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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

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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. Annexin A1 binding to glycans has been implicated in cell attachment and modulation of annexin A1 function. We characterized in detail the glycan binding preferences of annexin A1 using glycan arrays and surface plasmon resonance as a starting point to understand the role of glycan binding in annexin A1 function. Glycan array analysis identified a series of sulfated oligosaccharides, demonstrating for the first time annexin A1 binding to sulfated non-glycosaminoglycan carbohydrates. Using heparin/heparan sulfate microarrays, highly sulfated heparan sulfate/heparin were identified as preferential ligands of annexin A1. Binding of annexin A1 to heparin/heparan sulfate is calcium, but not magnesium dependent. The structure-activity relationship of annexin A1-heparan sulfate interactions were established in detail using chemically defined sugars and novel methods, being the first characterization of a calcium dependent heparin binding protein with such 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 twelve known mammalian annexins contain a conserved C-terminal annexin domain called 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

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Supporting Information Available

Supporting Information for annexin A1 glycan array binding, annexin sequence alignment and annexin A1-heparin oligosaccharide interactions are available free of charge via the internet at <http://pubs.acs.org>.

interactions and may 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 that reflect the intracellular situation, the *N*-terminal tail domain is buried in the core domain where it is inaccessible for interactions. The *N*-terminal tail is expelled at high calcium levels that 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 anti-inflammatory events (9). The range of these actions includes induction of anti-inflammatory cytokines, repression of cyclooxygenase-2 and inducible nitric oxide synthase, inhibition of phospholipase A₂ and blockage of leukocyte extravasion (8,10). Recently, annexin A1 has been identified as a specific tumor marker on endothelial cells beneath a tumor (11). Subsequent investigations revealed that annexin A1 is an angiogenic factor that is prominently involved in healing (12). blood vessel growth in adults and particularly important for angiogenesis in tumorigenesis and wound

None of the annexins carry signal sequences for the classical export pathway. Still, 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 (10,11,13). 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 co-crystal structures (16–19), but detailed biochemical characterization of heparin/heparan sulfate structure activity relationships is missing. Therefore, we analyzed annexin A1-glycan binding elaborately with a particular focus on heparin/heparan sulfate interactions that revealed to be important, preferential glycan ligands.

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). This non-template driven synthesis results in an extreme microheterogeneity regarding the modification pattern even on the same 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 at which the carbohydrate chain is extensively modified and partially degraded. Heparin is mainly

released from mast cells in the form of free polysaccharide chains. Heparin is commonly used to represent heparan sulfates in general due to its commercial availability, but contains only highly sulfated heparan sulfate sequences.

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).

Due to microheterogeneity, high electrostatically driven background binding and cross-reactivity (23,24), the determination of glycosaminoglycan binding preferences for a given protein is still far from routine and requires special screening tools (27). In most cases, activity assays to monitor heparan sulfate function are infeasible. Here, we investigated general glycan binding preferences of annexin A1 using carbohydrate and heparin microarrays. Microarrays (28,29) are particularly useful to study the structure-activity relationships of large numbers of the microheterogeneous glycosaminoglycans sequences (30,31). Heparin arrays have thus been exploited for the characterization of glycosaminoglycan-protein interactions (32,33). The results revealed that Annexin A1 binds broadly to sulfated glycans with highly charged heparin/heparan sulfate being the preferential ligands. We thus determined the structure-activity relationship of annexin A1 heparin/heparan sulfate binding using microarray, quantitative and qualitative surface plasmon resonance (34) approaches based on a collection of synthetic heparin oligosaccharides. This study characterized a calcium dependent binding of annexin A1 to structure-defined, chemically synthesized heparin/heparansulfate-related sugars, including the determination of dissociation constants for closely related heparan sulfate sequences. *N*-sulfation and 2-*O*-sulfation were identified as particularly important for binding, thereby explaining responsiveness of cells to annexin A1. The observations made by this study may be representative for other, closely related annexins. The biological relevance of annexin A1 heparin/heparan sulfate binding is discussed in detail.

