Glycan Recognition

Characterization and engineering of S100A12–heparan sulfate interactions

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Abstract

S100A12, an EF-hand calcium-binding protein, can be secreted by a variety of cell types and plays proinflammatory roles in a number of pathological conditions. Although S100A12 has been shown to interact with heparan sulfate (HS), the molecular detail of the interaction remains unclear. Here we investigate the structural basis of S100A12–HS interaction and how the interaction is regulated by the availability of divalent cations and the oligomeric states of S100A12. We discovered that S100A12–HS interaction requires calcium, while zinc can further enhance binding by inducing S100A12 hexamerization. In contrast, the apo form and zinc-induced tetramer form were unable to bind HS. Guided by the crystal structures of S100A12, we have identified the HS-binding site of S100A12 by site-directed mutagenesis. Characterization of the HS-binding site of S100A12 allowed us to convert the non-HS-binding apo and tetramer forms of S100A12 into a high affinity HS-binding variant by engineering a single-point mutation. Using a HS oligosaccharide microarray, we demonstrated that the N43K mutant displayed markedly enhanced selectivity toward longer HS oligosaccharides compared to the WT S100A12, likely due to the expanded dimension of the reengineered HS-binding site in the mutant. This unexpected finding strongly suggests that HS-binding sites of proteins might be amenable for engineering.

Key words: calcium, heparan sulfate, oligomerization, protein engineering, S100

Introduction

S100A12 is a member of the small calcium-binding S100 protein family. S100 proteins are involved in a variety of intracellular and extracellular activities such as cell proliferation, differentiation, apoptosis, inflammation, etc. (Donato 1999). It is known that the conformational and oligomeric states of S100A12 depend on ion concentration in its microenvironment (Moroz, Burkitt et al. 2009). Like many other S100 proteins, the ion-free apo form of S100A12 exists as a noncovalent homodimer. In the presence of calcium, S100A12 dimer coordinates calcium and undergoes a conformational change, but the oligomeric state of the protein remains the same (Moroz et al. 2003). In addition to calcium, S100A12 has been shown to bind zinc and form a stable tetramer. When both calcium and zinc

are present, S100A12 forms a stable hexamer (Moroz, Blagova et al. 2009; Moroz, Burkitt et al. 2009).

S100A12 is primarily secreted by neutrophils, macrophages and smooth muscle cells (Ilg et al. 1996). Both animal and human studies have shown that S100A12 is released during inflammatory conditions and functions as a proinflammatory molecule (Foell and Roth 2004). The extracellular functions of S100A12 include potent chemokine and cytokine activities (Foell et al. 2007; Yang et al. 2001). Several human studies found that S100A12 was strongly expressed in inflamed tissues of patients who were suffering from inflammatory diseases, such as rheumatoid or psoriatic arthritis (Foell, Kane et al. 2003; Rouleau et al. 2003; Yang et al. 2001), thoracic aortic aneurysm (Bowman et al. 2010), inflammatory bowel disease

(Foell et al. 2008; Sidler et al. 2008), Kawasaki disease (Foell, Ichida et al. 2003) and inflammatory lung diseases (Lorenz et al. 2008). In these reports, the expression levels of S100A12 generally correlated with tissue inflammatory activities. Thus, S100A12 might serve as an excellent diagnostic biomarker for a variety of inflammatory diseases. Although S100A12 is not present in mice, a number of studies have used murine model to demonstrate the proinflammatory function of S100A12 by simply injecting the protein to mice. Their results showed S100A12 was able to induce the migration of neutrophils and monocytes to the inflammatory sites (Rouleau et al. 2003; Yang et al. 2001). Another report employed a transgenic murine model that specifically expresses human S100A12 in vascular smooth muscle, where it was shown that S100A12 plays a role in triggering pathological vascular remodeling in mice (Bowman et al. 2010). Together, there is ample evidence suggests that S100A12 plays a crucial role in innate and acquired immune responses.

Several S100 proteins have been shown to bind heparan sulfate (HS), an abundant glycosaminoglycan found on the cell surface and in the extracellular matrix. S100A9, a protein abundantly expressed by neutrophils, has been reported to bind endothelial HS with high affinity in a calcium and zinc dependent manner (Robinson et al. 2002). S100A4, which is expressed mainly in nervous system, was shown to bind neuron and stimulate neurite outgrowth in a HS-dependent fashion (Kiryushko et al. 2006). In the same study, S100A12 was found to bind heparin and its stimulation of neurite outgrowth also depends on S100A12–HS interactions. Although these studies have clearly shown that selected extracellular S100 proteins bind HS and the interaction can have significant biological consequences, the structural requirement for the interaction remains unknown. For instance, it is unclear whether specific oligomeric states of S100 protein are required for HS interaction, what kind of HS structures are preferred by S100 proteins, and where the HS-binding site is located.

