THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 33, pp. 22041–22047, August 14, 2009

The First Draft of the Endostatin Interaction Network*

Received for publication, April 2, 2009, and in revised form, April 23, 2009 Published, JBC Papers in Press, June 19, 2009, DOI 10.1074/jbc.M109.002964

Clément Faye[‡1], **Emilie Chautard**[‡1], **Bjorn R. Olsen**[§], **and Sylvie Ricard-Blum**[‡2]

From the [‡]*Institut de Biologie et Chimie des Protéines, UMR 5086 CNRS, University Lyon 1, IFR 128 Biosciences Gerland Lyon Sud, 7 passage du Vercors, 69367 Lyon Cedex 07, France and the* [§]*Department of Developmental Biology, Harvard School of Dental Medicine and Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115*

Endostatin is a C-terminal proteolytic fragment of collagen XVIII that is localized in vascular basement membrane zones in various organs. It binds to heparin/heparan sulfate and to a number of proteins, but its molecular mechanisms of action are not fully elucidated. We have used surface plasmon resonance (SPR) arrays to identify new partners of endostatin, and to give further insights on its molecular mechanism of action. New partners of endostatin include glycosaminoglycans (chondroitin and dermatan sulfate), matricellular proteins (thrombospondin-1 and SPARC), collagens (I, IV, and VI), the amyloid peptide Aβ-(1–42), and transglutaminase-2. The biological functions of the endostatin network involve a number of extracellular proteins containing epidermal growth factor and epidermal growth factor-like domains, and able to bind calcium. Depending on the trigger event, and on the availability of its members in a given tissue at a given time, the endostatin network might be involved either in the control of angiogenesis, and tumor growth, or in neurogenesis and neurodegenerative diseases.

Endostatin is a C-terminal proteolytic fragment of collagen XVIII that is localized in vascular basement membrane zones in various organs. It inhibits angiogenesis and tumor growth (1–3). The effect of endostatin depends on its concentration (4, 5), on the length of exposure (6), on the type of endothelial cells (7), and on the growth factor inducing cell proliferation (fibroblast growth factor 2 or VEGF)[3] (8, 9).

Endostatin binds to several membrane proteins including $\alpha5\beta1$ and $\alpha v\beta3$ integrins (10, 11), heparan sulfate proteoglycans (glypican-1 and -4) (12), and KDR/Flk1/VEGFR2 (13). We have previously characterized the binding of endostatin to heparan sulfate chains (9), and of endostatin to integrins (11). Furthermore, we have shown that $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha v\beta5$ integrins bind to heparin/heparan sulfate (11).

The broad molecular targets of endostatin suggest that multiple signaling systems are involved in mediation of its antiangiogenic action. Endostatin is a broad spectrum angiogenesis inhibitor that suppresses angiogenesis by blocking general mechanisms that govern endothelial cell growth (14), and initiates a complex network of signaling at the gene level (15). However, its molecular mechanism of action is still a matter of debate.

An integrative view of the endostatin interaction network, including interactions between endostatin partners, is necessary to provide a clear understanding of how all these molecules work together to regulate angiogenesis, and tumor growth. This global approach places individual proteins into a functional context, and takes into account the fact that a single molecule such as endostatin can affect a wide range of other cell components. Indeed, most proteins and other components carry out their functions within a complex network of interactions and this approach based on protein-protein interaction networks has been developed for several years to give new clues on biological processes (16).

This study was thus designed to identify additional extracellular partners of endostatin in an attempt to obtain new insights into its mechanisms of action, and the biological processes in which it participates. For this purpose, we have developed protein and glycosaminoglycan arrays using an automated surface plasmon resonance (SPR) platform. Proteins and glycosaminoglycans selected for SPR analysis were present in the same tissues or structures, such as basement membranes (17), brain (18), cartilage (19), or they were involved in the same physiopathological processes (angiogenesis, neuro-degenerative diseases) as endostatin, and they were available as full-length molecules. Collagens I and VI, for example, have been selected because the $\alpha1$ and $\alpha2$ chains of collagen VI were determined to be potential pan-endothelial markers as was the $\alpha1$ chain of collagen XVIII containing endostatin (20), and because the genes coding for the $\alpha1$ and $\alpha2$ chains of collagen I, and the $\alpha3$ chain of collagen VI are up-regulated in angiogenic vessels and elevated in tumor endothelium (20). Some proteins and glycosaminoglycans were also included to serve as positive controls for well known interactions with the potential partners of endostatin. We report that endostatin binds to other endogenous angiogenesis inhibitor, the matricellular proteins thrombospondin-1 and SPARC, and to several collagens (I, IV, and VI). Other interacting partners of endostatin are transglutaminase-2, the amyloid peptide Aβ-(1–42), chondroitin, and dermatan sulfate.

EXPERIMENTAL PROCEDURES

Source of Proteins and Glycosaminoglycans—Recombinant human endostatin, the trimeric C-terminal domain of collagen XVIII called NC1 and several mutants were produced by

* This work was supported, in whole or in part, by National Institutes of Health Grant 36820 (to B. O.). This work was also supported by Association pour la Recherche contre le Cancer Grant 3652 (to S. R. B.), GIS Maladies Rares INSERM Grant A04115SP (to S. R. B.), grants from the CPER Rhône-Alpes (to C. F., E. C., and S. R. B.), Institut Rhône-Alpin des Systèmes Complexes (to C. F., E. C., and S. R. B.), the Emergence Research Program Région Rhône-Alpes (to S. R. B.), and a Bonus Qualité Recherche grant from the University Lyon-1 (to S. R. B.).

