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Characterization of Annexin A1 Glycan Binding Reveals Binding to Highly Sulfated Glycans with Preference for Highly Sulfated Heparan Sulfate and Heparin

T. Horlacher,[†,‡] C. Noti,[‡] J. L. de Paz,[‡,#] P. Bindschädler,[‡] M.-L. Hecht,[†,‡] D. F. Smith,[§] M. N. Fukuda,[∥] and P. H. Seeberger*[,†,‡,∥,⊥]

[†]Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, D-14476 Potsdam, Germany

[‡]Federal Institute of Technology (ETH) Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland

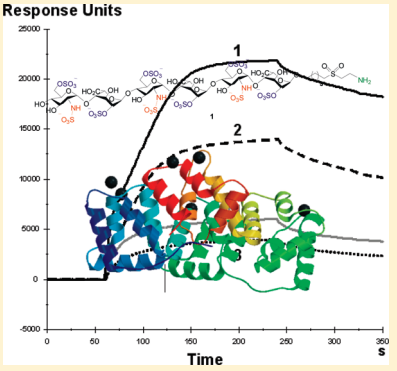
[§]Emory University School of Medicine, Atlanta, Georgia 30322, United States

[∥]Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, United States

[⊥]Freie Universität Berlin, Arnimallee 22, D-14195 Berlin, Germany

Supporting Information

ABSTRACT: Annexin A1 is a multifunctional, calcium-dependent phospholipid binding protein involved in a host of processes including inflammation, regulation of neuroendocrine signaling, apoptosis, and membrane trafficking. Binding of annexin A1 to glycans has been implicated in cell attachment and modulation of annexin A1 function. A detailed characterization of the glycan binding preferences of annexin A1 using carbohydrate microarrays and surface plasmon resonance served as a starting point to understand the role of glycan binding in annexin A1 function. Glycan array analysis identified annexin A1 binding to a series of sulfated oligosaccharides and revealed for the first time that annexin A1 binds to sulfated non-glycosaminoglycan carbohydrates. Using heparin/heparan sulfate microarrays, highly sulfated heparan sulfate/heparin were identified as preferred ligands of annexin A1. Binding of annexin A1 to heparin/heparan sulfate is calcium- but not magnesium-dependent. An in-depth structure−activity relationship of annexin A1−heparan sulfate interactions was established using chemically defined sugars. For the first time, a calcium-dependent heparin binding protein was characterized with such an approach. *N*-Sulfation and 2-*O*-sulfation were identified as particularly important for binding.



Annexins are a class of homologous, calcium-dependent phospholipid binding proteins and are present in many unicellular eukaryotes and most multicellular organisms.[1−3] All 12 known mammalian annexins contain a conserved C-terminal annexin domain termed the core that is comprised of four (in case of annexin A6, eight) characteristic repetitive domains.[2] This core domain contains the binding sites for calcium and mediates binding to phospholipids.[4] The preceding N-terminal domain (often called N-terminal tail) is variable in length and sequence for each annexin and is responsible for most unique functions of different annexins.[1] Annexin N-terminal tails mediate most protein interactions, and many contain phosphorylation sites. Annexins are involved in various biological processes including membrane trafficking and organization, membrane−cytoskeleton interactions, inflammation, coagulation, apoptosis, cell growth, and differentiation.[1]

Annexin A1 is a prominent, characteristic member of the annexin family containing an annexin core domain of four repeats that mediates binding to phospholipids in a calcium-dependent manner and a short N-terminal tail that mediates several protein interactions and contains phosphorylation sites.[5,6] At low calcium levels which reflect the intracellular situation, the N-terminal tail domain is buried in the core domain where it is inaccessible. The N-terminal tail is expelled at high calcium levels which are characteristic of extracellular or raised intracellular calcium levels.[7] Annexin A1 is a multifunctional protein acting inside and outside the cell and is involved in several, apparently unrelated processes including inflammation, regulation of neuroendocrine signaling, apoptosis, and membrane trafficking.[8] The exact molecular mode of action for most annexin A1 functions remains elusive. Originally, annexin A1 was identified as a mediator of glucocorticoid action and mediates many antiinflammatory events.[9] Recently, annexin A1 has been identified as a specific tumor marker on endothelial cells beneath a tumor.[10] Subsequent investigations revealed that annexin A1 is an angiogenic factor that is prominently involved in blood vessel growth in adults and particularly important for angiogenesis in tumorigenesis and wound healing.[11]

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None of the annexins carry signal sequences for the classical export pathway. Nevertheless, prominent extracellular localization and functions of several annexins have been established including annexin A1 blockage of leukocyte extravasion, neuroendocrine hormone regulation, and mediation of angiogenesis.[1] Extracellular annexin A1 often acts in an autocrine, paracrine, or juxtacrine manner and remains attached to cell surfaces.[10,12,13] Annexin A1 cell surface attachment might result from binding to phospholipids, but annexin A1 is specific for anionic phospholipids that are commonly not present on the extracellular membrane leaflet.[1] Thus, in most processes involving extracellular annexin A1, other ligands presumably mediate cell surface binding and localization. Glycans are apparent target ligands to mediate annexin A1 attachment to cell surfaces. Binding of annexin A1 to carboxylated glycans on endothelial cells has been demonstrated and participates in neutrophil adhesion and migration.[14] Annexin A1−heparin binding was used to purify the protein, but the fine specificities of the interaction have never been investigated.[15] Glycosaminoglycan interactions of other annexins have been elucidated more thoroughly including specificity analyses and cocrystal structures;[16−19] however, detailed biochemical characterization of heparin/heparan sulfate structure−activity relationships is missing. Therefore, we analyzed annexin A1−glycan binding in depth with a particular focus on heparin/heparan sulfate interactions which revealed to be important, preferred glycan ligands of annexin A1.

Heparan sulfate, a prominent glycosaminoglycan, is a linear polysaccharide comprised of the basic repeating unit of glucuronic acid and *N*-acetylglucosamine.[20] The polysaccharide is heavily modified in a cell type- and development-dependent manner, including epimerization, deacetylation, *N*-sulfation, and *O*-sulfations.[20] The nontemplate driven synthesis results in an extreme microheterogeneity regarding modification patterns even within one glycosaminoglycan chain. Heparan sulfate is attached to proteins forming proteoglycans and is present on most mammalian cells and in the extracellular matrix. The closely related heparin is a derivative of heparan sulfate whose carbohydrate chain is extensively modified and partially degraded. Heparin is mainly released from mast cells in the form of free polysaccharide chains.