In the current study, we investigate the structural details of the molecular interaction between S100A12 and HS. Our data show that S100A12–HS interaction is strictly Ca^{2+} dependent, while the optimum binding was obtained when both Ca^{2+} and Zn^{2+} are present. By HS oligosaccharide microarray we determined that the binding of S100A12 requires at least hexasaccharide and the binding is highly dependent on the density of sulfate groups. By site-directed mutagenesis we have also identified the HS-binding site of S100A12. Aided by this structural insight, we have shown that targeted engineering of the HS-binding site could convert non-HS-binding forms of S100A12 (apo and tetramer) into HS-binding forms. Remarkably, the engineered HS-binding site displays much higher selectivity toward longer oligosaccharides compared to the wild-type (WT) HS-binding site. To our knowledge, our study represents the first example that a novel HS-binding specificity can be engineered from a naturally occurring HS-binding site.

Results

S100A12 binds heparin Sepharose in a divalent cation and oligomeric states dependent manner

Using heparin Sepharose chromatography, we systematically examined the interaction between S100A12 and heparin in the absence or presence of different divalent cations (Table I). When running buffer is free from divalent cations, apo form of S100A12 did not bind heparin column at all and appeared in the flow through. If S100A12 was preincubated with 0.1 mM ZnCl_2 to allow stable

tetramer formation and then applied on heparin column, we still did not observe any binding. However, when S100A12 was preincubated with 1 mM CaCl_2 and then applied to heparin column, we found that S100A12 was retained on the column during the loading phase, but eventually was eluted during the washing phase with the cation-free running buffer. Presumably, this binding mode is due to the gradual stripping of calcium ions from S100A12, which converts it back to the apo form. In contrast, when S100A12 was preincubated with both CaCl_2 and ZnCl_2 to allow formation of stable hexameric complex, S100A12 hexamer bound well to heparin column and was not eluted until salt concentration reached 550 mM.

We next tested heparin binding when divalent cations were included in the running buffer. Interestingly, when Ca^{2+} was present in the running buffer, S100A12 (predominantly dimer) remained bound to the heparin column until salt concentration reached 660 mM. When both Ca^{2+} and Zn^{2+} are present in the running buffer, S100A12 (stable hexamer) binds even more avidly to heparin, which was eluted by 720 mM salt. In contrast, when running buffer only contains Zn^{2+} , S100A12 (stable tetramer) still did not show any binding to heparin column, suggesting that the tetrameric conformation is not compatible with heparin binding.

S100A12 binds cell surface in a calcium- and HS-dependent manner

As heparin is a highly sulfated variant of HS (average 2.5–3 sulfate groups per disaccharide unit), it is important to examine whether S100A12 truly binds HS expressed on the surface of cells. Unlike heparin, HS tends to be much sparsely sulfated (average 1–1.8 sulfate groups per disaccharide unit for most cell types). To this end, we performed S100A12 binding to CHO-K1 cells. In the presence of Ca^{2+} , S100A12 bound to CHO-K1 in a concentration-dependent manner (Figure 1A). In the presence of both Ca^{2+} and Zn^{2+} , the binding signal increased ~3-fold compared to Ca^{2+} only condition (relative fluorescence units (RFU) = 48 vs 13.5, Figure 1B), suggesting S100A12 hexamer had higher affinity for cell surface HS than the dimer.

To demonstrate that the binding is indeed mediated by HS, CHO cells were pretreated with heparin lyase III, which selectively remove cell surface HS. As expected, the cell surface binding almost entirely mediated by HS as 94% of binding was lost after heparin lyase treatment (RFU = 250 vs 15, Figure 1C). As another way to show that S100A12 predominantly binds cell surface HS, we tested S100A12 binding to pgsD CHO cells. pgsD cells have severe defect in HS biosynthesis and do not express any HS on cell surface (Lidholt et al. 1992). Again, S100A12 shown minimal binding to pgsD cells, amount to only 4.4% of its binding level to CHO-K1 (RFU = 223 vs 10, Figure 1D). Of note, pgsD cells are known to express 2–3 times more chondroitin sulfate on the surface to compensate for the loss of HS. The fact the S100A12 showed minimal binding to pgsD cells suggest that the protein does not bind to chondroitin sulfate.

S100A12 dimer and hexamer form stable complexes with HS

Our data so far suggest that both S100A12 dimer and hexamer can bind HS. As a common role of HS is to induce protein oligomerization, we investigated whether HS will further induce S100A12 oligomerization. We first tested whether heparin-derived octasaccharide could induce S100A12 dimer to form higher order oligomers in the presence of Ca^{2+} by size exclusion chromatography (SEC).

Table I. S100A12 binding to heparin Sepharose.

Divalent cation in running buffer*	Sample**	Bound	Elution NaCl concentration
No	S100A12	No	–
No	S100A12 + CaCl ₂	Yes	150 mM (during wash)
No	S100A12 + ZnCl ₂	No	–
No	S100A12 + CaCl ₂ + ZnCl ₂	Yes	550 mM
1 mM CaCl ₂	S100A12 + CaCl ₂	Yes	660 mM
1 mM CaCl ₂	S100A12 + CaCl ₂ + ZnCl ₂	Yes	720 mM
0.1 M ZnCl ₂	S100A12 + ZnCl ₂	No	–
1 mM CaCl ₂ , 0.1 mM ZnCl ₂	S100A12 + CaCl ₂ + ZnCl ₂	Yes	720 mM

*Running buffer A for heparin Sepharose contains 25 mM HEPES, 150 mM NaCl, pH 7.1. Running buffer B contains 25 mM HEPES, 1 M NaCl, pH 7.1.

