein secreted by platelets, leukocytes, endothelial cells, fibroblasts, and numerous other cell types into plasma and the extracellular matrix (ECM) and/or environment, is expressed at high levels during processes such as development, angiogenesis, and wound healing that involve changes in cell shape, adhesion, and motility. In support of the anti-adhesive role of SPARC in inflammation, Goldblum et al. [1994] demonstrated that SPARC reduces the adhesion of bovine aortic, retinal, and pulmonary endothelials cells in vitro, and induced cell rounding and the formation of intercellular gaps through which macromolecules could pass. More recently, the addition of SPARC to human umbilical vein endothelial cells (HUVEC) similarly enabled leukocytes to transmigrate through a formerly impermeable monolayer [Kelly et al., 2007]. As a necessary condition for the inflammatory response, permeabilization of the microvascular endothelial-cell barrier permits leukocytes, immunoglobulins, and other agents of immunity to proceed to sites of tissue injury, invasion by harmful substances, infection by microorganisms, and, in some cases, neoplasia. Conversely, faulty regulation of the barrier function is associated with chronic inflammation and autoimmunity [reviewed in Kumar et al., 2009].

The identities of cell-surface receptors capable of internalizing SPARC or by which SPARC could trigger intracellular signaling pathways were unknown until recent discoveries of SPARC interactions with the receptors integrin β1 [Weaver et al., 2008], vascular cell adhesion molecule 1 (VCAM-1) [Kelly et al., 2007], and stabilin-1 [Kzhyshkowska et al., 2006b]. Integrin β1, expressed on the cell surface in association with one of several other integrin alpha chains, affects intracellular junctions and mediates the attachment of cells to the surrounding ECM. In response to cellular stress, SPARC forms a complex with integrin β1 that promotes the survival of murine lens epithelial cells [Weaver et al., 2008]. The possibility that other functions of this SPARC-integrin interaction exist, particularly in the context of cell–cell adhesion, is yet unexplored.

VCAM-1, an immunoglobilin-like protein, is expressed on the surface of endothelial cells that have been activated by certain cytokines. Reorganization of the actin cytoskeleton and consequent transition of endothelial cells to a rounded shape, a well-known effect of SPARC termed ''intermediate adherence'' [Murphy-Ullrich, 2001], can be triggered by the interaction of SPARC and VCAM-1 [Kelly et al., 2007]. The resulting gaps between endothelial cells allow the passage of leukocytes and macromolecules to the

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extravascular space. The capacity of SPARC to abrogate focal adhesions in vitro also contributes to the permeability of endothelial cell monolayers [Goldblum et al., 1994], but whether the VCAM-1 pathway is involved in this effect is not known.

Stabilin-1, a scavenger receptor variably expressed on the surface of a subset of alternatively activated macrophages (M2), internalizes SPARC, targets it for degradation, and thereby potentially regulates its concentration in inflamed tissue [Kzhyshkowska et al., 2006b]. However, the expression of stabilin-1 is not limited to alternatively activated macrophages. It is agreed that stabilin-1 is at least transiently present in sinusoids, high endothelial venules, lymphatic endothelium, and stressed vascular endothelium [reviewed in Kzhyshkowska et al., 2006a]. However, its activity in these various endothelia is controversial, with some evidence that stabilin-1 provides a tether for leukocytes preparatory to extravasation [Salmi et al., 2004]. Alternatively, Prevo et al. [2004] contend that the promiscuity of stabilin-1 in binding to SPARC, acetylated low-density lipoprotein, advanced glycation end products, and gram-negative and -positive bacteria, as well as its rapid recycling between the surface and interior of the cell, argues for its sole function as a scavenger protein.