Heparin and heparan sulfate interact with dozens of proteins in organisms thereby modulating the activity of bound proteins.[21,22] The structure−activity relationship and the biological function of most heparin/HS protein interactions remain elusive.[23−26] However, in order to understand the effect of the glycan interaction and to identify respective target cells, the exact glycan structures that are bound by proteins have to be identified. Distinct modification patterns on heparan sulfate are only expressed on specific cell types, thereby regulating the responsiveness of cells toward heparin/heparan sulfate binding proteins.[23]

Determination of glycosaminoglycan binding preferences for a given protein is still far from routine due to microheterogeneity, high electrostatically driven background binding, and cross-reactivity[23,24] and requires special screening tools.[27] In most cases, activity assays to monitor heparan sulfate function are not feasible. Here, we investigated general glycan binding preferences of annexin A1 using carbohydrate and heparin microarrays.[28−33] The results revealed that annexin A1 broadly binds to sulfated glycans with highly charged heparin/heparan sulfate being among the preferred ligands. We thus determined the structure−activity relationship of annexin A1 heparin/heparan sulfate binding using microarrays as well as quantitative and qualitative surface plasmon resonance[34] approaches based on a collection of synthetic heparin oligosaccharides. This study characterized the calcium-dependent binding of annexin A1 to structurally defined, chemically synthesized heparin/heparan sulfate-related oligosaccharides. Dissociation constants for binding to closely related heparan sulfate sequences were also determined using SPR. *N*-Sulfation and 2-*O*-sulfation were identified as particularly important modifications for binding, thereby explaining distinct responsiveness of cells to annexin A1. The observations made for annexin A1 may be representative for other, closely related annexins.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), if not stated otherwise. Reagents for SPR measurements were acquired from GE Healthcare (Zurich, Switzerland). PAA-glycans were obtained from the Consortium of Functional Glycomics (San Diego, CA).

Purification of Annexin A1. Recombinant annexin A1 protein was produced as a C-terminal His6 tag fusion protein in bacteria. Full-length mouse annexin A1 cDNA was obtained from Invitrogen (Carlsbad, CA, USA). The cDNA was subcloned in pET29A vector for C-terminal His tag in frame, and annexin A1-His fusion protein was expressed in bacteria BL21(DE3) and induced by 1 mM isopropyl thiogalactoside. The fusion protein was extracted from bacterial pellet with 20 mM Tris buffer, pH 7.9, containing 500 mM NaCl and 4 mM imidazole. The extract was bound to Ni-NTA beads and washed with 20 mM Tris buffer containing 250 mM NaCl, and bound materials were eluted with 20 mM buffer containing 250 mM NaCl and 300 mM imidazole. Purified annexin A1-His protein was dialyzed against 20 mM Tris buffer, pH 7.9, containing 250 mM NaCl.

Synthesis of Heparin Oligosaccharides. Amine-functionalized heparin oligosaccharides were synthesized as described.[30,35,36] An amine-terminated linker was placed at the reducing end of the synthetic structures to allow for immobilization onto *N*-hydroxysuccinimide activated glass slides and on SPR chips. Key features of this modular synthesis are the use of 2-azidoglucose trichloroacetimidates as glycosylating agents for oligosaccharide assembly and the compatibility of the protecting group strategy with the sulfation−deprotection steps.

Glycan Arrays. Glycan microarrays were printed as described.[28,37,38] Version v2.1 of the printed microarray from the Consortium for Functional Glycomics (www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml) was used for analysis. Annexin A1 fused to His6 protein was diluted to 0.2 mg/mL in TBS-CM (20 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) containing 1% BSA and 0.05% Tween-20. An aliquot (70 μL) was applied to a microarray slide and incubated under a coverslip for 60 min in a humidified chamber at room temperature. Coverslips were then gently removed in a solution of TBS-CM/0.05% Tween-20 and subjected to the TBS standard wash procedure. To detect protein binding, the slide was similarly incubated in the dark with mouse anti-His IgG labeled with Alexa 488 (Qiagen) at 5 μg/mL, washed as described above, and washed with deionized water to remove salts. The slide was then spun in a slide centrifuge for approximately 15 s to dry and immediately scanned in a PerkinElmer ProScanArray scanner using an excitation

wavelength of 488 nm and ImaGene software (BioDiscovery, Inc., El Segundo, CA) to quantify fluorescence. Data are reported as average relative fluorescence units (RFU) of four of six replicates (after removal of highest and lowest values) for each glycan represented on the array.

Fabrication of Heparin Microarrays. Amine-linked heparin oligosaccharides were dissolved and diluted in 50 mM sodium phosphate (pH 8.5). Per spot, 1 nL of the oligosaccharide solution in according dilution was printed onto NHS-ester activated CodeLink slides (GE Healthcare, Switzerland) using an automated printing robot (Scienion, Berlin, Germany). Slides were incubated in a humid chamber to complete immobilization reactions and stored in a desiccator until usage.

Incubation of Heparin Micorarrays. Heparin microarray slides were washed three times with water and quenched in 100 mM ethanolamine and 50 mM sodium phosphate (pH 8.5) for 1 h at 50 °C. Quenched slides were washed three times with water and centrifuged to dryness. Slides were blocked with 2.5% BSA in HBS-N (10 mM HEPES, pH 7.4, 150 mM sodium chloride) with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% (v/v) Tween-20 for 1 h at room temperature, washed twice for each 10 min in TBS-CM (20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$), and centrifuged with 200*g* for 5 min at room temperature. His-tagged annexin A1 was dissolved in HBS-N with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% (v/v) Tween-20 and incubated on the slides for 1 h at room temperature. Afterward, slides were washed as described above and centrifuged with 200*g* for 5 min at room temperature. A fluorescence-labeled anti-His antibody (10 μg/mL; Quiagen, Hombrechtikon, Switzerland) was subsequently incubated on the slide in a same buffer (HBS-N with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.01% (v/v) Tween-20) and in a similar fashion. After two washes with TBS-CM and centrifugation with 200*g* for 5 min at room temperature, slides were scanned using a fluorescence microarray scanner (Tecan, Männedorf, Switzerland). Fluorescence intensities were evaluated using the Genespotter software (Microdiscovery, Berlin, Germany).

Heparin Oligosaccharide Immobilization of SPR CM5 Chips. Heparin oligosaccharides were immobilized on CM5 chips (GE Healthcare, Switzerland) using primary amine coupling according to manufacturer's instructions (GE Healthcare, Switzerland). HBS-N (10 mM HEPES, pH 7.4, 150 mM sodium chloride) was used as running buffer. In brief, the chip surface was activated by an injection of a solution of 50 mM *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide for 8 min at a flow rate of 10 μL/min. The oligosaccharides (10 μM in PBS with 1 mM hexadecyltrimethylammonium chloride) were flowed over the activated surface at a flow rate of 5 μL/min until the desired change in the response units was reached. Remaining active groups on the chip surface were quenched by an injection of 1 M ethanolamine hydrochloride (pH 8.5) for 7 min at a flow rate of 10 μL/min.