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 33-0-4-37-65-29-26; Fax: 33-0-4-72-72-26-04; E-mail: s.ricard-blum@ibcp.fr.

[3] The abbreviations used are: VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; NC1, non-collagenous 1; SPARC, secreted protein acidic and rich in cysteine; SPR, surface plasmon resonance.

human embryonic kidney cells expressing Epstein-Barr virus nuclear antigen (293-EBNA cells) according to established protocols (8, 9, 11). Amino acid residues were numbered starting from the first amino acid residue of endostatin (His^1, also referred to as His^{132} when numbering starts from the first amino acid of the entire C-terminal domain NC1 of collagen XVIII). Laminin (L2020) isolated from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, human collagen I (C5483), human amyloid β protein fragment 1–42 (A9810), chondroitin sulfate from bovine trachea (C8529), heparin (H3393), and dermatan sulfate (C3788) from porcine intestinal mucosa, murine SPARC (S5174), guinea pig transglutaminase (T5398), bovine biglycan (B8041), and human fibronectin (F2006) were purchased from Sigma. Sodium hyaluronate was from Acros Organics (Geel, Belgium). Heparan sulfate from porcine intestinal mucosa was from Celsus (OH). Full-length human $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha v\beta5$ integrins were from Chemicon (Millipore, France), and recombinant human thrombospondin-1 was from R&D Systems (Minneapolis, MN). Pepsinized collagens IV and VI from human placenta were a generous gift from Dr. Florence Ruggiero (UMR 5086, CNRS, University Lyon 1, France). Bovine collagen XI was a generous gift from Dr. Marie-Claire Ronzière (UMR 5086, CNRS, University Lyon 1, France).

SPR Arrays—SPR arrays were handled in a Biacore Flexchip system (GE Healthcare), a commercially available high-density array platform that is capable of analyzing one analyte against 400 target spots at a time. Proteins or glycosaminoglycans were printed directly in triplicate at two different concentrations onto the gold surface of a Gold Affinity chip (GE Healthcare) using a non-contact PiezoArray spotter (PerkinElmer Life Sciences). The spotted matrix (15 × 15) comprised 225 spots. Proteins were spotted at concentrations varying from 50 to 200 μg/ml and glycosaminoglycans at 0.5 and 1 mg/ml. Six drops of 330 pl each were delivered to the surface of the chip (total spotted volume, 2.2 nl; spot diameter, 250–300 μm; spotted amount, 100–400 pg/spot). The chips were then dried at room temperature and stored under vacuum at 4 °C until their insertion into the Biacore Flexchip. The regions of interest of the chip were defined when the chip was dry. Each region of interest had four associated reference spots that were used to correct bulk refractive index changes as well as nonspecific binding of the analyte to the chip. The chip was blocked with a buffer containing mammalian proteins (Superblock, Pierce) for 5 times for 5 min. The blocked chip was then equilibrated with phosphate-buffered saline, 0.05% Tween 20 at 500 μl/min for 90 min. The analyte was flowed over the chip surface at 25 °C at a concentration ranging from 50 nM to 5 μM for 25 min at the same flow rate. The dissociation was monitored during injection of phosphate-buffered saline, 0.05% Tween for 40 min. Injected proteins were diluted in phosphate-buffered saline, 0.05% Tween, except full-length integrins, which were diluted in 10 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MnCl_2$, and 50 mM octyl β-D-glucopyranoside. Data collected from reference spots (gold surface) and buffer spots were subtracted from those collected on spotted proteins or glycosaminoglycans to obtain specific binding curves.

Building and Visualization of the Endostatin Interaction Network—Cytoscape, a software environment for integrated models of biomolecular interaction networks (21), was used to visualize the endostatin interaction network built with the tools provided on the website of MatrixDB (22).

In Silico Analysis of Endostatin Network—UniProtKB entries (Universal Protein Resource) (23) were tagged with 10 categories of keywords (biological process, cellular component, coding sequence diversity, developmental stage, disease, domain, ligand, molecular function, post-translational modification, and technical term). The keywords associated to the 26 protein partners of endostatin were ranked according to their occurrence. The percentage of keyword use in the endostatin network was compared with the keyword use in the 19,398 annotated human protein entries of UniProtKB (January 20, 2009). UniProt data were stored into a data base based on AceDB (24) to ease the counting of UniProt keywords and InterPro annotations.

InterPro (Integrated Resource of Protein Domains and Functional Sites) is a data base of protein families, domains, regions, repeats, and sites (25). UniProtKB cross-references to InterPro were used to identify the domains present in the protein partners of endostatin. The percentage of occurrence of a particular domain was calculated as described above for UniProtKB keywords, except that cross-references were available for 16,774 annotated human protein entries.

RESULTS

We looked for additional partners of endostatin using SPR arrays. This approach was selected because the yeast two-hybrid assay, which is widely used for high throughput discovery of protein interactions, is not the method of choice for studying extracellular interactions. Extracellular proteins are not optimally expressed in the nucleus, they are sticky and this may lead to false positive results. Furthermore, two-hybrid assays are not adapted to identify protein-polysaccharide interactions, which are of major importance for cell-matrix interactions and for the organization of the extracellular matrix. Known interactions were analyzed to validate SPR arrays and the Biacore Flexchip as reliable tools for the investigation of interactions established by endostatin. We confirmed by this technique previously described interactions between heparin/heparan sulfate and several extracellular proteins including endostatin (8, 9, 26), collagens I and V (27, 28), fibronectin, and transglutaminase-2. Protein-protein interactions between endostatin and laminin (29), endostatin and $\alpha5\beta1$ integrin (10, 11), or between tissue transglutaminase and $\alpha5\beta1$ integrin (30) were also confirmed, as were interactions between proteins and proteoglycans such as the collagen VI-biglycan interaction (31) (Table 1).