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).

Purification of Annexin A1

Recombinant annexin A1 protein was produced as C-terminal His6 tag fusion protein in bacteria. Full-length mouse annexin A1 cDNA was obtained from Invitrogen (Carlsbad, California, 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-thio-galactoside. 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, 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). 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 TBSCM (20 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 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 cover slip for 60 min in a humidified chamber at room temperature. Cover slips were then gently removed in a solution of TBSCM/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 sec 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 dessicator until usage.

Incubation of Heparin Micorarrays

Heparin microarray slides were washed three times with water and quenched in 100 mM ethanolamine, 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 MgCl₂, 1 mM CaCl₂ 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 MgCl₂ and 1 mM CaCl₂) and centrifuged with 200 × g for 5 min at room temperature. HIS-tagged annexin A1 was dissolved in HBS-N with 1 mM MgCl₂, 1 mM CaCl₂ and 0.1% (v/v) Tween-20 and incubated on the slides for 1 h at room temperature. Afterwards, 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 MgCl₂, 1 mM CaCl₂ 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 μ L/min. The oligosaccharides (10 μ M in PBS with 1 mM hexadecyltrimethylammonium chloride) were flowed over the activated surface at a flow rate of 5 μ L/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 μ L/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 μ g/mL final concentration), chondroitin sulfate (10 μ g/mL final concentration), heparan sulfate (10 μ g/mL final concentration) or arixtra (fondaparinux sodium, GlaxoSmithKline, Germany; 10 μ g/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 manufacturer's protocol (GE Healthcare, Switzerland). HBS-EP (10 mM HEPES, pH 7.4, 150 mM sodium chloride, 50 μ M EDTA, 0.05% v/v Tween-20) was used as running buffer. In brief, the chip surface was pre-primed by an injection of 500 μ M nickel chloride in running buffer. Next, the protein (1 μ M 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 S1). General binding themes for annexin A1 glycan interactions observed were as follows: annexin A1 bound several highly sulfated glycans, mainly β 1-4 linked *N*-acetyllactosamine (type 2 LacNAc) or lactose that are sulfated two or three times at the 3' and/or 6' hydroxyl groups. A triple sulfated LacNAc ($[\text{3OSO}_3^-][\text{6OSO}_3^-]\text{Gal}\beta\text{1-4}[\text{6OSO}_3^-]\text{GlcNAc}\beta$) revealed to be the best binder. In addition, annexin A1 also bound to sialylated and neutral glycans, which is consistent with the previous findings that annexin A1 bound to carboxylated glycans (Srikrishna, et al *J Immunol* 166, 4678–4688) and selectin ligand mimicry I-peptide (Hatakeyama, *Proc Natl Acad Sci U S A* 106, 3095–3100 (2009)). Hence, annexin A1 binds two types glycans: highly sulfated glycans and weakly acidic and fucosylated glycans, probably through each distinct

binding mechanism. Here after, we will describe the annexin A1 binding to sulfated glycans, in particular to heparin/heparan sulfates.

Annexin A1 Binds to Heparin/Heparan Sulfate

Next to sulfated glycans, glucuronic acid was among the best recognized glycans on the glycan array (Figure 1 and Supporting Information S1), suggesting that annexin A1 binds glycosaminoglycans, particularly to heparin/HS since annexin A1 binding to heparin columns has been exploited to purify the protein. Furthermore, binding of annexin 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 S2), with only one amino acid of the heparin-binding region of annexin A2 (Lys²⁸⁰ – 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 a subsequent incubation step. Annexin A1 bound several heparin oligosaccharides as well as isolated heparin on the array (Figure 3A). Co-incubation with heparin blocked binding, thereby demonstrating sugar specific binding (inlay Figure 3A). Before the heparin/heparan sulfate sequence specificity of annexin A1 was analyzed in detail (discussed in a subsequent section), we first validated binding to heparin /heparan sulfate of annexin A1 and determined general annexin A1-glycosaminoglycan preferences and 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). HIS-tagged Annexin A1 was immobilized on nitrilotriacetic acid (NTA) modified SPR chips. Heparin was flowed over immobilized annexin A1. (This part is very fragmental, causing problem. I will delete). Only 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, the PAA immobilized glycans, including LacNAc that was well bound on the glycan array, did not interact with the immobilized annexin A1. 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