Sample Injection and Surface Regeneration. If not stated otherwise, HBS-N (10 mM HEPES, pH 7.4, 150 mM sodium chloride) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was used as running buffer and sample buffer. In case of the determination of the annexin A1 calcium dependency, HBS-N was used as running and sample buffer. Annexin A1 was diluted in sample buffer with or without heparin (10 μg/mL final concentration), chondroitin sulfate (10 μg/mL final concentration), heparan sulfate (10 μg/mL final concentration), or arixtra (fondaparinux sodium; GlaxoSmithKline, Germany; 10 μg/mL final concentration). For the determination of the calcium and sodium chloride dependency of annexin A1−heparin binding, the concentration of $CaCl_2$ and NaCl was varied in the respective sample buffers. Samples were injected for 3−5 min to facilitate binding. After the injection and a dissociation period, solutions of 0.2% (w/v) sodium dodecyl sulfate or 1 M sodium chloride were injected to remove all bound proteins.

Annexin A1 Immobilization and Injections Using NTA Chips. His-tagged annexin A1 was immobilized onto NTA chip (GE Healthcare, Switzerland) surfaces according to the manufacturer's protocol (GE Healthcare, Switzerland). HBS-EP (10 mM HEPES, pH 7.4, 150 mM sodium chloride, 50 μM EDTA, 0.05% (v/v) Tween-20) was used as running buffer. In brief, the chip surface was preprimed by an injection of 500 μM nickel chloride in running buffer. Next, the protein (1 μM annexin A1 in running buffer) was flowed over the surface to facilitate binding. Carbohydrates in HBS-N with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were injected for 3 min to test binding. After the injections and a dissociation period, the surface was regenerated by injection of regeneration solution (10 mM HEPES, pH 7.4, 150 mM sodium chloride, 35 mM ethylenediaminetetraacetic acid (EDTA), 0.05% (v/v) Tween-20) that removes all His-tag bound protein from the chip.

RESULTS

Annexin A1 Glycan Binding. Annexin A1 was incubated on glycan arrays to determine the general annexin A1 glycan binding preferences (Figure 1 and Supporting Information Figure S1). The following general binding themes for annexin A1−glycan interactions were observed: annexin A1 bound several highly sulfated glycans, mainly β1−4-linked *N*-acetyllactosamine (type 2 LacNAc) and lactose that are sulfated two or three times at the 3′- and/or 6′-hydroxyl groups. A triple sulfated LacNAc ($[3OSO_3^-][6OSO_3^-]$Galβ1−4$[6OSO_3^-]$GlcNAcβ) was revealed to be the best binder among the pure carbohydrate ligands. Annexin A1 also bound to sialylated and neutral glycans. This finding is consistent with previous reports on annexin A1 binding to carboxylated glycans[14] and to the selectin ligand mimicking I-peptide.[39] Hence, annexin A1 binds two types of glycans probably via distinct binding mechanisms: highly sulfated glycans and weakly acidic and fucosylated glycans. Here we describe annexin A1 binding to sulfated glycan, in particular to heparin/heparan sulfates.

Annexin A1 Binds to Heparin/Heparan Sulfate. Besides sulfated glycans, glucuronic acid was among the best recognized glycans on the array (Figure 1 and Supporting Information Figure S1), suggesting that annexin A1 binds glycosaminoglycans and in particular to heparin/HS since annexin A1 binding to heparin columns has been exploited to purify the protein. Furthermore, binding of annexins A2 and A5 to heparin is well established.[16,19] Sequence comparison of human annexin A1 with human annexin A2 and rat annexin A5 revealed that annexins A1 and A2 are highly conserved in the amino acid sequence relevant to heparin binding (for alignment see Supporting Information Figure S2) with only one amino acid of the heparin binding region of annexin A2 (Lys^{280}-Val) being altered in annexin A1.

To determine the binding preference of annexin A1 for heparin experimentally, we incubated the His-tagged protein on heparin microarrays in the presence or absence of heparin. A fluorescence-labeled anti-His antibody was used for detection in

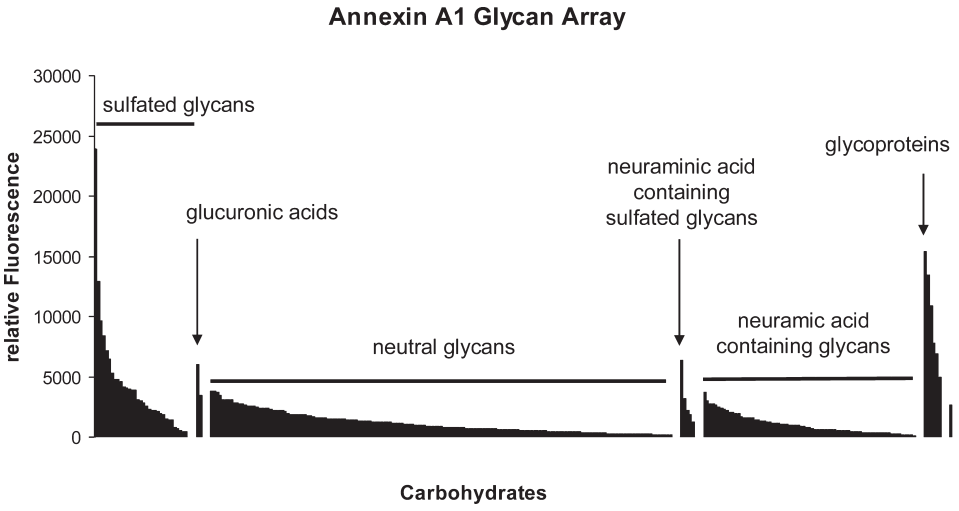

Figure 1. Annexin A1 binds sulfated oligosaccharides. Binding of glycan arrays by annexin A1-His. Glycan structures listed starting from the strongest binder are as follows: [3OSO_3^-][6OSO_3^-]Galβ1−4[6OSO_3^-]GlcNAcβ-Sp0; ceruloplasmin; AGP-βl; [3OSO_3^-]Galβ1−4(6OSO_3^-)Glcβ−Sp8; transferrin; [6OSO_3^-]Galβ1−4[6OSO_3^-]Glcβ-Sp8; [3OSO_3^-]Galβ1−4(6OSO_3^-)Glcβ−Sp0; AGP; [3OSO_3^-]Galβ1−4[6OSO_3^-]GlcNAcβ-Sp8; AGP-A; 39, [4OSO_3^-][6OSO_3^-]Galβ1−4GlcNAcβ-Sp0; Neu5Acα2−6Galβ1−4[6OSO_3^-]GlcNAcβ-Sp8.

a subsequent incubation step. Annexin A1 bound several heparin oligosaccharides (Figure 2) as well as isolated heparin on the array (Figure 3A). Coincubation with heparin blocked binding, thereby demonstrating sugar-specific binding (inlay, Figure 3A). Before the heparin/heparan sulfate sequence specificity of annexin A1 was analyzed (discussed in a subsequent section), we first validated binding of annexin A1 to heparin/heparan sulfate of annexin A1 and determined general annexin A1−glycosaminoglycan binding preferences and the corresponding binding conditions. Protein binding to heparin needs careful examination and verification due to high cross-reactivity and common background binding which result from the high negative charge of heparin.