Whether stabilin-1 and SPARC, both highly expressed in microvascular endothelium during inflammatory events, interact in such a way as to modulate vascular permeability or permit the extravasation of leukocytes from the blood into inflamed tissue is an important question. Little is known about the interaction(s) between SPARC and stabilin-1 as a scavenger receptor expressed by M2 in the several other systems in which both proteins are present. These include ECM remodeling, angiogenesis, tumor growth and metastasis, arthritis, and prenatal osteogenesis [Brekken and Sage, 2001; Kzhyshkowska et al., 2006a]. As a large, complex, multi-domain protein, stabilin-1 is not well characterized in terms of its tertiary structure. Indeed, its individual domains have proved refractory to purification for study in vitro (Dr. Julia Kzhyshkowska, personal communication). To overcome this difficulty, we selected the domains of stabilin-1 that might interact with SPARC according to phage display analysis and arrayed them as overlapping 10- to 12-residue peptides for ELISA-type binding assays. We identified a site on SPARC that recognizes stabilin-1, as well as a monoclonal antibody that binds to this site and another monoclonal antibody that does not interfere with the SPARC-stabilin-1 interaction. We propose that these results will lead to further discoveries about the functions of SPARC, a matricellular protein, and stabilin-1, a scavenger receptor, in the contexts of normal cell function and pathology.

EXPERIMENTAL PROCEDURES

Recombinant human SPARC was made with Sf9 cells (specifically, Sf9 Cells Adapted in Sf-900™ III SFM (Invitrogen, Carlsbad, CA)) as described [Sage, 2003]. Monoclonal mouse anti-SPARC antibodies (mAbs) 255, 293, and 303 have been described [Sweetwyne et al., 2004; Weaver et al., 2008].

SPARC binding was tested on a Multipin peptide array from Mimotopes International (Clayton, Victoria, Australia). The array presented an overlapping library of 49 peptides identical to EGF-like domains 7 and 8 and laminin EGF-like domain 1 of human stabilin-1. The positions of these domains within the overall domain structure of stabilin-1 are shown in Figure 1. Most of the peptides were 10-mers with an overlap of three residues. These parameters were altered slightly wherever doing so resulted in bracketing of the peptides by two cysteines (i.e., CX_7C or CX_9C) to facilitate presentation of these peptides by formation of disulfide-bonded loops. The manufacturer synthesized the peptides and covalently attached them to gear-shaped disks mounted at the ends of plastic pins for submersion in 96-well ELISA plates. The other 47 positions of the peptide array (the rack of 96 pins) were occupied by controls and by peptides for a different experiment.

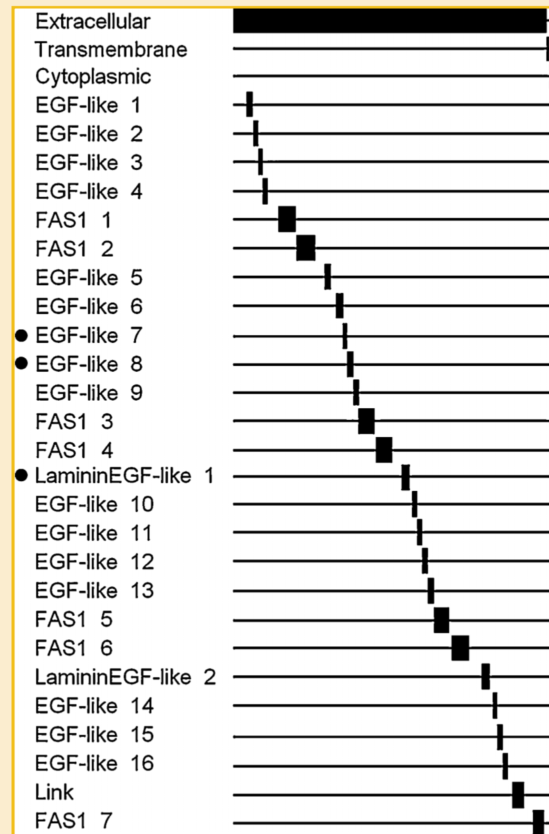

Fig. 1. Domains of human stabilin-1 (adapted from an image by SwissProt, http://expasy.org/sprot/). Dots indicate the domains identified by phage display that might participate in interactions with SPARC. The sequences of the three domains were parsed into overlapping peptides for ELISA-type assays to test SPARC binding. Bars represent entire sequence; rectangles represent domains.