Apart from heparin, other sulfated glycosaminoglycans might interact functionally with annexin A1. The general 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 (39) – 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 **1** was well recognized by annexin A1 on 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. Annexin A1 bound hexasaccharide **1** to result in a strong, readily detectable signal (Figure 4B). Only heparin reduced binding of annexin A1 to background level when co-injected while arixtra acted only as a weak inhibitor. 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. Furthermore, the immobilization of heparin oligosaccharides and injection of protein (34) proved superior to the immobilization of annexin A1 and injection of the heparin oligosaccharides. Lower baseline drift, excellent reproducibility, simple regeneration and higher detection signal due to the increased size of the solution-based analyte were observed.

Annexin A1 Heparin/HS Binding Conditions

We determined the conditions required for annexin A1 to bind heparin oligosaccharide **1**. These conditions are presumably representative for interactions of this protein with glycosaminoglycans and other sulfated glycans. Calcium dependency of the annexin A1-heparin interaction was tested, since heparin binding of other annexins is calcium dependent. 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). Annexin A1 injected in standard binding buffer (10 mM HEPES, 150 mM NaCl with 1 mM CaCl₂ and 1 mM MgCl₂) 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 EC₅₀ of 281 μ M for calcium was determined (Figure 5B). These experiments demonstrated pronounced calcium dependent binding of annexin A1 to heparin.

SPR experiments utilizing annexin A1 at pH 5.0 (10 mM HEPES, pH 5.0, 150 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂) showed reduced binding (Figure 5A). At elevated ion strength (0.5 M NaCl with 10 mM HEPES, pH 7.4, 1 mM CaCl₂ and 1 mM MgCl₂) annexin A1 bound only poorly to **1** (Figure 5A). Injection of annexin A1 with increasing sodium chloride concentrations revealed an IC₅₀ = 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). The oligosaccharides resemble highly modified heparan sulfate or heparin as the backbone is comprised of the repeating unit L-iduronic acid and *N*-substituted glucosamine. Some members of the oligosaccharide library (hexasaccharides **2** and **4**) are not or only very rarely found in nature, since the epimerase generally does not act upon glucuronic acid moieties vicinal to *N*-acetylglucosamine. Nonetheless, the synthetic heparin oligosaccharides are of great value because they allow for directly determining the contribution of general sulfation types without the need to consider any backbone effects.

Protein binding to heparin saccharides must be carefully examined due to the high charge of these molecules. Cross-reactivity and background binding are common and inconsistencies are often observed. While microarrays allowed a facile, rapid screening of annexin A1-heparin/HS binding preferences regarding the full set of heparin oligosaccharides of the

library (Figure 2) to identify common binding themes, background binding on arrays is high. Therefore, distinct binding events needed to be verified using complementary techniques. SPR enables such precise, in-depth analysis of heparin/HS interactions and, using carefully chosen controls, background binding and pure charge interactions can be identified. SPR thus yielded commonly more reliable results in case of conflicting results between these microarray and SPR experiments, since SPR allowed identifying and subtracting background binding. In addition, the 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 which might result in different binding strength and preferences.

The heparin/HS binding preference of human annexin A1 was first analyzed using heparin microarrays. 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 **1**, **2**, **3**, **5**, **6** and **8**. Shorter heparin saccharides (**7**, **10**, **11**) were poorly recognized, indicating a length requirement for sufficient binding. Hexasaccharide **5** was particularly well recognized emphasizing the importance of *N*-sulfation and 2-*O*-sulfation for binding.