Binding of annexin A1 to heparin/heparan sulfate was validated using surface plasmon resonance (SPR) measurements. His-tagged annexin A1 was immobilized on nitrilotriacetic acid (NTA) modified SPR chips. Heparin was flowed over immobilized annexin A1. Heparin bound annexin A1 in a concentration-dependent manner (Figure 3B) and confirmed the interaction of annexin A1 with highly sulfated heparan sulfate/heparin. In addition, annexin A1 bound to immobilized heparin oligosaccharides (see subsequent sections), thereby further validating annexin A1−heparin binding. In contrast, other free carbohydrates (LacNAc, Le^X, Le^A, Le^Y, blood group antigen H1 and H2; data not shown) as well as PAA immobilized Le^A and LacNAc, which were well bound on the glycan array, did not interact with the immobilized annexin A1 as determined by SPR (Figure 3B). In the following, we thus focused on annexin A1−glycosaminoglycan interactions, whereas other potential annexin A1−glycan interactions were not further investigated.

Annexin A1−Glycosaminoglycan Binding Preferences. The glycosaminoglycan binding preferences of annexin A1 were determined using SPR to obtain the glycosaminoglycan specificity of annexin A1 and to analyze potential cross-reactivities. Initially, His-tagged annexin A1 was immobilized on a NTA chip before solutions of different glycosaminoglycans (5 or 50 μg/mL) were flowed over the chip (Figure 4A). Heparin bound strongly to annexin A1 even at low concentrations (5 μg/mL), whereas chondroitin sulfate, heparan sulfate, and arixtra,[40] a highly sulfated heparin pentasaccharide, bound less well even at higher concentrations. To verify these results, we immobilized hexasaccharide **1** on a SPR chip since this oligosaccharide was well recognized by annexin A1 on the heparin microarrays. Annexin A1 was injected in the presence or absence of 10 μg/mL heparin, heparan sulfate, chondroitin sulfate, and the heparin-like pentasaccharide arixtra, respectively. Annexin A1 bound hexasaccharide **1** to result in a strong, readily detectable signal (Figure 4B). Heparin reduced binding of annexin A1 to background level when coinjected while arixtra, a highly sulfated synthetic pentasaccharide anticoagulant, acted only as a weak inhibitor. Hence, not only the number of sulfates but also spacing and backbone conformation are critical for heparin−annexin A1 binding. Heparan sulfate and chondroitin sulfate moderately decreased binding to the immobilized hexasaccharide (Figure 4B). These findings underscore annexin A1's preference for highly sulfated heparin-like glycosaminoglycans.

Annexin A1 Heparin/HS Binding Conditions. We determined the conditions required for annexin A1 to bind heparin oligosaccharide **1**. Presumably, these conditions are representative for interactions of this protein with glycosaminoglycans and other types of sulfated glycans. To this end, heparin hexasaccharide **1** and monosaccharide **11** (as control) were immobilized on a SPR chip. Annexin A1 was flowed over the surfaces under various conditions (Figure 5A) revealing calcium-, pH-, and salt-dependent binding. Annexin A1 injected in standard binding buffer (10 mM HEPES and 150 mM NaCl with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) bound strongly to the immobilized hexasaccharide **1**. In the absence of divalent cations and the presence of EDTA, annexin A1 binding was abolished. Addition of calcium restored annexin A1 binding, while annexin A1 did not bind **1** in the presence of magnesium ions alone. By injecting annexin A1 with varying calcium concentrations, an EC_{50} of 281 μM for calcium was determined (Figure 5B).

SPR experiments measuring annexin A1 binding at pH 5.0 (10 mM HEPES, pH 5.0, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) revealed reduced binding levels (Figure 5A). At elevated

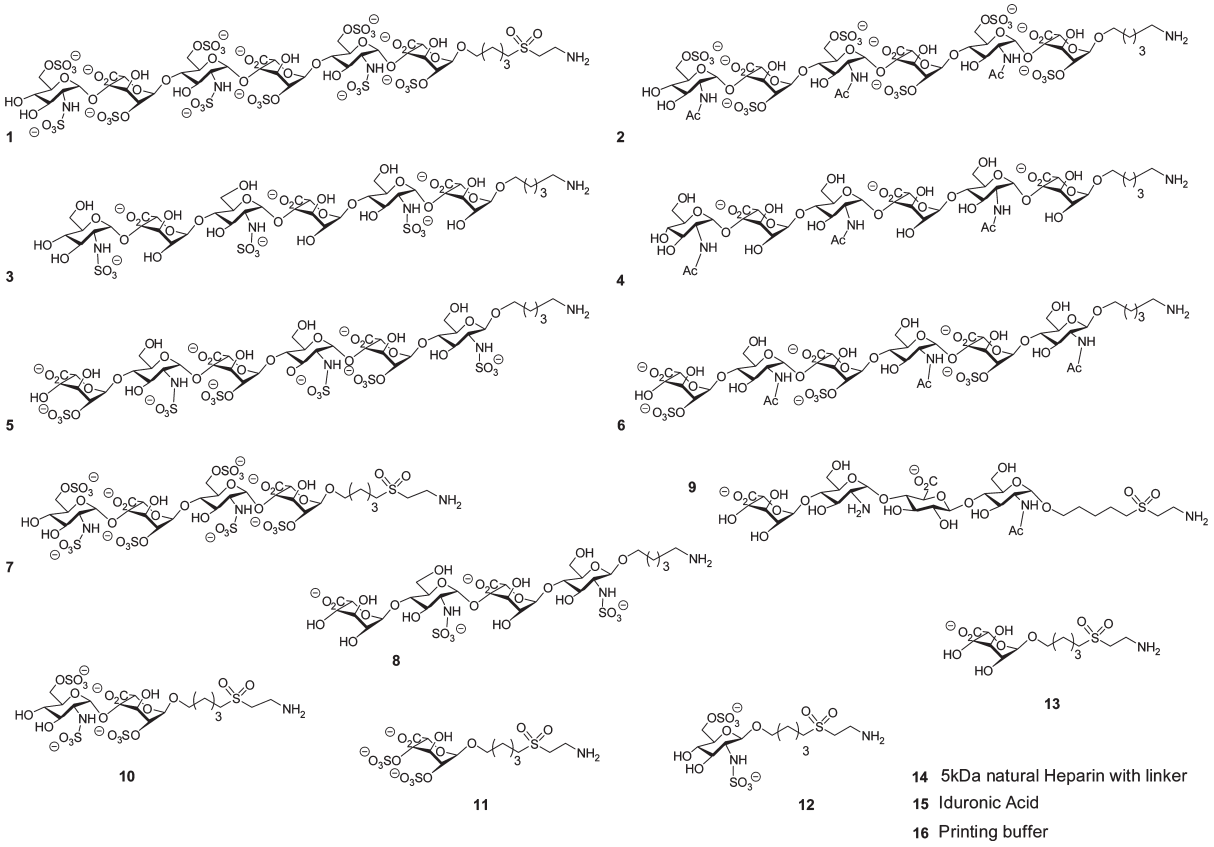

Figure 2. Heparin oligosaccharide library containing 13 synthetic heparin oligosaccharides and heparin from natural sources.