**If indicated, sample was preincubated with divalent ions for 10 min at RT before applying onto the heparin column.

Fig. 1. S100A12 binds cell-surface HS. (A) Biotinylated S100A12 at 2, 5 and 10 µg/mL was incubated with CHO cells in HBS containing 1 mM CaCl₂ and stained with streptavidin-PE. The shaded histogram is from cells stained with only streptavidin-PE. (B) Binding of 2 µg/mL biotin-S100A12 to CHO-K1 cells in HBS containing 1 mM CaCl₂ only or with 1 mM CaCl₂ plus 0.1 mM ZnCl₂. (C) Binding of 10 µg/mL biotin-S100A12 to CHO-K1 cells pretreated with 5 mU/mL heparin lyase III (HL-III). (D) Binding of 10 µg/mL biotin-S100A12 to CHO-K1 cells or HS-deficient pgsD CHO cells.

Compared to S100A12 dimer alone, S100A12-octasaccharide mixture showed a small (0.05 mL) but reproducible forward shift in elution position (Figure 2A). This small shift suggests that the octasaccharide formed a stable complex with S100A12 dimer but did not change the oligomeric state of S100A12. We next performed a similar experiment for S100A12 hexamer in the presence of Ca²⁺ and Zn²⁺. Similarly, heparin-derived octasaccharide induced a 0.05 mL forward shift on elution position (Figure 2B), again suggesting formation of a stable complex between S100A12 hexamer and octasaccharide but no change in oligomeric states.

HS oligosaccharide microarray analysis of S100A12–HS interaction

To explore the structure specificity of HS–S100A12 interactions, we performed a HS oligosaccharide microarray analysis of S100A12 binding. The HS oligosaccharide array contains 52 structure-defined HS oligosaccharides with various lengths and sulfation patterns (Figure 3A; Hippensteel et al. 2019; Nguyen et al. 2019). We have performed HS microarray analysis of both S100A12 dimer (in the presence of CaCl₂, Figure 3B) and S100A12 hexamer in the presence of CaCl₂ and ZnCl₂ (Figure 3C). The overall binding patterns to

Fig. 2. S100A12 dimer and hexamer form stable complexes with heparin octasaccharide. (A) 20 μ g of S100A12 dimer was incubated with 4.5 μ g of heparin-derived octasaccharide (H8) at room temperature for 2 h and resolved on a high-performance liquid chromatography–size-exclusion chromatography (HPLC-SEC) column with a running buffer containing 1 mM CaCl_2 . The elution positions of the MW standards BSA (66.5 kD) and soluble RAGE (32.7 kD) are marked with black triangles (11.4 and 12.3 mL, respectively). (B) Twenty microgram of S100A12 hexamer was incubated with 4.5 μ g of heparin-derived octasaccharide at room temperature for 2 h and resolved on a HPLC-SEC column with a running buffer containing 1 mM CaCl_2 and 0.1 mM ZnCl_2 .

HS oligosaccharides are quite similar between S100A12 dimer and hexamer, suggesting dimer and hexamer interact with HS in a similar fashion. The first conclusion can be made from the array analysis is that hexasaccharide is sufficient for interacting with S100A12 (oligo #16, 34 and 40). Secondly, it appears that S100A12–HS interaction does not strictly require either 6-O- or 2-O-sulfation. Rather, the interaction appears to largely correlate with the overall sulfation level, with the highly sulfated structures bind significantly better than the less sulfated structures. This point is best illustrated in a group of heptasaccharides (oligo 4–12) that bears 3–8 sulfate groups (Figure 3D). Although oligos containing only three sulfate groups (oligo 9 and 12) did not bind S100A12, oligos showed gradually enhanced binding with increased sulfation level (#6 with 4 sulfates; #7 with 5 sulfates; #8 and #11 with 6 sulfates; #5 and #10 with 7 sulfates; and #4 with 8 sulfates). At last, we found that the binding of hexasaccharides was most sensitive to minor changes in the sulfation pattern. As shown in Figure 3E, oligo #35 contains a total of six sulfate groups but did not show any binding to S100A12, while oligo #34 showed good binding with only one extra 6-O-sulfate at the non-reducing-end glucosamine, suggesting that this particular 6-O-sulfate group is crucial for binding. Oligo #39 also has very similar structure as #34 with only one less 2-O-sulfate at the middle glucuronic acid, yet #39 showed much weaker binding compared to #34. The most striking observation in terms of structural specificity of S100A12 binding can be found between oligos #39 and #40. Although #40 has one less N-sulfate groups than #39, it binds significantly better than #39, suggesting that for oligosaccharides of this particular structure,

removing the N-sulfate from the reducing-end glucosamine actually promotes binding to S100A12.