Identification by SPR Arrays of New Partners to Build the Interaction Network of Endostatin—New partners of endostatin, either as a monomer or as a trimer within the NC1 domain of collagen XVIII, were identified (Figs. 1 and 2). They include the amyloid peptide Aβ-(1–42), thrombospondin-1, SPARC, transglutaminase-2, chondroitin sulfate, dermatan sulfate, and collagens I, IV, and VI. Because collagen IV and VI preparations used in this study were solubilized by pepsin treatment, it is likely that endostatin bind to the triple-helical part of these

TABLE 1
Interactions identified using surface plasmon resonance arrays

Molecule injected in buffer flow (analyte)	Molecule spotted on the array (ligand)
Interactions identified using SPR arrays	
Heparin	Endostatin
Heparin	Collagen XI
Heparan sulfate	Endostatin
Laminin-1	Endostatin
Laminin-1	NC1(XVIII)
Laminin-1	Heparin
Laminin-1	Heparan sulfate
Laminin-1	Dermatan sulfate
Laminin-1	Transglutaminase-2
Integrin $\alpha5\beta1$	Endostatin
Integrin $\alpha5\beta1$	NC1(XVIII)
Integrin $\alpha5\beta1$	Heparan sulfate
Integrin $\alpha5\beta1$	Transglutaminase-2
Integrin $\alpha5\beta1$	Heparin
Integrin $\alpha5\beta1$	Hyaluronan
Integrin $\alpha v\beta3$	Endostatin
Integrin $\alpha v\beta3$	NC1(XVIII)
Integrin $\alpha v\beta3$	Heparin
Integrin $\alpha v\beta3$	Collagen XI
Integrin $\alpha v\beta3$	Collagen VI
Integrin $\alpha v\beta3$	Transglutaminase-2
Collagen I	Biglycan
Collagen I	Transglutaminase-2
Collagen I	Endostatin
Collagen I	Heparan sulfate
Collagen I	Dermatan sulfate
Collagen I	Heparin
Collagen I	Chondroitin sulfate
Collagen IV	Transglutaminase-2
Collagen IV	Endostatin
Collagen IV	NC1(XVIII)
Collagen IV	Heparin
Collagen IV	Heparan sulfate
Collagen IV	Dermatan sulfate
Collagen IV	Biglycan
Collagen IV	Chondroitin sulfate
Collagen VI	Transglutaminase-2
Collagen VI	Endostatin
Collagen VI	NC1(XVIII)
Collagen VI	Heparin
Collagen VI	Heparan sulfate
Collagen VI	Dermatan sulfate
Collagen VI	Biglycan
Collagen VI	Chondroitin sulfate
Fibronectin	Heparin
SPARC	Endostatin
SPARC	NC1(XVIII)
SPARC	Heparin
SPARC	Dermatan sulfate
SPARC	Collagen I
SPARC	Collagen XI
Transglutaminase-2	Heparin
Transglutaminase-2	Collagen XI
Transglutaminase-2	Endostatin
Transglutaminase-2	NC1(XVIII)
Transglutaminase-2	Heparan sulfate
Endostatin	Collagen I
Endostatin	Chondroitin sulfate
Endostatin	Collagen VI
Endostatin	Heparin
Endostatin	Heparan sulfate
Endostatin	Amyloid peptide β-(1–42)
Endostatin	Dermatan sulfate
Endostatin	Transglutaminase-2
Endostatin	Biglycan
Amyloid peptide β-(1–42)	NC1(XVIII)
Amyloid peptide β-(1–42)	Biglycan
Amyloid peptide β-(1–42)	Collagen XI
Amyloid peptide β-(1–42)	Endostatin
Amyloid peptide β-(1–42)	Transglutaminase-2
Amyloid peptide β-(1–42)	Hyaluronan
Amyloid peptide β-(1–42)	Heparan sulfate
Amyloid peptide β-(1–42)	Heparin
Amyloid peptide β-(1–42)	Chondroitin sulfate
Amyloid peptide β-(1–42)	Dermatan sulfate
Thrombospondin-1	Collagen I
Thrombospondin-1	Collagen VI
Thrombospondin-1	Collagen XI
Thrombospondin-1	Chondroitin sulfate
Thrombospondin-1	Dermatan sulfate
Thrombospondin-1	Heparin
Thrombospondin-1	Heparan sulfate
Thrombospondin-1	Endostatin
Thrombospondin-1	NC1(XVIII)
Thrombospondin-1	Transglutaminase-2
Thrombospondin-1	Fibronectin
Thrombospondin-1	Biglycan
Thrombospondin-1	Amyloid peptide β-(1–42)
No interaction detected using SPR arrays	
Endostatin	Collagen XI
Endostatin	Fibronectin
Endostatin	Hyaluronan

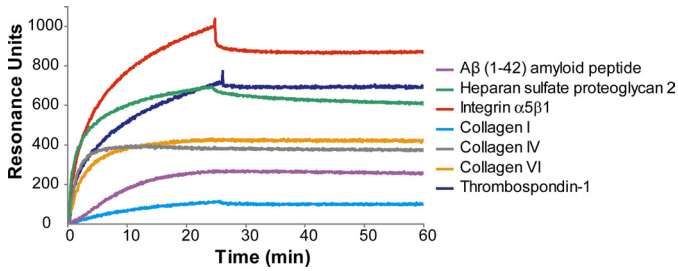

FIGURE 1. **SPR arrays.** Injection of collagens I (50 nM), IV (250 nM), and VI (250 nM), thrombospondin-1 (70 nM), heparan sulfate proteoglycan-2 (155 nM), and amyloid peptide (5 μM) over immobilized endostatin spotted onto a Gold Affinity chip.

collagen molecules. Endostatin did not bind to fibronectin, collagen XI, or hyaluronan (Table 1).