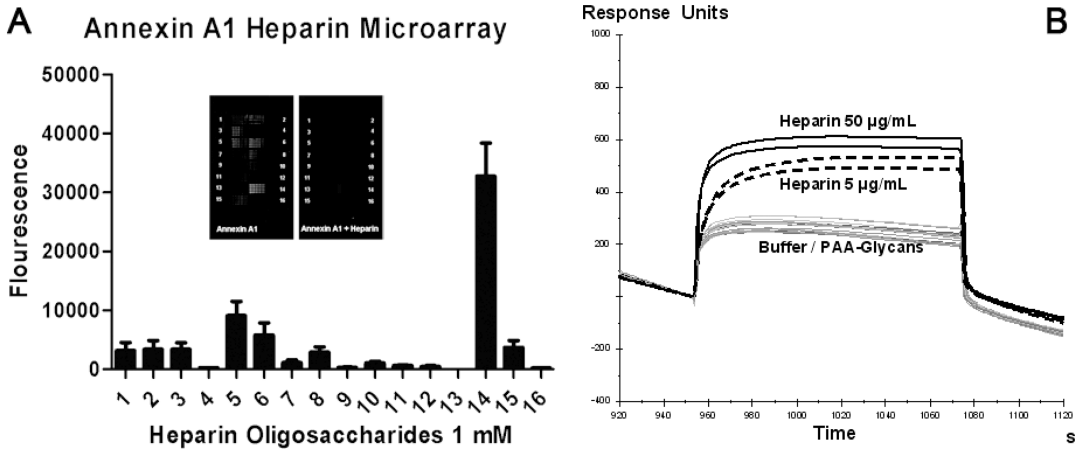

Figure 3. Annexin A1 binds to heparin/heparan sulfate oligosaccharides. (A) Annexin A1 binds to heparin and heparan sulfate oligosaccharides immobilized on microarrays. Annexin A1 (10 μg/mL) was incubated on heparin microarrays bearing heparin oligosaccharides **1**−**16**. Bound protein was detected using a fluorescence-labeled anti-His-tag antibody. Mean fluorescence intensities for all 1 mM spots of heparin oligosaccharides **1**−**16** from seven independent experiments are shown. Bars indicate standard error. (B) Binding of heparin to annexin A1 immobilized on SPR chips. Annexin A1 (1 μM) was immobilized onto NTA chips, and heparin, polyacrylamide (PAA) coupled glycans (LeA, LeX, and a biotin control; each 5 or 50 μg/mL), or buffer were flowed over the surface.

ion strength (0.5 M NaCl with 10 mM HEPES, pH 7.4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) annexin A1 bound only poorly to hexasaccharide **1** (Figure 5A). Injections of annexin A1 with increasing sodium chloride concentrations revealed an IC_{50} = 207 mM for sodium chloride (Figure 5C). These findings indicate a partly ionic nature of the annexin A1−heparin interaction that can be inhibited effectively by sodium chloride.

Annexin A1 Binding Preferences for Heparin/Heparan Sulfate Modifications. Using the collection of synthetic heparin oligosaccharides (Figure 2), the recognition of distinct sulfation types on heparin/HS chains by annexin A1 was analyzed. The library contains synthetic heparin-like oligosaccharides ranging from mono- to hexasaccharides that exhibit different sulfation patterns regarding 2-*O*-, *N*-, and 6-*O*-sulfation (Figure 2). This

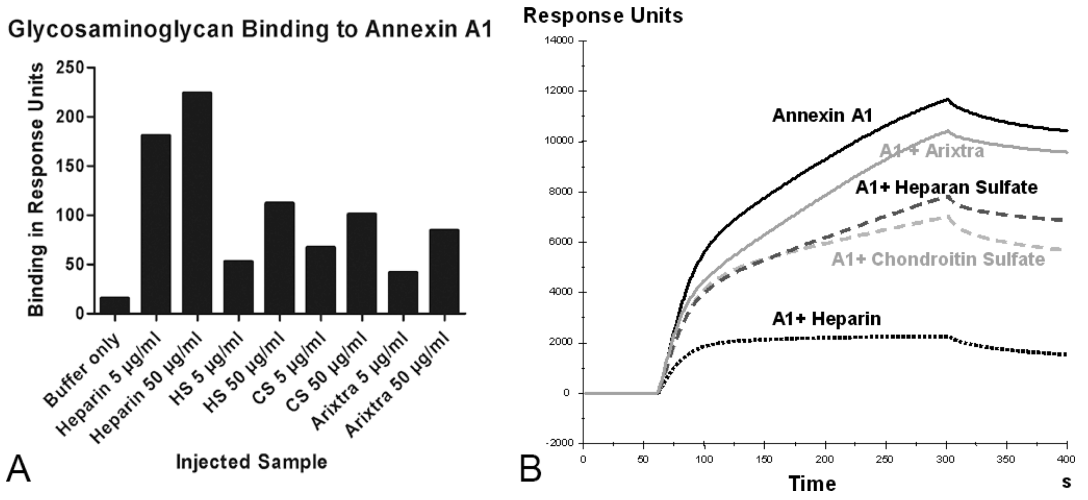

Figure 4. Glycosaminoglycan specificity of annexin A1. (A) Annexin A1 (1 μM) was immobilized on NTA chips, and glycosaminoglycans (each 5 or 50 μg/mL) in running buffer were flowed over the surface-bound annexin A1. Binding levels at the end of injection are shown. HS: heparan sulfate; CS: chondroitin sulfate. (B) Annexin A1 (1 μM) was flowed over the surface functionalized with heparin oligosaccharide **1** and **11** in the presence or absence of various glycosaminoglycans (each 10 μg/mL). The differential binding level of annexin A1 to hexasaccharide **1** (with monosaccharide **11** binding subtracted) at the end of the injection is shown.

way, the synthetic heparin oligosaccharides allow for directly determining the contribution of general sulfation types. While most oligosaccharides of the library display natural modification patterns, some members of the oligosaccharide library (hexasaccharides **2** and **4**) are not or only very rarely found in nature, since the epimerase that synthesizes iduronic acid moieties generally does not act upon glucuronic acid moieties vicinal to *N*-acetylglucosamine.