Identification of the HS-binding site of S100A12

As crystal structures of S100A12 dimer, tetramer and hexamer are all available (Moroz et al. 2002; Moroz et al. 2001; Moroz, Blagova et al. 2009), we set out to map the HS-binding site of S100A12. A surface charge representation of S100A12 dimer in complex with calcium identified a positively charged patch centered around helix-II (H-II) and helix-III (H-III) (Figure 4). The positive charges were contributed by six lysine residues including K30, K34 and K39 located in H-II, K46 and K49 in the coil connecting H-II and H-III, and K51 in H-III. By site-directed mutagenesis we mutated each of these lysines into alanines and examined the impact on binding to heparin Sepharose. As shown in Table II, with the exception of K46A, all other lysine mutants showed 100–200 mM reduction in salt concentration required for elution. This result suggests that the HS-binding site of S100A12 likely constitutes K30, K34, K39, K49 and K51. As expected, double mutants K30A–K34A and K34A–K49A had additive effect on heparin binding, resulting in a 330–370 mM reduction in salt concentration required for elution. Of note, all single and double mutants were able to form hexamer in the presence of Ca^{2+} and Zn^{2+} (data not shown), suggesting the introduced mutations did not affect divalent ion binding and oligomerization. To further examine whether these residues are truly involved in HS binding, we performed the cell surface binding assay. As shown in Figure 5, K34A bind to cell surface at only 7% of the WT level (RFU = 45 vs 630), while K30A–K34A binds at merely 1.3% of WT level (RFU = 5.6 vs 416), which suggests that these residues likely direct interact with HS.

To try to understand why the apo form of S100A12 dimer and the Zn-mediated S100A12 tetramer did not bind HS, we compared the structure of their H-II and H-III elements to that of the Ca^{2+} -bound dimer and $\text{Ca}^{2+}/\text{Zn}^{2+}$ -bound hexamer. It turned out for both apo and tetramer forms of S100A12, an additional small helix (H-IIa) is present right next to H-II (Figure 6C and D). H-IIa replaces a large portion of the long flexible coil linker that is present in the Ca^{2+} -dimer and $\text{Ca}^{2+}/\text{Zn}^{2+}$ -hexamer (Figure 6A and B), which substantially changes the relative orientation of H-III to H-II. Such conformational change results in substantially increased distance among HS-binding residues located in different structural elements. For instance, the distance between K39 and K49 almost doubled from 13 Å in the Ca^{2+} -dimer and $\text{Ca}^{2+}/\text{Zn}^{2+}$ -hexamer (Figure 6A and B) to 24 and 26 Å in the apo form and the tetramer, respectively (Figure 6C and D). Likewise, the distance between K51 and K34 is 8 Å in the Ca^{2+} -dimer and $\text{Ca}^{2+}/\text{Zn}^{2+}$ -hexamer (Figure 6A and B), and it becomes 16 Å in the apo form and the tetramer (Figure 6C and D). As the dimension of sulfated sugar ring of HS is roughly between 8 and 12 Å, the HS-binding residues in the Ca-dimer and Ca/Zn-hexamer are perfectly aligned for HS binding. In contrast, the same residues are poorly arranged in the apo and tetramer forms of S100A12 with excessive distances among the lysines located in the H-II and loop/H-III, which might be the main reason that these forms failed to interact with HS.

Engineering the HS-binding site of S100A12

It is remarkable that in S100A12 the same set of five basic residues (Lys30, Lys34, Lys39, Lys49 and Lys51) can be arranged in conformations that display either no binding or strong binding to HS.

Fig. 3. Binding of biotin-S100A12 to HS oligosaccharide microarray. (A) List of all 52 structure-defined oligosaccharides that are immobilized on the microarray chip. (B) Microarray was probed with 1 $\mu\text{g/mL}$ biotin-S100A12 in a binding buffer containing 1 mM CaCl_2 , followed by avidin conjugated with Alexafluor-488. The averaged fluorescence intensity was plotted. (C) Microarray was probed with 1 $\mu\text{g/mL}$ biotin-S100A12 in a binding buffer containing 1 mM CaCl_2 and 0.1 mM ZnCl_2 . (D) Replot of S100A12 binding to heptasaccharides #4–12 in the presence of Ca^{2+} and Zn^{2+} (data from panel C). Heptasaccharides are ranked by the number of sulfate groups they carry. (E) Structures of hexasaccharides #34, 35, 39 and 40. For each pair of structures, the differences in sulfation pattern are highlighted with a red box.

As mentioned in last section, the main factor that might contribute to the absence of interaction between the apo and tetramer forms of S100A12 and HS could be the large distance between K39 and K49 (24 Å), an arrangement that is incompatible with engaging multiple sulfate groups (Figure 6C and D). If this prediction is correct, installing one additional lysine residue between K39 and K49, which

would bridge the positive charges offered by K39 and K49, might facilitate restoration of HS-binding of the apo and tetramer forms. Based on the crystal structures, the best location to introduce this additional lysine would be Asn43, which is located within H-IIa and is roughly midway between K39 and K49 (Figure 7A). In theory, mutating Asn43 to lysine would greatly increase the chance of HS

Fig. 4. Potential HS-binding site of S100A12. (A) Surface charge representation of S100A12 dimer with bound calcium (PDB: 1E8A). Blue represents positive charges while red represents negative charges. (B) Cartoon representation S100A12 dimer in the same orientation as in A. Side chains of basic residues that are potentially involved in HS binding are shown in sticks. Helix-II and helix-III (H-II and H-III) are marked in bold. Two EF hands of one S100A12 monomer are shown in magenta and salmon, respectively. Calcium ions are shown in yellow spheres.