Next, hexasaccharides **1** to **6** were immobilized on two different SPR chips to validate annexin A1 binding preferences. Using this technique, a direct binding comparison 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 control on both chips. Monosaccharide **11** is a highly charged non-natural analog and reflects general charge interactions. Due to the relation to heparin, binding to **11** also reflects binding events between the protein binding pocket and sulfate groups. Monosaccharide **11** thus constitutes a very stringent control and binding levels above that of **11** should reflect only specific binding events.

Annexin A1 was flowed over the chip surfaces and binding was measured in parallel (Figure 6). Fair to very strong 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 strengths greatly differed for the immobilized saccharides (Figures 6A and 6B). Particularly hexasaccharides **1**, **2** and **5** were bound very strongly. 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) was not recognized above background level (given by control **11**). High affinity binding to hexasaccharides **1**, **2** and **5**, all carrying several sulfate groups of different types and all also good binders on the microarrays, was particularly pronounced when analyzing differential binding with binding to control **11** being subtracted (Figures 6C and 6D). These preferences underscore the importance of several different sulfations for annexin A1 binding. 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 strength of annexin A1. Annexin A1 in varying concentrations was flowed over the surfaces containing immobilized heparin hexasaccharides to measure binding by SPR. The dissociation constants (K_D) were determined assuming a 1:1 binding model by plotting binding against the protein concentration and relative affinity constants were calculated (Table 1 and Supporting Information S3). Annexin A1 bound the heparin hexasaccharides in the low micromolar range whereby hexasaccharide **1** was the best binder

($K_D = 3.1 \mu\text{M}$). Binding strength comparison revealed preferential binding of annexin A1 to hexasaccharides **1**, **2** and **5** for which the relative binding affinity roughly correlates with the qualitative SPR results. Binding to the hexasaccharides **3**, **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 results from all experiments to determine annexin A1-heparin/HS binding preferences, annexin A1 binds preferentially to highly sulfated heparin-like oligosaccharides, but has also low affinity for other glycosaminoglycans. Heparin hexasaccharides **1**, **2** and **5** that contain two or three sulfation types were bound better than hexasaccharides containing only one sulfation type (hexasaccharides **3** and **6**). Non-sulfated hexasaccharide **4** was bound by annexin A1 just above background. 2-*O*-Sulfate groups as well as *N*-sulfates are 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, because hexasaccharide **2** that is 2-*O*- and 6-*O*-sulfated bound better than 2-*O*-sulfated hexasaccharide **6**. Accordingly, 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, particularly highly sulfated HS/heparin. Sulfated non-glycosaminoglycan sugars and non-sulfated carboxylated glycans were bound as well, but not further investigated. Sulfates are key binding motifs for annexin A1 on glycans. Strong binding is mediated by concurrent interactions with several sulfate groups, because binding strengths correlates roughly with the number of sulfate groups on a glycan. High affinity annexin A1 interactions presumably results from binding to correctly spaced sulfate groups on one glycan chain corresponding to the annexin A1 binding pocket. In addition, annexin A1 might interact with sulfate groups from different chains, thereby strengthening weak single interactions.

Binding of annexin A1 to heparin/heparan sulfate depends on the pH and ionic strength - typical features of 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 highly specific interaction. The determined binding conditions demonstrate that annexin A1 interacts with heparin at physiological extracellular conditions. These established binding conditions agree -insofar available- with published observations regarding other annexin-heparin interactions, including heparin vs. heparan sulfate preference, calcium- and pH-dependence and dissociation constants (17,18,40). Among the glycosaminoglycans, annexin A1 binds preferentially to highly sulfated heparin. Chondroitin sulfate and heparan sulfate are bound to a lesser extent. Annexin A1 binds the highly charged heparin-like pentasaccharide arixtra only poorly, thereby indicating that not only the sulfation extent but also correct spacing of sulfate groups and presumably backbone structure is important for annexin A1 binding. Highly charged heparan sulfate is likely a main target for annexin A1, but cross-reactivity with other glycosaminoglycans and sulfated glycans is probable on the organism level. These determined binding conditions and annexin A1 glycosaminoglycan binding preferences indicate targeted interactions of annexin A1 with highly sulfated glycans. Using the heparin oligosaccharide library, the structure-activity relationship of annexin A1 regarding heparin/heparan sulfate modifications was analyzed to define the heparan sulfate