Direct interactions were also found between transglutaminase-2 and the β-amyloid peptide or transglutaminase-2 and $\alpha v\beta3$ integrin. Furthermore, collagen XI was identified as a new ligand of $\alpha v\beta3$ integrin, and was shown to bind the amyloid peptide Aβ-(1–42) (Table 1).

SPR assays were performed with mutants of the NC1 domain to determine the influence of well characterized mutations on the binding of these new partners to the trimeric NC1 domain. Mutations of two arginine residues Arg^{27} and Arg^{139} of the NC1 domain abolish the binding to heparin (32), whereas individuals homozygous for the D104N polymorphism might have a high risk of occurrence of sporadic breast cancer (33). Mutations of Arg^{27} and Arg^{139} decreased binding of the NC1 domain to collagen IV, collagen VI, and the amyloid peptide, suggesting that these two residues participate in these interactions. The D104N mutation, whether in monomeric endostatin or within the NC1 domain, did not significantly alter the ability to bind to heparin, collagens IV and VI, $\alpha5\beta1$ integrin, and heparan sulfate proteoglycan 2.

Analysis of Endostatin Network—To determine the major structural and functional features of the endostatin interaction network, the network was analyzed using the annotations provided by UniProtKB and InterPro. Annotations were available for the 26 protein partners of endostatin. Endostatin partners lacking annotations (endorepellin, zinc, glycosaminoglycans, and multimolecular complexes) were excluded from the analysis because they are not annotated in UniProtKB.

UniProtKB keywords were found to be present four times on average in the protein partners of endostatin. Only keywords

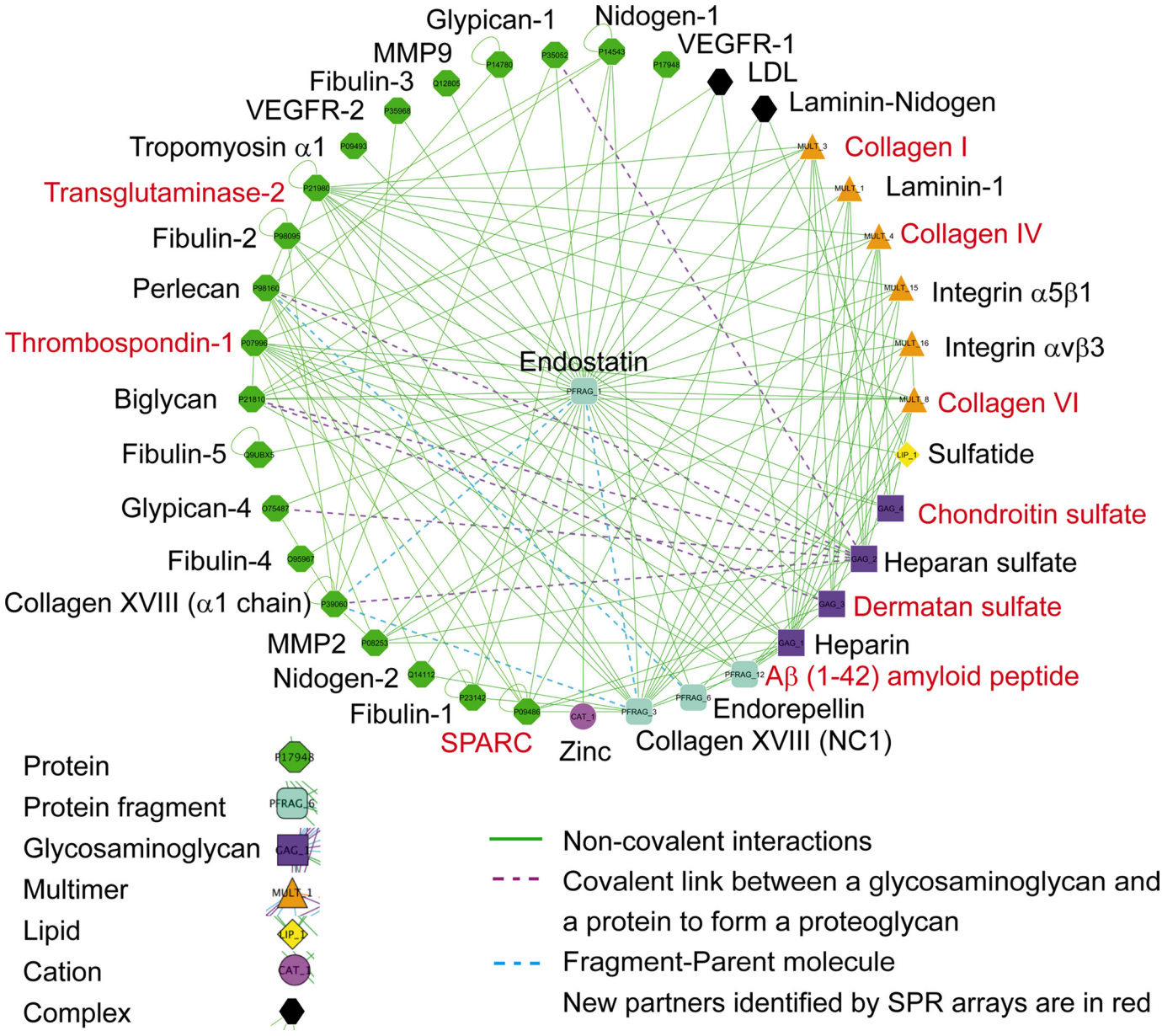

FIGURE 2. **The interaction network of endostatin.** New partners identified by SPR arrays are in *red*.