Table II. S100A12 mutants binding to heparin Sepharose column.

Mutant	Elution NaCl concentration (mM)
WT	720
K30A	590
K34A	500
K39A	560
K46A	700
K49A	610
K51A	580
K30A-K34A	290
K34A-K49A	250

binding because K43 would be only ~ 10 Å from K39 and ~ 14 Å from K49 (Figure 7A). To this end, we have prepared N43K mutant and tested its HS-binding capacity. Indeed, both apo and tetramer forms of N43K showed strong binding to heparin Sepharose. The elution positions of the apo and tetramer forms of N43K are at 650 and 730 mM NaCl (Table III), respectively, which are very similar to the elution position of the Ca-dimer and Ca/Zn hexamer forms of WT S100A12. This result is quite surprising due to the fact that only a single additional basic residue could restore a completely dysfunctional HS-binding site. We further tested binding of N43K mutant to cell-surface HS under various buffer conditions including without divalent cations, with zinc only, and with both zinc and calcium. Consistent with our observation with heparin Sepharose, N43K bound well to MC3T3 cells when no divalent cation was present, whereas WT S100A12 showed minimal binding when used at the same concentration (Figure 7B). Likewise, when only zinc was present, N43K showed strong binding to cell surface even at 1 $\mu\text{g/mL}$, whereas WT S100A12 again showed almost no binding (Figure 7C). In contrast, when both calcium and zinc were present, WT and N43K showed comparable binding to MC3T3 cells (Figure 7C).

Fig. 5. Binding of S100A12 mutants to cell-surface HS. (A) Biotinylated WT S100A12 or K34A mutant to MC3T3 cells at 5 $\mu\text{g/mL}$ in HBS containing 1 mM CaCl_2 and 0.1 mM ZnCl_2 . (B) Binding of K30A-K34A double mutant to MC3T3 cells at 5 $\mu\text{g/mL}$ in HBS containing 1 mM CaCl_2 and 0.1 mM ZnCl_2 .

Due to the differences in geometry of the HS-binding sites between N43K and WT S100A12, we predict that the mutant might display different preferences for HS oligosaccharides. To test this, we examined binding specificity of N43K mutant by HS oligosaccharide

Fig. 6. Geometry of HS-binding residues in calcium-bound S100A12 dimer (PDB:1E8A) (A); calcium/zinc-bound S100A12 hexamer (PDB:1GQM) (B); S100A12 dimer apo form (PDB:2WCE) (C) and zinc-bound S100A12 tetramer (PDB: 2WCB) (D). Only one dimer is shown in each structure with the two monomers in green and salmon, respectively. Distance between HS-binding residues are marked with red dash line and helices H-II, H-IIa and H-III are marked in bold.

array in the presence of Zn^{2+} only. Interestingly, we found that the mutant showed much greater selectivity compared to WT S100A12 (Figure 7D, compared to Figure 3B and 3C). Specifically, the N43K mutant much prefers oligosaccharides that are longer than dodecasaccharide (oligo #18, 19, 20, 22 and 27) and showed either no binding or very weak binding to the hexasaccharides and heptasaccharides (oligo# 4–17 and 28–40). Our finding strongly suggests that we have engineered a novel HS-binding site by fine tuning the existing structural elements of S100A12.

DISCUSSION

In the current study we have shown that S100A12, a small EF-hand calcium-binding protein often associated with inflammatory conditions, binds HS in a divalent cation- and oligomeric state-dependent manner. As the extracellular calcium concentration is normally between 1 and 1.5 mM (Hofer and Brown 2003; Rossol et al. 2012), which is the same concentration as what we used in our biochemical studies, our data suggests the majority of the extracellular S100A12 are occupied by calcium and should be able to binding HS on the cell surface and in the matrix. In contrast, the extracellular concentration of zinc ion is much more fluctuant, which can range between low μM and 300 μM (Buxaderas and Farre-Rovira 1985; Vallee and Falchuk 1993). The extracellular concentrations

of calcium and zinc ions suggest that under normal physiological conditions S100A12 mostly exists in dimer and hexamer states. Although both forms are capable of binding HS, the binding properties are different between dimer and hexamer (Figure 1B). Our results suggest that the fluctuation of calcium and zinc concentrations during various pathophysiological conditions might greatly impact on the avidity of S100A12–HS interaction and consequently affect the biological function of S100A12.

The HS-binding site of S100A12 consists of five basic residues and has a dimension of 15 Å by 25 Å (Figure 6A). Such dimension is typical of many HS-binding proteins such as FGF2 and antithrombin (Xu and Esko 2014). The length of the binding site is roughly equivalent to the length of a hexasaccharide, which is consistent with our microarray analysis suggesting that the hexasaccharide is sufficient to provide good binding (Figure 3). In terms of the geometry of the HS-binding site, S100A12 bears strong similarity to the well-characterized antithrombin. The HS-binding site of antithrombin mainly consists of five basic residues, with R46 and R47 residues at the beginning part of an α -helix and the adjacent loop, and R114, R125 and R129 residues in a neighboring α -helix (Jin et al. 1997; Skinner et al. 1997). The arrangement of the HS-binding residues in S100A12 is quite similar in that K30, K34 and K39 are located in H-II, whereas K49 and K51 are located at the beginning part of H-III and the adjacent loop. The relative orientations of the two neighboring helices in S100A12 and antithrombin are also