ELISAs were performed according to instructions provided by the manufacturer. Briefly, the disks of the peptide array were blocked 1 h in PBS containing 0.1% Tween-20 and 2% bovine serum albumin (BSA) (Roche Diagnostics, Mannheim, Germany) and washed 3×10 min in PBS. Next, the disks were incubated 1 h with 0.82 μg SPARC (diluted in Hank's Buffered Saline Solution (HBSS)) per well and washed 3×10 min in wash buffer (PBS containing 0.05% Tween-20). They were next incubated 1 h in polyclonal (pAb) goat anti-SPARC IgG (R&D Systems, Minneapolis, MN) at

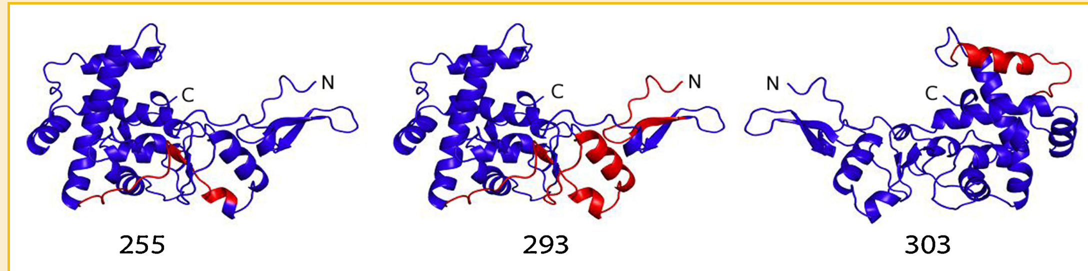

Fig. 2. Epitopes (in red) of mAbs in human SPARC. Note that the cartoon at the right side of the figure was rotated 180° to improve the view of the 303 epitope. Images were created with POLYVIEW-3D [Porollo and Meller, 2007]. N, N-terminus; C, C-terminus.

0.2 μg/ml or monoclonal mouse anti-SPARC IgG at 1 μg/ml in antibody diluent (HBSS containing 0.1% BSA and 0.05% Tween-20). The disks were washed 3 × 10 min with wash buffer and were subsequently incubated with horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG or goat anti-mouse IgG (Jackson ImmunoResearch) at 0.2 μg/ml in antibody diluent. After three more washes, the disks were incubated in tetramethylbenzidine (TMB) substrate (Thermo Scientific, Rockford, IL) until color intensity clearly differed among wells, at which time the reaction was stopped with 10% H_3PO_4. Color intensity was measured by absorbance at 450 nm. The reaction time for the assays varied from 1 to 30 min, depending on the antibody in use and the number of times the peptide array had been regenerated. The assays were also performed without SPARC to measure the background reactivity of the antibodies with the peptides. Each assay was repeated at least three times. The peptide array was regenerated after each assay by ultrasonication at 65°C in 100 mM (10×) PBS containing 1% sodium dodecyl sulfate and 0.1% beta-mercaptoethanol, after which the array was washed with water at 65°C, immersed in methanol, and allowed to dry at room temp.

Results from each assay were adjusted by subtraction of the lowest reading from each data point. Averages of replicates were calculated after elimination of the outliers according to Grubb's test ($P < 0.05$). Average background was subtracted to yield a final measurement for the binding of SPARC to each peptide. Because Grubb's test rewards variability, we discounted the results as unreliable whenever the value of at least one of the replicates fell below that of any of the background replicates.

RESULTS

The region of SPARC that binds to stabilin-1 was elucidated as follows. In an ELISA-type assay, SPARC was allowed to bind to overlapping peptides that are identical to the EGF-like 7, EGF-like 8, and laminin EGF-like 1 domains of stabilin-1 (Fig. 1). First, we detected SPARC binding to the peptides with our SPARC-binding mAbs 255, 293, and 303, the epitopes of which are illustrated in Figure 2. We expected that, in cases in which a particular epitope of SPARC was occupied by the binding interaction, the mAb corresponding to that epitope would be hindered from detecting SPARC and would therefore provide a false negative. We repeated the assays with a SPARC-reactive goat pAb from R&D Systems as a positive control and in order to detect all binding of SPARC to the stabilin-1 peptides, regardless of which epitopes were occupied by the interaction. We compensated for the binding of the antibodies alone to the peptides by repetition of the assays without SPARC and by subtraction of the resulting figures from the data for SPARC binding (Table I).