used more than four times in the network were thus taken into account for further analysis. We checked that the over-representation of a keyword within the network was not due to an over-representation in UniProtKB human entries. Protein partners of endostatin were frequently and significantly associated to the following keywords "basement membrane," "extracellular matrix," and "secreted" for the "cellular component category," to "calcium" for the "ligand category," to "cell adhesion" for the "biological process category," and to "EGF-like domain" for the "domain" category (Table 2).

The procedure described above was applied to InterPro to analyze the domain structure of the protein partners of endostatin (Table 3). InterPro domains were found to be present two times on average in the endostatin network. Only keywords used more than twice in the network were taken into account for further analysis. The most represented domains in the endostatin network were the EGF domain and its variants (EGF calcium-binding, EGF-like, EGF-like calcium-binding, EGF-like region conserved site, EGF-like type 3, and EGF-type aspartate/asparagine hydroxylation conserved site). They were present in 12 proteins over 26 (46%). Although numerous extracellular matrix proteins comprise EGF or EGF-like modules, the number of these modules was increased by at least a factor of 2 in the endostatin network. The network was also enriched in the concanavalin A-like lectin/glucanase domain (found in laminin-1, thrombospondin-1, and perlecan), in collagen triple helix repeats, and in the von Willebrand factor A domain.

DISCUSSION

A number of new endostatin partners have been identified by SPR arrays. Of course we cannot rule out the existence of further partners of endostatin as discussed below, but the coverage of interactomes (interaction networks) remains difficult to assess, and is a general concern for existing data sets (34).

TABLE 2
Use of keywords from UniProtKB in the protein partners of endostatin

Keyword category (UniProtKB)	Keyword	Number of annotations in endostatin partners (26)	Number of annotations in human proteins (19,398)	Use in endostatin partners	Use in human proteins	Ratio between endostatin partners and human proteins
				%		
Technical term	Three-dimensional structure	13	3,471	50.00	17.89	2.79
	Direct protein sequencing	17	2,608	65.38	13.44	4.86
Coding sequence diversity	Alternative splicing	10	7,307	38.46	37.67	1.02
	Polymorphism	21	10,670	80.77	55.01	1.47
Cellular component	Basement membrane	6	35	23.08	0.18	127.90
	Extracellular matrix	13	221	50.00	1.14	43.89
	Membrane	7	6,427	26.92	33.13	0.81
	Secreted	20	1,722	76.92	8.88	8.67
Ligand	Calcium	14	800	53.85	4.12	13.06
Biological process	Cell adhesion	9	425	34.62	2.19	15.80
Disease	Disease mutation	12	1,526	46.15	7.87	5.87
Domain	EGF-like domain	9	233	34.62	1.20	28.82
	Signal	23	3,459	88.46	17.83	4.96
Post-translation modification	Glycoprotein	24	4,425	92.31	22.81	4.05
	Phosphoprotein	8	6,540	30.77	33.71	0.91

TABLE 3
Use of InterPro keywords of the protein partners of endostatin

Term	Number of occurrence in endostatin partners (26)	Number of occurrence in human proteins (16,774)	Use in endostatin partners	Use in human proteins	Ratio between endostatin partners and human proteins
			%		
Collagen triple helix repeat	3	82	11.54	0.49	23.60
Concanavalin A-like lectin/glucanase, subgroup	3	76	11.54	0.45	25.47
EGF	7	206	26.92	1.23	21.92
EGF calcium binding	7	74	26.92	0.44	61.03
EGF-like	4	146	15.38	0.87	17.68
EGF-like calcium binding	7	101	26.92	0.60	44.71
EGF-like region, conserved site	12	276	46.15	1.65	28.05
EGF-like, type 3	9	232	34.62	1.38	25.03
EGF-type aspartate/asparagine hydroxylation conserved site	7	99	26.92	0.59	45.62
Immunoglobulin I-set	3	136	11.54	0.81	14.23
Immunoglobulin subtype 2	3	211	11.54	1.26	9.17
Immunoglobulin-like	3	709	11.54	4.23	2.73
Immunoglobulin-like fold	4	706	15.38	4.21	3.66
von Willebrand factor, type A	3	90	11.54	0.54	21.51

Endostatin is a component of basement membrane, and it interacts with collagen IV, a major component of basement membrane, and with collagen VI. Endostatin may link elastic fibers to collagen VI microfibrils in elastic tissues such as the aortic wall where endostatin is present (35). The binding of endostatin to collagen VI is in agreement with the fact that collagen VI is in contact with endothelial basement membranes. Collagen VI might anchor endothelial basement membranes not only by interacting with collagen IV (36), but also by interacting with endostatin.