Due to the high charge of heparin, background binding and inconsistencies are commonly observed. We thus analyzed annexin A1 interactions with the heparin library using several, complementary techniques to verify binding. The heparin arrays were used for a facile, rapid screening of annexin A1−heparin/HS binding preferences regarding the full set of heparin oligosaccharides of the library (Figure 2), but background binding on arrays was high. Therefore, binding was validated using SPR that enables an in-depth analysis of heparin/HS interactions and the identification of background binding via internal controls. In case of conflicting results between heparin array and SPR experiments, SPR yielded typically more reliable results. Heparin oligosaccharides were immobilized with low density on the SPR chips to exactly measure 1:1 interactions. On microarrays, the sugars were immobilized with high density, thereby allowing for multivalent binding that might result in different binding strengths and preferences.

For the analysis of heparin/HS binding preferences using heparin arrays, annexin A1 was incubated on the microarrays. Bound protein was detected using secondary fluorescence-labeled anti-His antibodies, and fluorescence intensities were evaluated (Figure 3A). Annexin A1 bound best to isolated heparin and well to several synthetic heparin oligosaccharides, including oligosaccharides **1**, **2**, **3**, **5**, **6**, and **8**. Shorter heparin saccharides (**7**, **10**, **11**) were poorly recognized, indicating a length requirement for sufficient binding.

Annexin A1 binding preferences were validated using qualitative SPR. Using this technique, a direct comparison of binding preferences between different sulfation patterns is possible. Hexasaccharides **1**−**3** and monosaccharide **11** were immobilized on one chip while a second chip carried hexasaccharides **4**−**6** and monosaccharide **11**. Equal amounts of saccharides were coupled to the SPR chips to directly compare annexin A1 binding. Monosaccharide **11** was used as stringent control on both chips, because monosaccharide **11** is a highly charged non-natural analogue and reflects general charge interactions, but due to its similarity to heparin, binding to **11** might also reflect natural binding events between the protein binding pocket and sulfate groups.

Annexin A1 was flowed over the chip surfaces, and binding was measured in parallel (Figure 6). Annexin A1 binding to all heparin oligosaccharides, including monosaccharide **11**, was observed, indicating a broad specificity of annexin A1 toward heparin/heparan sulfate due to strong electrostatic interactions. Binding levels greatly differed between the immobilized saccharides (Figures 6). Particularly, hexasaccharides **1**, **2**, and **5** that all carry several sulfate groups at different positions and all bound well on the microarrays were bound strongly. These preferences underscore the importance of several sulfates at different positions for annexin A1 binding. In addition, hexasaccharide **6** was bound well, indicating a particular importance of 2-*O*-sulfation for binding since hexasaccharide **3** (bearing only *N*-sulfate groups) did not interact above background level (as determined by control **11**).

Because hexasaccharide **3** was not recognized by annexin A1 using SPR above background but bound well on microarrays, we determined the binding constants for heparin hexasaccharides **1**−**6** by SPR to resolve conflicting results and to quantify the binding strengths for annexin A1 interactions. Annexin A1 in varying concentrations was flowed over the surfaces which contained immobilized heparin hexasaccharides to measure the binding strength by SPR. The dissociation constants (K_D) were determined assuming a 1:1 binding model, and relative affinity constants were calculated (Table 1 and Supporting Information Table S3). Annexin A1 bound the heparin hexasaccharides in the low micromolar range whereby hexasaccharide **1** was the best binder (K_D = 3.1 μM). Binding strength comparison revealed preferential binding of annexin A1 to hexasaccharides **1**, **2**, and **5** for which the respective binding preferences roughly correlated with the qualitative SPR results. Binding to the hexasaccharides **3**,

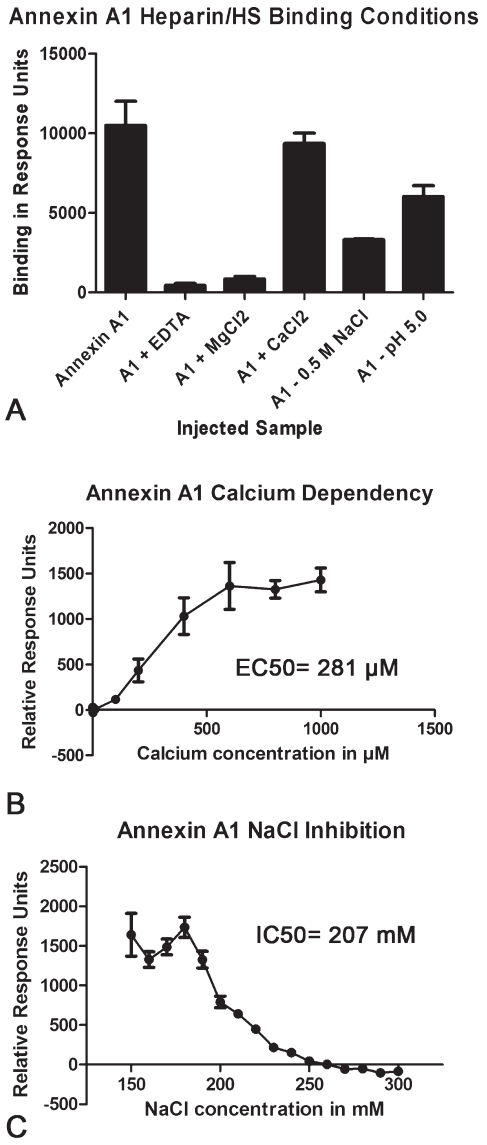

Figure 5. Heparin/heparan sulfate binding conditions of annexin A1. (A) Annexin A1 (1 μM) was flowed over the surface-coupled oligosaccharide **1** and monosaccharide **11** under various conditions: $MgCl_2$ + CaCl, in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 1 mM $MgCl_2$ and 1 mM $CaCl_2$; EDTA, in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 5 mM EDTA; $MgCl_2$, in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 1 mM $MgCl_2$; $CaCl_2$, in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 1 mM $CaCl_2$; 0.5 M NaCl, in 10 mM HEPES (pH 7.4) and 500 mM NaCl with 1 mM $MgCl_2$ and 1 mM $CaCl_2$; pH 5.0, in 10 mM HEPES (pH 5.0) and 150 mM NaCl with 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The differential binding level of annexin A1 to hexasaccharide **1** (with monosaccharide **11** binding subtracted) at the end of the injection is shown. Error bars indicate standard error. Apparent residual binding of annexin A1 under high salt conditions is an artifact induced by the density shift caused through the high salt concentration. (B) Annexin A1 (0.5 μM) in HBS-N was flowed over the surface-coupled oligosaccharide **1** and monosaccharide **11** in the presence of varying $CaCl_2$ concentrations. Differential binding in relative response units at the end of injection is plotted against the calcium concentration. (C) Annexin A1 (0.5 μM) in 10 mM HEPES (pH 7.4) with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and varying NaCl concentrations was flowed over the surface-coupled oligosaccharide **1** and monosaccharide **11**. Differential binding in relative response units at the end of injection is plotted against the sodium chloride concentration.