Because there was a fair degree of variability within some of the replicates (Supplemental Material), we deemed these results as qualitative and created an arbitrary rule to assess the data, namely, that binding was indicated by a series of contiguous overlapping peptides to which the pAb and at least one of the mAbs bound in a peak-like pattern. Upon application of this rule, it was apparent that the pAb detected significant SPARC binding to three distinct regions of stabilin-1 (Fig. 3). Two of the regions of the stabilin-1 peptides to which SPARC bound in these assays contained sequences similar to peptides present in stabilin-1 that were positive for SPARC binding by phage display: GRVC (our unpublished data) in the EGF-like domain 7, and C(S)YVGP[1] in EGF-like domain 8 (Dr. Kimberly Kelly, personal communication). The third region of SPARC binding was in the laminin EGF-like 1 domain, N-terminal to FHGTAC, the phage display sequence that originally led to the discovery that SPARC and stabilin-1 interact with each other. There is some indication that SPARC bound to FHGTAC in this assay, but the background binding of the antibodies to the peptides that contained all or part of this sequence was high in this region and may have confounded the results. It is also possible that among the peptides that contained all or part of FHGTAC, the one with the clearest affinity to SPARC (CHEGFHGTAC) may have formed a loop by disulfide bonding to which SPARC could bind more easily.

To determine the binding site on SPARC for the stabilin-1 peptides, we compared the reactivity of the mAbs to regions of stabilin-1 that were shown by the control pAb to bind SPARC. The 303 mAb clearly detected SPARC binding to each of the three stabilin-1 regions identified as SPARC-binding sites by the pAb, whereas the 255 mAb did not. Indeed, the 255 mAb did not unambiguously detect SPARC binding to any of the stabilin-1 peptides. The 293 mAb can be confidently said to have detected SPARC binding to only one of these three regions of stabilin-1.

The differences in detection of SPARC bound to the stabilin-1 peptides by the three mAbs can be explained by the positions and

[1]The (S) in C(S)YVGP is present in stabilin-1, but not in the corresponding phage display sequence, CGYVGP.

TABLE I. SPARC Was Allowed to Bind to Stabilin-1 Peptides in ELISA-Type Assays With Detection by Sequential Incubation in the Given Antibodies, the Appropriate HRP-Conjugated Secondary Antibodies, and TMB

Amino acid sequence	Domain	Goat pAb	mAb 255	mAb 293	mAb 303
TLCEPCPGGL	Laminin EGF-like 1	0.065			
EPCPGGLGGV		0.041			
CPGGLGGVC		**0.109**			
GLGGVCSGHG		**0.145**			0.051
GVCSGHGQCQ		**0.094**	0.010		0.047
SGHGQCQDRF		**0.108**	0.018	0.011	**0.135**
GQCQDRFLGS		**0.089**			0.063
CQDRFLGSGEC		0.179			
RFLGSGECHC		**0.140**	0.076	0.026	
GSGECHHEG			0.013		
ECHCHEGFHG		0.068	0.017	0.010	
CHEGFHGTAC		**0.147**	0.069	0.057	0.015
GFHGTACEVC		0.101	0.030		
GTACEVCELG		0.015			
CEVCELGRYG		0.010	0.075		
CELGRYGPNC		0.082	0.019	0.054	0.098
GRYGPNCTGV		**0.215**		0.042	0.044
GPNCTGVCDC					
CTGVCDCAHG		0.042	0.021		
VCDCAHGLCQ		0.029	0.016		