Fig. 7. Characterization of the HS-binding specificity of N43K mutant. (A) Structure of zinc-bound S100A12 tetramer (PDB: 2WCB) with Asn43 (green stick) mutated to Lys43 (white stick). Distance between HS-binding residues is marked with dashed lines. Only one dimer is shown with the two monomers in green and salmon. (B) Binding of biotinylated WT S100A12 or N43K mutant to MC3T3 cells at 10 µg/mL in HBS containing no divalent ions. (C) Binding of biotinylated WT S100A12 or N43K mutant to MC3T3 cells at 1 µg/mL in HBS containing 0.1 mM ZnCl₂ only, or in HBS containing 1 mM CaCl₂ and 0.1 mM ZnCl₂. (D) HS oligosaccharide microarray (as shown in Figure 3A) was probed with 1 µg/mL biotin-N43K mutant in a binding buffer containing 0.1 mM ZnCl₂, followed by avidin conjugated with Alexafluor-488. The averaged fluorescence intensity was plotted.

similar. In both proteins the helices intersect with each other at a roughly 60° angle (Figure 4). Because antithrombin displays a strong dependence on 3-O-sulfated glucosamine for interaction with HS, the structural similarity of S100A12 to antithrombin prompted us to wonder whether S100A12 also displays a preference for 3-O-sulfated structures. Although we did observe enhanced binding of S100A12 toward 3-O-sulfated oligosaccharides (Figure 3, comparing oligo 4 vs 5 and 19 vs 20), the enhancement in binding is likely incremental, in contrast to the all-or-none effect of 3-O-sulfation on antithrombin-HS interactions (Richard et al. 2009).

One of the most interesting discoveries is that the apo dimer and zinc-bound tetramer forms of S100A12 showed no binding to HS. This fact is quite intriguing in both biological and structural perspectives. Although the extracellular calcium concentration is normally above 1 mM (Hofer and Brown 2003; Hofer et al. 2000), in some occasions the concentration of calcium can dip below 100 µM (Launikonis et al. 2003; Pumain and Heinemann 1985). As in some inflammatory conditions the concentration of S100A12 can reach close to 1 mM (Rouleau et al. 2003), the majority of S100A12 would remain in apo form when calcium concentration drops below 100 µM. Quite possibly, converting extracellular S100A12 to apo

Table III. Comparison of WT S100A12 and N43K mutant binding to heparin Sepharose column

Divalent cation in running buffer*	N43K	WT S100A12
No	650 mM	No binding
Zn	730 mM	No binding
Ca	730 mM	660 mM

*Running buffer A for heparin Sepharose contains 25 mM HEPES, 150 mM NaCl, pH 7.1. Running buffer B contains 25 mM HEPES, 1 M NaCl, pH 7.1.

form would allow S100A12 to quickly diffuse into tissues when it is free from tethering by HS. Structure wise, it is quite striking that a calcium-induced conformational change renders an all-or-none binding to HS. Analysis of the structures of the apo form and zinc-induced tetramer suggests that the calcium-induced conformational change brings several dispersed lysine residues into a close-knit web of positive charges, which becomes conducive for HS binding. Build on this discovery, we report here for the first time that a non-HS-binding protein can be converted to a HS-binding protein by introducing a single point mutation. The key of our success was

restoration of a web of positive charges that is conducive for HS binding. It happens that a single additional lysine, installed at the loop connecting H-II and H-III, is sufficient to restore the web of positive charges (Figure 7A).

Interestingly, our engineered HS-binding site displayed a higher level of selectivity compared to WT S100A12. Although the WT S100A12 binds well to a large number of hexasaccharides and heptasaccharides, the N43K mutant binds selectively to oligosaccharides that are dodecasaccharides or longer (Figure 3A and B and Figure 7D). One likely reason for this preference for longer oligosaccharide could be the increased dimension of the HS-binding site of N43K. The distance between K30 and K49 (which defines the long axis of the HS-binding site, Figure 4B and Figure 7A) is increased from 25 Å in Ca²⁺-bound WT dimer to 32 Å in Zn²⁺-bound N43K tetramer. As the length of the HS heptasaccharide is around 26.2 Å, it is sufficient to cover the whole HS-binding site in WT S100A12 but unlikely to reach the outmost basic residues in the HS-binding site of N43K. To cover the 32 Å distance, a minimal of nonasaccharide (33.7 Å) or decasaccharide (37.5 Å) is likely required. Attributable to the fact that our HS oligo array does not contain any decasaccharide and all included nonasaccharides (oligo #41–47) do not bear sulfates on the two terminal glucuronic acids (Figure 3A), the only oligosaccharides on the array that bound well were the dodecasaccharides.