Endostatin binds to several molecules participating in the control of angiogenesis. Besides integrins and heparan sulfate, it binds to an endogenous inhibitor of angiogenesis, endorepellin, a C-terminal domain of perlecan (37). Heparan sulfate proteoglycan-2 or perlecan, which plays a dual role in angiogenesis (38), also interacts with endostatin. Transglutaminase-2 is a new interacting partner of endostatin that is also involved in angiogenesis. The formation of a complex between this enzyme and VEGFR-2 has been proposed as a mechanism for modulation of endothelial cell response to VEGF (39). We have identified another endogenous inhibitor of angiogenesis, thrombospondin-1, as an interacting partner of endostatin. Thrombospondin-1 and endostatin share several properties. Both are found in platelets, bind to heparin, and are endogenous inhibitors of angiogenesis. Furthermore, the expression of thrombospondin-1 is up-regulated by endostatin (15), and thrombospondin-1 may act as a mediator of anti-angiogenic therapy (40). Endostatin also binds to SPARC, which blocks angiogenesis (41, 42) and is found as well as endostatin in platelets and basement membranes.

Interestingly, SPARC and thrombospondin-1 belong to the matricellular protein family. The term "matricellular" refers to a group of modular, extracellular proteins whose functions are achieved by binding to matrix proteins as well as to cell surface receptors, or to other molecules such as cytokines and proteases that interact, in turn, with the cell surface (43). Members of this protein class serve as biological mediators of cell function by interacting directly with cells or by modulating the activity of growth factors, proteases, and other extracellular matrix proteins (44). Endostatin might regulate cell adhesion not only via integrin signaling, but also in association with thrombospondin-1 and SPARC. Other matricellular proteins, such as members of the tenascin protein family and osteopontin, might also be able to interact with endostatin to control cell adhesion. The participation of the endostatin network to the control of cell adhesion is further supported by the fact that 46% of the protein partners of endostatin are annotated with a Gene Ontology term (45) referring to cell adhesion (data not shown).

We demonstrated that endostatin binds to the amyloid Aβ peptide, which has been previously shown to be co-localized with endostatin in amyloid plaques of patients with Alzheimer disease (18). Several other partners of endostatin are involved in neurodegenerative diseases. They include transglutaminase-2, which catalyzes the cross-linking of the amyloid peptide β (46), fibulin-1 (47), laminin-1 (48), thrombospondin-1 (49), heparan sulfate and heparan sulfate proteoglycans (50), perlecan (51), $\alpha5\beta1$ integrin (52), collagen IV (53), and collagen VI, an important component of the neuronal injury response (54). The binding of endostatin to the amyloid peptide could have a protective effect, as suggested for transthyretin (55), and for other extracellular components such as collagens VI (54) and XXV (56). Depending on the trigger event (*e.g.* increase in VEGF expression), and on the availability of its members in a given tissue at a given time, the endostatin network might be involved either in the control of angiogenesis, and tumor growth, or in neurogenesis and neurodegenerative diseases.

At the molecular level, the major features of the endostatin interaction network are the presence of EGF modules (*e.g.* in fibulins, nidogens, laminin-1, perlecan, thrombospondin-1, and integrins), and the ability of several partners to bind calcium (*e.g.* transglutaminase-2). This finding is supported by the fact that 50% of the protein partners of endostatin are annotated with the Gene Ontology term "calcium ion binding" (data not shown). Other extracellular proteins containing EGF modules might be potential partners of endostatin. We are currently studying the structure of EGF domains found in endostatin partners to identify possible common features, which will be helpful to select EGF domains of multidomain proteins for further interaction studies.

The interaction network will be useful for mimicking gene silencing studies and suppression *in silico* of glycosaminoglycans. This will be of special interest for glycosaminoglycans, which can be "suppressed" *in vivo* only by silencing several genes involved in their biosynthesis. It will also be of interest for the set-up of combination therapies, in which drugs targeting different pathways are simultaneously administered. The multifaceted nature of the angiogenic process suggests that the combination of antiangiogenic drugs might be more effective than single-agent therapies (57).

We combined extracellular protein-protein and protein-glycosaminoglycan interactions involving endostatin without discriminating their spatio-temporal expression. The next step will be to switch from a static to a dynamic extracellular interaction network by (i) putting weight on the interactions using kinetic and affinity constants to define the hierarchy of interactions according to their rate of formation and their stability, (ii) determining mutually exclusive interactions from three-dimensional structures and/or docking experiments, and (iii) integrating expression data. The building of a dynamic extracellular interaction network will be of interest to understand how information/signaling is conveyed through the network and to predict the consequences of perturbations due to changes in the expression of pro- and anti-angiogenic factors. The integrated network will be used as a framework to build a mathematical model of endostatin mechanism of action in various physio-pathological processes such as basement membrane assembly, angiogenesis, neurogenesis, and neurodegenerative diseases.

Acknowledgments—We thank C. Marquette and L. Blum (ICBMS, UMR 5246 CNRS, University Lyon 1, France) for providing access to the spotter. The support of the GE Healthcare staff (C. Quétard, P. Linden, T. Salomon, X. Rousselot, and A. Sylvan), who provided access to the Biacore Flexchip for the first experiments is gratefully acknowledged. We thank F. Ruggiero and M. C. Ronzière (UMR 5086, CNRS, University Lyon 1, France) for their generous gift of collagens IV, VI, and XI.