Amino acid sequence	Domain	Goat pAb	mAb 255	mAb 293	mAb 303
PSNPCSHPDR	EGF-like 7	0.074			0.038
CSHPDRGGC		0.079	0.024	0.031	0.019
PDRGGCSENA		0.050			
GGCSENAECV		0.044			
SENAECVPGS					
AECVPGSLGT					
CVPGSLGTHHC		0.049	0.028		
GSLGTHHCTC		0.099	0.013		0.033
GTHHCTCHKG		**0.105**	0.010	0.030	**0.140**
HCTCHKGWSG		0.083	0.035		0.062
CHKGWSGDGR		**0.223**			
GWSGDGRVCV		**0.114**			0.039
GDGRVCV AID	EGF-like 7 and 8	0.036			
RVCV AIDECE		0.012			
V AIDECELDV		0.013			
DECELDVGGG	EGF-like 8	0.012			
CELDVGGGC		0.032			
DVGGGCHTDA					
GGCHTDALCS		0.021			
HTDALCSYVG					
ALCSYVGPGQ		0.094			0.054
CSYVGPGQSRC			0.024		
YVGPGQSRCT		**0.205**		**0.112**	0.079
PGQSRCTCKL		**0.107**	0.036	**0.110**	0.053
SRCTCKLGFA		0.081	0.035	**0.111**	**0.119**
TCKLGFAGDG					0.065
LGFAGDGYQC					
AGDGYQCSPI					
GYQCSPIDPC		0.026			

Data represent A_{450} after neutralization of the TMB reaction, adjusted by subtracting the values for control assays that did not include SPARC. Each assay was repeated at least three times. Values below 0.01 were not reported. Amino acids in red represent sequences with potential for SPARC binding by phage display. (Spaces between residues represent transitions between stabilin-1 domains.) Green, data were considered reliable in that none of the values for the experimental replicates fell below any value for the control replicates. Gray, values for the experimental and control replicates overlapped, indicating that data were unreliable.

sizes of their respective epitopes (Fig. 2). The epitope for the 255 mAb centers on a beta strand that links the two well-characterized modules of SPARC, the follistatin-like (FS) and EF-hand calcium-binding (EC) domains [Hohenester et al., 1997]. This epitope includes the binding sequence GHK and overlaps a peptide of 18 residues (peptide 2.3) that is known to stimulate angiogenesis and cell proliferation [Lane and Sage, 1990]. This highly reactive site could be expected not only to participate in protein–protein interactions but also to serve as a potent antigen. Indeed, our mAbs 236 and 293 have been found to react with this sequence.

However, the 293 epitope includes residues that are separated from the 255 epitope and that are brought into proximity with that epitope by the tertiary structure of SPARC. This could explain why the 293 and not the 255 mAb was able to detect SPARC binding to

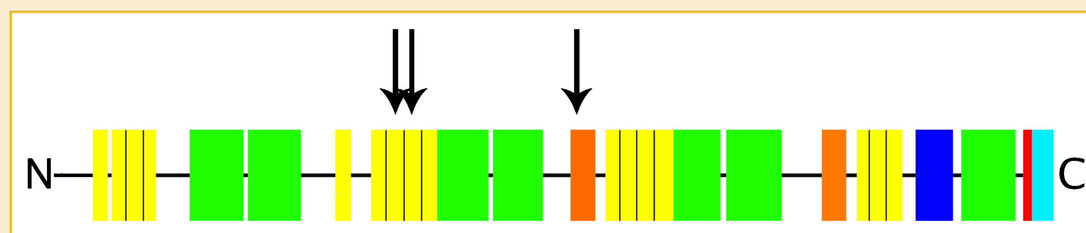

Fig. 3. Arrows indicate known binding sites for SPARC on stabilin-1. The types of domains in stabilin-1 are indicated by color: yellow, EGF-like; green, fasciclin-like; orange, laminin EGF-like; blue, link; red, transmembrane; aqua, cytoplasmic. N, N-terminus; C, C-terminus.

the laminin EGF-like 1 domain of stabilin-1. The 293 epitope might be extensive enough to accommodate the binding of the 293 mAb to SPARC even when the 255 epitope is occupied by the interaction with this particular sequence of stabilin-1.

The 303 mAb occupies a site distant from that recognized by the 255 mAb. Because it was not sterically hindered by the SPARC-stabilin-1 interactions, the 303 mAb detected SPARC binding to all three regions of stabilin-1 identified by the pAb. We conclude that (1) the binding site on SPARC for stabilin-1 is the 255 epitope; (2) the 255 mAb should be tested as a possible reagent for abrogating the interaction between the two molecules, both in vitro and in vivo; and (3) the 303 epitope, located on a beta-coil in the EC domain of SPARC, does not bind to any of the sequences of stabilin-1 that were selected for the assays. These results do not preclude the possibility that SPARC could bind to other sequences in stabilin-1 or that other epitopes of SPARC could be involved in binding interactions with this complex, multi-domain protein.