4, and **6** was observed, in agreement with heparin microarray binding and qualitative SPR. The observed differences in binding strength, particularly for heparin oligosaccharide **3**, between microarrays and SPR experiments might result from different immobilization levels (the high density coupling on microarrays might facilitate multivalent interactions, thereby strengthening weak interactions) and technique-specific differences.

In summary, considering the results from all experiments to determine annexin A1−heparin/HS binding preferences, annexin A1 binds preferentially to highly sulfated heparin-like oligosaccharides, but also to other glycosaminoglycans. Heparin hexasaccharides **1**, **2**, and **5** which contain two or three sulfation types were bound better than hexasaccharides containing only one sulfation type (hexasaccharides **3** and **6**). Nonsulfated hexasaccharide **4** was bound by annexin A1 just above background. 2-*O*-Sulfate groups as well as *N*-sulfates are thus apparently particularly important for annexin A1 binding, because hexasaccharides **1** and **5** containing *N*- and 2-*O*-sulfation bound best. 6-*O*-Sulfation also contributes to annexin A1 binding as indicated by the fact that hexasaccharide **2** which is 2-*O*- and 6-*O*-sulfated bound better than only 2-*O*-sulfated hexasaccharide **6**. In agreement with this finding, hexasaccharide **1** carrying 2-*O*-, *N*-, and 6-*O*-sulfation bound better than hexasaccharide **5** that is only 2-*O*- and *N*-sulfated.

DISCUSSION

Annexin A1 binds to highly sulfated glycans and in particular to highly sulfated HS/heparin. Sulfated non-glycosaminoglycan sugars and nonsulfated carboxylated glycans were bound as well, revealing broad specificity toward several glycan classes. Sulfates were revealed to constitute important binding motifs for annexin A1 on glycans. Strong binding is mediated by concurrent interactions with several sulfate groups, because binding strength correlates roughly with the number of sulfate groups on a glycan. Strong annexin A1 interactions presumably result from binding to correctly spaced sulfate groups on one glycan chain corresponding to the annexin A1 binding pocket. In addition, annexin A1 might interact multivalently with sulfate groups from different chains, thereby strengthening weak single interactions.

Under physiological conditions, annexin A1 interacts with heparin/heparan sulfate. Binding depends on the pH and ionic strength, typical features important for ionic interactions. Most importantly, annexin A1 binding to heparin/heparan sulfate and likely all sulfated glycans strongly depends on calcium ions and cannot be substituted by magnesium ions, thereby indicating a targeted interaction. These established binding conditions agree, insofar as available, with published observations regarding other annexin−heparin interactions, including heparin vs heparan sulfate preference, calcium and pH dependence, and dissociation constants.[17,18,41]

Among the glycosaminoglycans, annexin A1 binds preferentially to highly sulfated heparin. Chondroitin sulfate and heparan sulfate are bound to a lesser extent. Highly charged heparan sulfate seem to be a particularly important target for annexin A1, but binding of other glycosaminoglycans and sulfated glycans is probable on the organism level. Still, many types of glycosaminoglycans are bound, and electrostatically driven background binding to common glycosaminoglycan motifs is high. Such low level binding might initially anchor annexin A1 to vicinal cells and/or guide annexin A1 on target cells by repetitive annexin A1