This unexpected finding might change how we comprehend the evolution of HS-binding proteins. As we know the HS-binding proteins can arise from all kinds of protein folds that bear no structural homology to each other (Xu and Esko 2014). Our finding indicates that gaining robust HS-binding capacity can be as simple as introducing a single additional basic charge, which could happen instantly in evolutionary terms. Such an easy route to acquire HS-binding capability might partially explain why so many structurally diverse proteins evolved to be HS-binding proteins. In addition, the fact that our reengineered HS-binding site displays a structural selectivity that is different from the WT S100A12 strongly suggests that the HS-binding specificity can be tailored. For example, different versions of the same HS-binding growth factor could potentially be engineered to control its bioavailability (which is affected by its binding to HS) in tissue engineering projects.

In conclusion, we have thoroughly investigated the structural elements that govern S100A12–HS interactions. As many other S100 family proteins are either known HS-binding proteins or potential HS-binding proteins (Kiryushko et al. 2006; Robinson et al. 2002), our study could serve as a foundation to further investigate the role of HS in the extracellular function of S100 proteins in general. Engineering of a non-HS-binding form of S100A12 into a HS-binding form provides a conceptual advance in our understanding of how HS-binding site is constructed, which might have direct impact on protein engineering should alternative HS-binding specificity is desired.

Materials and methods

Expression and purification of full length human S100A12

The complete open reading frame of human S100A12 was cloned into pET21b (Novagen) using Nde I and Xho I sites. Recombinant S100A12 was expressed in *Escherichia coli* at 37°C. After sonication to lyse cells, CaCl₂ was added to the supernatant to reach final concentration of 10 mM, and the lysate was incubated at room temperature (RT) for 10 min. Purification was carried out using

HiScreen Phenyl FF (High Sub) column (GE Healthcare) at pH 7.5 (Tris buffer), followed by anion exchange chromatography on a 1 mL HiTrap Q-Sepharose column (GE Healthcare) at pH 8 (Tris buffer). After purification, S100A12 was >9% pure as examined by silver staining. The biological activity of purified S100A12 was verified by its binding to human soluble RAGE, which displays an apparent binding affinity of 67 nM (data not shown), very similar to what was reported (Hofmann et al. 1999).

Heparin Sepharose chromatography

To characterize the binding of WT S100A12 and S100A12 mutants to heparin, 500 µg of purified WT or mutant S100A12 were applied to a 1 mL HiTrap heparin-Sepharose column (GE Healthcare) and eluted with a salt gradient from 150 mM to 1 M NaCl at pH 7.1 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer with or without 1 mM CaCl₂ and/or 0.1 mM ZnCl₂). In some experiments, the mutants were incubated with divalent ions for 10 min at room temperature (RT) before loading onto the column. The conductivity measurements at the peak of the elution were converted to the concentration of NaCl based on a standard curve.

Biotinylation of S100A12

WT or mutant S100A12 was diluted in 1 mL buffer containing 25 mM HEPES, 150 mM NaCl, 1 mM CaCl₂ and 0.1 mM ZnCl₂, pH 7.1, followed by loading onto a 200 µL heparin-Sepharose column. Sulfo-NHS-LC-biotin in a buffer (500 µL of a 0.33 mM solution) containing 25 mM HEPES, 150 mM NaCl, 1 mM CaCl₂ and 0.1 mM ZnCl₂, pH 7.1 was then applied to the column and biotinylation was allowed to proceed for 30 min at RT. The reaction was stopped by applying 2 mL of 50 mM Tris, pH 7.7. Then the column was eluted with 25 mM HEPES, 1 M NaCl, 1 mM CaCl₂ and 0.1 mM ZnCl₂, pH 7.1. The biotinylated S100A12 was repurified by applying onto a 1 mL HiTrap heparin-Sepharose column, which also verified that the heparin binding property of S100A12 was not altered by biotinylation.

Flow cytometry-based binding assay

CHO-K1 cells or MC3T3 cells were incubated with biotinylated WT or mutant S100A12 in 100 µL of PBS containing 1 mM CaCl₂ or 1 mM CaCl₂ and 0.1 mM ZnCl₂ for 45 min at 4°C. After rinsing, the cells were stained with streptavidin-phycoerythrin (BioLegend) at a dilution of 1:1000 for 15 min and analyzed by flow cytometry. In some experiments, cells were pretreated with recombinant heparin lyases III (5 mU/mL, produced as a recombinant protein in *E. coli*) for 15 min at RT prior to binding experiments.

Analytical SEC

For analysis of S100A12 dimer and heparin-derived octasaccharide complex, 20 µg purified S100A12 were incubated with 4.5 µg octasaccharide in 25 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, pH 7.1 at RT for 2 h. The complex was resolved on an SRT SEC-300 column (8.7/200 mm, Sepax) using 25 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, pH 7.1 at 4°C. The analysis of S100A12 hexamer-octasaccharide complex was performed the same way except that the incubation buffer and running buffer were 25 mM HEPES, 150 mM NaCl, 1 mM CaCl₂ and 0.1 mM ZnCl₂, pH 7.1.

Site-directed mutagenesis

S100A12 mutants were prepared using published method (Zheng et al. 2004). Mutations were confirmed by sequencing, and recombinant protein was expressed and purified as described for WT S100A12.