REFERENCES

1. Nyberg, P., Xie, L., and Kalluri, R. (2005) *Cancer Res.* **65,** 3967–3979
2. Folkman, J. (2006) *Exp. Cell Res.* **312,** 594–607
3. Digtyar, A. V., Pozdnyakova, N. V., Feldman, N. B., Lutsenko, S. V., and Severin, S. E. (2007) *Biochemistry* **72,** 235–246
4. Celik, I., Sürücü, O., Dietz, C., Heymach, J. V., Force, J., Höschele, I., Becker, C. M., Folkman, J., and Kisker, O. (2005) *Cancer Res.* **65,** 11044–11050
5. Tjin Tham Sjin, R. M., Naspinski, J., Birsner, A. E., Li, C., Chan, R., Lo, K. M., Gillies, S., Zurakowski, D., Folkman, J., Samulski, J., and Javaherian, K. (2006) *Cancer Gene Ther.* **13,** 619–627
6. Li, C., Harris, M. B., Venema, V. J., and Venema, R. C. (2005) *Biochem. Biophys. Res. Commun.* **329,** 873–878
7. Schmidt, A., Wenzel, D., Ferring, I., Kazemi, S., Sasaki, T., Hescheler, J., Timpl, R., Addicks, K., Fleischmann, B. K., and Bloch, W. (2004) *Dev. Dyn.* **230,** 468–480
8. Yamaguchi, N., Anand-Apte, B., Lee, M., Sasaki, T., Fukai, N., Shapiro, R., Que, I., Lowik, C., Timpl, R., and Olsen, B. R. (1999) *EMBO J.* **18,** 4414–4423
9. Ricard-Blum, S., Féraud, O., Lortat-Jacob, H., Rencurosi, A., Fukai, N., Dkhissi, F., Vittet, D., Imberty, A., Olsen, B. R., and van der Rest, M. (2004) *J. Biol. Chem.* **279,** 2927–2936
10. Rehn, M., Veikkola, T., Kukk-Valdre, E., Nakamura, H., Ilmonen, M., Lombardo, C., Pihlajaniemi, T., Alitalo, K., and Vuori, K. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 1024–1029
11. Faye, C., Moreau, C., Chautard, E., Jetne, R., Fukai, N., Ruggiero, F., Humphries, M. J., Olsen, B. R., and Ricard-Blum, S. (2009) *J. Biol. Chem.*, in press
12. Karumanchi, S. A., Jha, V., Ramchandran, R., Karihaloo, A., Tsiokas, L., Chan, B., Dhanabal, M., Hanai, J. I., Venkataraman, G., Shriver, Z., Keiser, N., Kalluri, R., Zeng, H., Mukhopadhyay, D., Chen, R. L., Lander, A. D., Hagihara, K., Yamaguchi, Y., Sasisekharan, R., Cantley, L., and Sukhatme, V. P. (2001) *Mol. Cell* **7,** 811–822
13. Kim, Y. M., Hwang, S., Kim, Y. M., Pyun, B. J., Kim, T. Y., Lee, S. T., Gho, Y. S., and Kwon, Y. G. (2002) *J. Biol. Chem.* **277,** 27872–27879
14. Cao, Y. (2008) *Adv. Cancer Res.* **100,** 113–131
15. Abdollahi, A., Hahnfeldt, P., Maercker, C., Gröne, H. J., Debus, J., Ansorge, W., Folkman, J., Hlatky, L., and Huber, P. E. (2004) *Mol. Cell* **13,** 649–663
16. Chautard, E., Thierry-Mieg, N., and Ricard-Blum, S. (2009) *Pathol. Biol.* **57,** 324–333
17. Marneros, A. G., and Olsen, B. R. (2005) *FASEB J.* **19,** 716–728
18. Deininger, M. H., Fimmen, B. A., Thal, D. R., Schluesener, H. J., and Meyermann, R. (2002) *J. Neurosci.* **22,** 10621–10626
19. Pufe, T., Petersen, W. J., Miosge, N., Goldring, M. B., Mentlein, R., Varoga, D. J., and Tillmann, B. N. (2004) *Matrix Biol.* **2,** 267–276
20. St. Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., and Kinzler, K. W. (2000) *Science* **289,** 1197–1202
21. Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003) *Genome Res.* **13,** 2498–2504
22. Chautard, E., Ballut, L., Thierry-Mieg, N., and Ricard-Blum, S. (2009) *Bioinformatics* **25,** 690–691