requirements for annexin A1 binding. Heparin microarrays were used for a general analysis of annexin A1-heparin saccharide interactions. Binding of selected heparin oligosaccharides was validated using the more precise SPR, because background binding is common on microarrays. In general, heparin microarrays and SPR results correlate, but differences, particularly for heparin oligosaccharide **3** which is well bound on the array but binds annexin A1 poorly on SPR chips, were observed. These differences might result from different immobilization levels allowing for multivalent interactions on the array, thereby strengthening such weak interactions. Microarray results revealed that annexin A1 binds best to isolated heparin (**14**). 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 to indicate that the preferential natural glycosaminoglycan motif bound by annexin A1 is at least partially reflected by the highly bound synthetic hexasaccharides (**1** and **5**).

Sulfation patterns on heparin/heparan sulfate that are recognized by annexin A1 were determined. No single type of modification is sufficient for strong interactions. High affinity binding is mediated by interactions with 6-*O*-, 2-*O*- and *N*-sulfate groups. Likely, not all sulfate groups present on hexasaccharide chains are bound by annexin A1. More highly sulfated hexasaccharides such as **1**, **2** or **5** bind better than hexasaccharides **3** or **6** and demonstrate that additional, non-binding 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, whereas additional non-binding sulfate groups are generally tolerated. This binding mode presumably reflects the non-template driven synthesis of heparin/heparan sulfate. Cells can only regulate the extent of heparin/heparan sulfate modifications on the sugar chain, but not the expression of distinct 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.

Based on sequence homology, annexin A1 might bind to heparin with a binding site homologous to annexin A2. Most amino acids that interact with heparin in annexin A2 are conserved in annexin A1. One amino acid (Lys²⁸⁰) binding to heparin in annexin A2 is altered in annexin A1 to valine (Val²⁸⁸). The importance of this alteration will be the target of further investigations. Important natural glycosaminoglycan ligands of annexin A1 are likely highly sulfated heparan sulfate/heparin motifs due to preferential, enhanced binding to such motifs. Still, many types of glycosaminoglycans are bound and electrostatically driven background binding to common glycosaminoglycan motifs is high. This less specific binding might initially anchor annexin A1 to vicinal cells and/or guide annexin A1 on target cells by repetitive annexin A1 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 subject of further investigations.

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 (10,12,41). In these processes, annexin A1 generally acts in an autocrine or paracrine/juxtacrine manner

(10,11,13). It is likely that heparan sulfate is involved in annexin A1 anchoring to cells in order to direct the protein's distribution. Annexin A1 binding to heparan sulfate and other glycans might be particularly important during annexin A1 functioning in the blood stream in order to prevent dilution in the blood system. Specific carboxylated glycans were identified to be involved in neutrophil extravasation and annexin A1 was identified in a subsequent screen for binders to these glycans (14,41). Binding to heparan sulfate might prevent dispersion of annexin A1 in the blood stream and might present the protein on endothelial cells and leukocytes for signaling to leukocytes resulting in decreased extravasation. Annexin A1 might compete with heparan binding sites for cytokines on the inflamed endothelium as an additional function in the anti-inflammatory action of annexin A1. Furthermore, annexin A1 has been identified as a tumor marker on endothelial cell adjacent to a tumor and an important role of annexin A1 in adult angiogenesis was discovered (11,12). 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' action on target cells (42,43). 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.