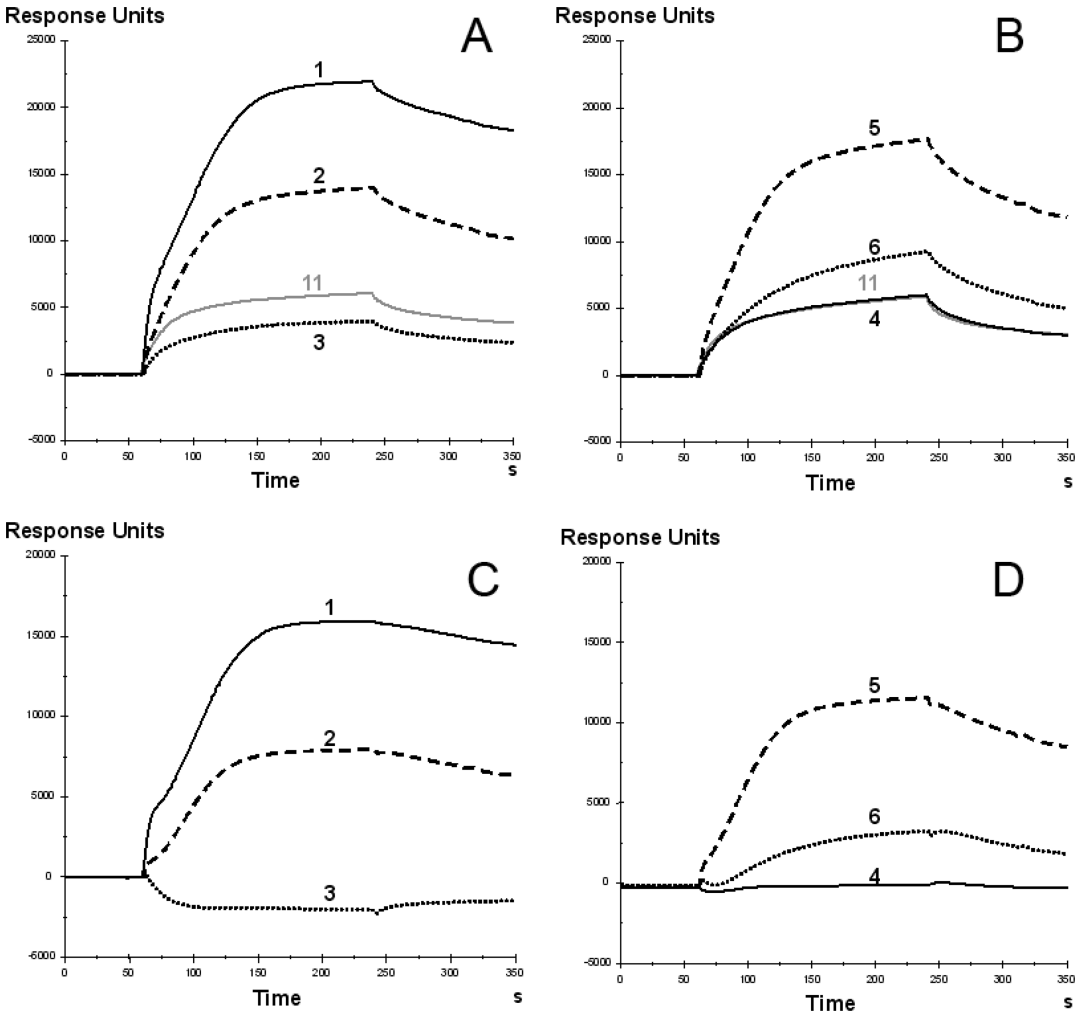

Figure 6. Annexin A1 binding preference for heparin hexasaccharides **1**−**6** determined by SPR. Annexin A1 (8 μM) was flowed over the surface of a SPR chip bearing heparin oligosaccharides **1**, **2**, **3**, and **11** (A, C) or **4**, **5**, **6**, and **11** (B, D). Annexin A1 binding to hexasaccharides and monosaccharide **11** is shown in (A) and (B). Differential binding of annexin A1 to hexasaccharides (with monosaccharide **11** binding subtracted) is shown in (C) and (D). Legends A and C: black line, hexasaccharide **1**; black dashed line, hexasaccharide **2**; black dotted line, hexasaccharide **3**; gray line, monosaccharide **11**. Legends B and D: black line, hexasaccharide **4**; black dashed line, hexasaccharide **5**; black dotted line, hexasaccharide **6**; gray line, monosaccharide **11**.

dissociation from and association with glycosaminoglycan chains until a specific sequence is bound. In addition, distinct other interacting glycosaminoglycans or sulfated glycans may also affect annexin A1 functions. Interestingly, this investigation revealed substantial annexin A1 binding to highly sulfated non-glycosaminoglycan sugars which are the subject of further investigations.

Structure−activity relationship (SAR) determination of annexin A1 regarding heparin/heparan sulfate modifications revealed that annexin A1 binds best to isolated heparin. Either the optimal heparin binding motif is not present in the synthetic oligosaccharides or the longer, isolated heparin chain can be better accessed by annexin A1. Accordingly, among the synthetic oligosaccharides, longer, highly sulfated saccharides are bound best, indicating a minimal chain length requirement of four to six sugar residues for detectable binding. Highly sulfated hexasaccharides are bound strongly, and binding is not blocked by glycosaminoglycans other than heparin, indicating that the preferential natural glycosaminoglycan motif bound by annexin A1 is at least partially reflected by the highly bound synthetic hexasaccharides (**1** and **5**).

Table 1. Dissociation Constants for Annexin A1−Heparin Hexasaccharide Interactions[a]

heparin hexasaccharide	dissociation constant (K_D), μM	relative affinity
1	3.1	1
2	4.0	0.78
3	12.7	0.24
4	7.9	0.39
5	3.4	0.91
6	7.9	0.39

[a] Annexin A1 in varying concentrations was flowed over SPR chips with immobilized heparin hexasaccharides **1**−**6** (Figure 2). Plotting of the response units versus the concentration allowed for determination of the apparent dissociation constants assuming a 1:1 binding mode.

Furthermore, the SAR analysis demonstrated that annexin A1 binding to heparin/heparane sulfate correlates with the extent of sulfation on the glycosaminoglycan chain. However, not only the sulfation level but also correct spacing of sulfate groups and

presumably the backbone structure are important for annexin A1 binding, since annexin A1 binds the highly charged heparin-like pentasaccharide arixtra only weakly. In addition, the type of sulfation influences annexin A1−HS/heparin interactions. For instance, hexasaccharide **6** (bearing only 2-*O*-sulfation) is bound better than hexasaccharide **3** (bearing only *N*-sulfate groups), although both hexasaccharides bear the same number of sulfate groups. Whereas some sulfate types appear to contribute more to binding strength, no single type of modification is sufficient for strong interactions. High level binding is mediated by interactions with 6-*O*-, 2-*O*-, and *N*-sulfate groups, but not all sulfate groups present on a hexasaccharide chain are likely bound by annexin A1. Highly sulfated hexasaccharides such as **1**, **2**, or **5** bind better than hexasaccharides **3** or **6** and demonstrate that additional, nonbinding sulfate groups are tolerated by annexin A1. Therefore, this protein binds apparently to several distinct sulfate groups within a heparin/heparan sulfate binding motif. Each interaction contributes additively to the binding strength and is partially redundant; additional nonbinding sulfate groups are generally tolerated. This binding mode presumably reflects the nontemplate driven synthesis of heparin/heparan sulfate. Cells can only regulate the extent of distinct heparin/heparan sulfate modifications on the sugar chain but not the expression of unique binding motifs. Binding of annexin A1 to several different sulfate groups that contribute additively to binding strength guarantees that annexin A1 interacts with heparan sulfate on cells that targeted express high levels of certain sulfotransferases.

The biological role of annexin A1 binding to heparin/heparan sulfate, and most other glycan interactions, remains elusive. However, prominent functions of extracellular annexin A1 have been established, including mediation of angiogenesis, blockage of leukocyte extravasion, and regulation of neuroendocrine hormone release.[11,13,42] In these processes, annexin A1 generally acts in an autocrine or paracrine/juxtacrine manner.[10,12,13] It is likely that heparan sulfate is involved in annexin A1 anchoring to cells in order to direct the protein's distribution and might prevent dilution of the protein, for instance in the bloodstream. Furthermore, annexin A1 might compete with other proteins for binding sites on heparan sulfate, thereby modulating their action, e.g., with cytokines on the inflamed endothelium as an additional function in the antiinflammatory action of annexin A1.

Specific carboxylated glycans were identified to be involved in neutrophil extravasion, and annexin A1 was identified in a subsequent screen for binders to these glycans.[14,42] Furthermore, annexin A1 has been identified as a tumor marker on endothelial cells adjacent to a tumor, and an important role of annexin A1 in adult angiogenesis was discovered.[10,11] Remarkably, heparan sulfate has prominent functions in leukocyte adhesion, wound healing, tumorigenesis, and angiogenesis since heparan sulfate interacts with several key proteins to modulate the proteins' actions on target cells.[43,44] We propose that heparan sulfate interactions also affect annexin A1 function in these processes, since binding to heparan sulfate is highly likely due to the high affinity and specificity of the annexin A1 heparin/HS interaction. The exact function of these annexin A1−heparan sulfate interactions will be addressed in further investigations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Annexin A1 glycan array binding, annexin sequence alignment, and annexin A1−heparin oligosaccharide interactions. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +49 (0) 30-838-59300. Fax: +49 331 567-9302. E-mail: Peter.Seeberger@mpikg.mpg.de.

Present Addresses

#Instituto de Investigaciones Químicas, Centro de Investigaciones Científicas Isla de La Cartuja, CSIC-US, Americo Vespucio, 49, 41092 Sevilla, Spain.

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