23. UniProt Consortium (2009) *Nucleic Acids Res.* **37,** D169–D174
24. Durbin, R., and Thierry-Mieg, J. (1994) in *Computational Methods in Genome Research* (Suhai, S., ed) pp. 45–55, Plenum Press, New York
25. Hunter, S., Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Binns, D., Bork, P., Das, U., Daugherty, L., Duquenne, L., Finn, R. D., Gough, J., Haft, D., Hulo, N., Kahn, D., Kelly, E., Laugraud, A., Letunic, I., Lonsdale, D., Lopez, R., Madera, M., Maslen, J., McAnulla, C., McDowall, J., Mistry, J., Mitchell, A., Mulder, N., Natale, D., Orengo, C., Quinn, A. F., Selengut, J. D., Sigrist, C. J., Thimma, M., Thomas, P. D., Valentin, F., Wilson, D., Wu, C. H., and Yeats, C. (2009) *Nucleic Acids Res.* **37,** D211–D215
26. Sasaki, T., Fukai, N., Mann, K., Göhring, W., Olsen, B. R., and Timpl, R. (1998) *EMBO J.* **17,** 4249–4256
27. Ricard-Blum, S., Peel, L. L., Ruggiero, F., and Freeman, N. J. (2006) *Anal. Biochem.* **352,** 252–259
28. Ricard-Blum, S., Beraud, M., Raynal, N., Farndale, R. W., and Ruggiero, F. (2006) *J. Biol. Chem.* **281,** 25195–25204
29. Javaherian, K., Park, S. Y., Pickl, W. F., LaMontagne, K. R., Sjin, R. T., Gillies, S., and Lo, K. M. (2002) *J. Biol. Chem.* **277,** 45211–45218
30. Akimov, S. S., Krylov, D., Fleischman, L. F., and Belkin, A. M. (2000) *J. Cell Biol.* **148,** 825–838
31. Wiberg, C., Heinegård, D., Wenglén, C., Timpl, R., and Mörgelin, M. (2002) *J. Biol. Chem.* **277,** 49120–49126
32. Sasaki, T., Larsson, H., Kreuger, J., Salmivirta, M., Claesson-Welsh, L., Lindahl, U., Hohenester, E., and Timpl, R. (1999) *EMBO J.* **18,** 6240–6428
33. Lourenço, G. J., Cardoso-Filho, C., Gonçales, N. S., Shinzato, J. Y., Zeferino, L. C., Nascimento, H., Costa, F. F., Gurgel, M. S., and Lima, C. S. (2006) *Breast Cancer Res. Treat.* **100,** 335–338
34. Venkatesan, K., Rual, J. F., Vazquez, A., Stelzl, U., Lemmens, I., Hirozane-Kishikawa, T., Hao, T., Zenkner, M., Xin, X., Goh, K. I., Yildirim, M. A., Simonis, N., Heinzmann, K., Gebreab, F., Sahalie, J. M., Cevik, S., Simon, C., de Smet, A. S., Dann, E., Smolyar, A., Vinayagam, A., Yu, H., Szeto, D., Borick, H., Dricot, A., Klitgord, N., Murray, R. R., Lin, C., Lalowski, M., Timm, J., Rau, K., Boone, C., Braun, P., Cusick, M. E., Roth, F. P., Hill, D. E., Tavernier, J., Wanker, E. E., Barabási, A. L., and Vidal, M. (2009) *Nat. Methods* **6,** 83–90
35. Miosge, N., Sasaki, T., and Timpl, R. (1999) *FASEB J.* **13,** 1743–1750
36. Kuo, H. J., Maslen, C. L., Keene, D. R., and Glanville, R. W. (1997) *J. Biol. Chem.* **272,** 26522–26529
37. Mongiat, M., Sweeney, S. M., San Antonio, J. D., Fu, J., and Iozzo, R. V. (2003) *J. Biol. Chem.* **278,** 4238–4249
38. Bix, G., and Iozzo, R. V. (2008) *Microsc. Res. Tech.* **71,** 339–348
39. Dardik, R., and Inbal, A. (2006) *Exp. Cell Res.* **312,** 2973–2982
40. Bocci, G., Francia, G., Man, S., Lawler, J., and Kerbel, R. S. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 12917–12922
41. Chlenski, A., Liu, S., Crawford, S. E., Volpert, O. V., DeVries, G. H., Evangelista, A., Yang, Q., Salwen, H. R., Farrer, R., Bray, J., and Cohn, S. L. (2002) *Cancer Res.* **62,** 7357–7363
42. Chlenski, A., Liu, S., Guerrero, L. J., Yang, Q., Tian, Y., Salwen, H. R., Zage, P., and Cohn, S. L. (2006) *Int. J. Cancer* **118,** 310–316
43. Bornstein, P. (1995) *J. Cell Biol.* **130,** 503–506
44. Alford, A. I., and Hankenson, K. D. (2006) *Bone* **38,** 749–757
45. Ashburner, M., Ball, C. A., Blake, J. A., Botsein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., Sherlock, G. (2000) *Nat. Genet.* **25,** 25–29
46. Wang, D. S., Dickson, D. W., and Malter, J. S. (2008) *Int. J. Clin. Exp. Pathol.* **1,** 5–18
47. Timpl, R., Sasaki, T., Kostka, G., and Chu, M. L. (2003) *Nat. Rev. Mol. Cell Biol.* **4,** 479–489
48. Morgan, C., Bugueño, M. P., Garrido, J., and Inestrosa, N. C. (2002) *Peptides* **23,** 1229–1240
49. Buée, L., Hof, P. R., Roberts, D. D., Delacourte, A., Morrison, J. H., and Fillit, H. M. (1992) *Am. J. Pathol.* **141,** 783–788
50. van Horssen, J., Wesseling, P., van den Heuvel, L. P., de Waal, R. M., and Verbeek, M. M. (2003) *Lancet Neurol.* **2,** 482–492
51. Castillo, G. M., Ngo, C., Cummings, J., Wight, T. N., and Snow, A. D. (1997) *J. Neurochem.* **69,** 2452–2465
52. Matter, M. L., Zhang, Z., Nordstedt, C., and Ruoslahti, E. (1998) *J. Cell Biol.* **141,** 1019–1030
53. Kiuchi, Y., Isobe, Y., and Fukushima, K. (2002) *Life Sci.* **70,** 1555–1564
54. Cheng, J. S., Dubal, D. B., Kim, D. H., Legleiter, J., Cheng, I. H., Yu, G. Q., Tesseur, I., Wyss-Coray, T., Bonaldo, P., and Mucke, L. (2009) *Nat. Neurosci.* **12,** 119–121
55. Buxbaum, J. N., Ye, Z., Reixach, N., Friske, L., Levy, C., Das, P., Golde, T., Masliah, E., Roberts, A. R., and Bartfai, T. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 2681–2686
56. Osada, Y., Hashimoto, T., Nishimura, A., Matsuo, Y., Wakabayashi, T., and Iwatsubo, T. (2005) *J. Biol. Chem.* **280,** 8596–8605
57. Abdollahi, A., Lipson, K. E., Sckell, A., Zieher, H., Klenke, F., Poerschke, D., Roth, A., Han, X., Krix, M., Bischof, M., Hahnfeldt, P., Grone, H. J., Debus, J., Hlatky, L., and Huber, P. E. (2003) *Cancer Res.* **63,** 8890–8898