DISCUSSION

Stabilin-1, a scavenger receptor on a subpopulation of alternatively activated macrophages (M2), removes acetylated low-density lipoproteins, advanced glycation end products, and SPARC from the intracellular space by endocytosis [Kzhyshkowska et al., 2006a]. Stabilin-1 is also expressed by microvascular endothelial cells during inflammation [Salmi et al., 2004]. Further study is required to determine whether SPARC and stabilin-1 interact in this context and if so, whether stabilin-1 internalizes SPARC in endothelial cells, similar to its activity in M2.

SPARC is thought to promote inflammation via its contribution to the permeability of the microvasculature, a process allowing leukocytes to pass into extravascular tissues. It is speculated that stabilin-1 also promotes inflammation by its arresting of the flow of leukocytes in the blood and positioning them for extravasation. In chronic inflammation and autoimmunity, excessive concentrations of immune cells and cytokines in the tissue can damage healthy cells (e.g., in atherosclerosis and diabetes [Kim et al., 2006]), or trigger abnormal cell proliferation (e.g., in psoriatic skin [Racz and Prens, 2009]). Methods by which vascular permeability and leukocyte extravasation can be modulated are therefore of considerable interest clinically. The treatment of autoimmune conditions by humanized monoclonal antibodies has had mixed success, yet holds promise, as is evident by the development of MLN0002, a mAb for the treatment of active Crohn's disease that likely blocks the recruitment of leukocytes to inflamed tissue by integrin $\alpha4\beta7$ [Feagan et al., 2008]. Unlike its predecessor natalizumab, which is associated with an increase in the incidence of a rare brain disorder, progressive multifocal leukoencephalopathy [Fontoura, 2010], MLN0002 has been associated with no serious side effects.

Regardless of possible therapeutic implications, the identification and development of mAbs for the study of protein–protein interactions is of value in itself. We have examined the binding interaction of stabilin-1 and SPARC and have identified a pair of mAbs that can be used for further study of this interaction. Phage display analysis indicated three potential binding sites for SPARC in the EGF-like 7, EGF-like 8, and laminin EGF-like 1 domains of stabilin-1. A goat pAb reactive with recombinant human SPARC detected binding of SPARC to limited sequences in each of the three domains. We compared this result with the detection of SPARC binding to the same stabilin-1 sequences by our mAbs 303, 255, and 293. We found that the 303 mAb detected SPARC binding to the same sites as the pAb, but the 255 mAb did not, data indicating that the 255 epitope is occupied when SPARC binds to stabilin-1. The 293 epitope is similar to the 255 epitope. Indeed, in the case of two of the stabilin-1 sequences to which SPARC bound, the 293 mAb, like the 255 mAb, did not detect SPARC. However, the 293 mAb did detect SPARC binding to one of the identified sequences, a region within the laminin EGF-like 1 domain of stabilin-1. This result could be explained by the nature of the 293 epitope. It is more extensive than the 255 epitope and includes a discontinuous segment of SPARC that might provide sufficient affinity for the recognition of the 293 mAb despite the occupation of the 255 epitope by the interaction of SPARC with the aforementioned sequence.

In summation, our research indicates that the 255 mAb should be tested for applications requiring the abrogation of the SPARC-stabilin-1 interaction. Moreover, the 255 mAb could have a dual role in the reduction of inflammation via its abrogation of the proinflammatory activity of SPARC and, simultaneously, its prevention of the binding of SPARC to stabilin-1. In both cases, the capacity of stabilin-1 to endocytose proinflammatory advanced glycation end products would be increased. We further speculate that the 303 mAb could form a trimeric complex with SPARC and stabilin-1 at the cell surface and thereby interfere with the internalization of SPARC and other target molecules by cells that express stabilin-1.

The characterization of the binding interaction between SPARC and stabilin-1 and the discovery of two monoclonal antibodies with complementary effects on this interaction provide tools for extending our knowledge of some of the mechanisms involved in endothelial permeability and the clearance of harmful or superfluous biomolecules.

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