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Figure 1. Annexin A1 binds sulfated oligosaccharides

Binding of glycan arrays by annexin A1-His. Glycan structures listed starting from the strongest binder are: 26. $[3\text{OSO}_3^-][6\text{OSO}_3^-]\text{Gal}\beta 1-4[6\text{OSO}_3^-]\text{GlcNAc}\beta\text{-Sp}0$; 4. Ceruloplasmine; 3. AGP- β 1; 30. $[3\text{OSO}_3^-]\text{Gal}\beta 1-4(6\text{OSO}_3^-)\text{Glc}\beta\text{-Sp}8$; 6. Transferrin; 45. $[6\text{OSO}_3^-]\text{Gal}\beta 1-4[6\text{OSO}_3^-]\text{Glc}\beta\text{-Sp}8$; 29. $[3\text{OSO}_3^-]\text{Gal}\beta 1-4(6\text{OSO}_3^-)\text{Glc}\beta\text{-Sp}0$; 1. AGP; 35. $[3\text{OSO}_3^-]\text{Gal}\beta 1-4[6\text{OSO}_3^-]\text{GlcNAc}\beta\text{-Sp}8$; 2. AGP-A; 39. $[4\text{OSO}_3^-][6\text{OSO}_3^-]\text{Gal}\beta 1-4\text{GlcNAc}\beta\text{-Sp}0$; 244. Neu5Ac α 2-6Gal β 1-4 $[6\text{OSO}_3^-]\text{GlcNAc}\beta\text{-Sp}8$.

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** to **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** to **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, or buffer were flowed over the surface.

Figure 4. Glycosaminoglycan specificity of annexin A1

A) Annexin A1 (1 μ M) was immobilized on NTA chips and glycosaminoglycans (each 5 or 50 μ g/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 μ M) was flowed over the surface functionalized with heparin oligosaccharide **1** and **11** in the presence or absence of various glycosaminoglycans (each 10 μ g/mL). The differential binding level of annexin A1 to hexasaccharide **1** (with monosaccharide **11** binding subtracted) at the end of the injection is shown.

Figure 5. Heparin/heparan sulfate binding conditions of annexin A1

A) Annexin A1 (1 μ M) was flowed over the surface-coupled oligosaccharide **1** and monosaccharide **11** under various conditions: **MgCl₂ + CaCl₂**: in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 1 mM MgCl₂ and 1 mM CaCl₂; **EDTA**: in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 5 mM EDTA; **MgCl₂**: in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 1 mM MgCl₂; **CaCl₂**: in 10 mM HEPES (pH 7.4) and 150 mM NaCl with 1 mM CaCl₂; **0.5 M NaCl**: in 10 mM HEPES (pH 7.4) and 500 mM NaCl with 1 mM MgCl₂ and 1 mM CaCl₂; **pH 5.0**: in 10 mM HEPES (pH 5.0) and 150 mM NaCl with 1 mM MgCl₂ and 1 mM CaCl₂. 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 μ M) in HBS-N was flowed over the surface-coupled oligosaccharide **1** and monosaccharide **11** in presence of varying CaCl₂ concentrations. Differential binding in relative response units at the end of injection is plotted against the calcium concentration. C) Annexin A1 (0.5 μ M) in 10 mM HEPES (pH 7.4) with 1 mM MgCl₂ and 1 mM CaCl₂ 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.

Figure 6. Annexin A1 binding preference for heparin hexasaccharides 1 to 6 determined by SPR
 Annexin A1 (8 μ M) was flowed over 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). Legend A, C: black line hexasaccharide **1**; black dashed line: hexasaccharide **2**; black dotted line: hexasaccharide **3**, gray line: monosaccharide **11**. Legend B, D: black line: hexasaccharide **4**; black dashed line: hexasaccharide **5**; black dotted line: hexasaccharide **6**, gray line: monosaccharide **11**.

Table 1
Dissociation Constants for Annexin A1 – Heparin Hexasaccharide Interactions

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.

Heparin Hexasaccharide	Dissociation Constant (K_D) in μ M	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