Glycan array fabrication

Microarrays were printed by sciFLEXARRAYER S5 of various amine-containing HS oligosaccharide (50 μ M) in print buffer (50 mM phosphate, pH 8.5 containing 0.005% Tween-20) onto NHS-activated glass slides. Each compound was printed at 6×6 pattern. Printed slides were allowed to react in an atmosphere of 80% humidity for 24 h and non-reacted HS oligosaccharide was removed from slides by rinsing with deionized water. Remaining NHS groups were blocked by immersion in buffer (50 mM ethanolamine in 50 mM borate buffer, pH 9.2) for 1 h at 50°C. Slides were rinsed with water, dried and stored before use. Of note, at position #1 of microarray, we immobilized commercial heparin, which was intended to serve as a positive control. However, due to the fact that heparin was immobilized through its internal free amines, heparin likely lay flat on the microarray surface. In contrast, all HS oligosaccharides were immobilized through their reducing-end and the compounds stand up on the surface. As a result, position #1 always show poor binding to HS-binding proteins (Hippensteel et al. 2019; Nguyen et al. 2019).

Glycan array binding assay

Printed slides were analyzed without any further modification of the surface. Slides were incubated in a sandwich procedure, in which the Biotinylated S100 A12 was incubated first, followed by Alexa 488 conjugated Avidin incubation. Biotinylated S100 A12 was added at a concentration of 1 μ g/mL and Alexa 488 conjugated Avidin was added at a concentration of 10 μ g/mL in buffer (25 mM HEPES, 150 mM NaCl, 0.05% Tween-20, 10% BSA, pH 7.1). The samples (100 μ L) were applied directly onto the surface of a single slide and covered with a microscope and then incubated in a humidified chamber for 60 min at room temperature. Slides were subsequently washed by successive rinses in (i) 25 mM HEPES, 150 mM NaCl, 0.05% Tween-20, 1% BSA, pH 7.1 and (ii) deionized water, dried and then immediately subjected to imaging. For S100A12 dimer binding, 1 mM CaCl_2 was added to the incubation and washing buffer. For S100A12 hexamer binding 1 mM CaCl_2 and 0.1 mM ZnCl_2 were added to the incubation and washing buffer. For N43K tetramer binding 0.1 mM ZnCl_2 were added to the incubation and washing buffer. The images were acquired using the excitation wavelength of 488 nm on GenePix 4300 A scanner. The intensity data is the mean value $\pm$ SD of 36 individual spots.

Author contributions

X.Z. and D.X. designed the research, X.Z. C.O and G.S. performed the work, X.Z. C.O and G.S. J.L. and D.X. analyzed and interpreted data, X.Z and D.X. wrote the manuscript. J.L revised the paper.

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Conflict of interest statement

None declared.

Abbreviations

HS, heparan sulfate; SEC, size exclusion chromatography; WT, wild-type; HBS, HEPES buffered saline; Strep-PE, streptavidin-phycoerythrin.

References

- Bowman HM, Wilk J, Heydemann A, Kim G, Rehman J, Lodato JA, Raman J, McNally EM. 2010. S100A12 mediates aortic wall remodeling and aortic aneurysm. *Circ Res*. 106:145–154.
- Buxaderas SC, Farre-Rovira R. 1985. Whole blood and serum zinc levels in relation to sex and age. *Rev Esp Fisiol*. 41:463–470.
- Donato R. 1999. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta*. 1450:191–231.
- Foell D, Ichida F, Vogl T, Yu X, Chen R, Miyawaki T, Sorg C, Roth J. 2003a. S100A12 (EN-RAGE) in monitoring Kawasaki disease. *Lancet*. 361:1270–1272.
- Foell D, Kane D, Bresnihan B, Vogl T, Nacken W, Sorg C, Fitzgerald O, Roth J. 2003b. Expression of the pro-inflammatory protein S100A12 (EN-RAGE) in rheumatoid and psoriatic arthritis. *Rheumatology (Oxford)*. 42:1383–1389.
- Foell D, Roth J. 2004. Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis Rheum*. 50:3762–3771.
- Foell D, Wittkowski H, Ren Z, Turton J, Pang G, Daebritz J, Ehrchen J, Heydemann J, Borody T, Roth J et al. 2008. Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. *J Pathol*. 216:183–192.
- Foell D, Wittkowski H, Roth J. 2007. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Pract Rheumatol*. 3:382–390.
- Hippensteel JA, Anderson BJ, Orfila JE, McMurtry SA, Dietz RM, Su G, Ford JA, Oshima K, Yang Y, Zhang F et al. 2019. Circulating heparan sulfate fragments mediate septic cognitive dysfunction. *J Clin Invest*. 129:1779–1784.
- Hofer AM, Brown EM. 2003. Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol*. 4:530–538.
- Hofer AM, Curci S, Doble MA, Brown EM, Soybel DI. 2000. Intercellular communication mediated by the extracellular calcium-sensing receptor. *Nat Cell Biol*. 2:392–398.
- Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P et al. 1999. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell*. 97:889–901.
- Ilg EC, Troxler H, Burgisser DM, Kuster T, Markert M, Guignard F, Hunziker P, Birchler N, Heizmann CW. 1996. Amino acid sequence determination of human S100A12 (P6, calgranulin C, CGRP, CAAF1) by tandem mass spectrometry. *Biochem Biophys Res Commun*. 225:146–150.
- Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. 1997. The anticoagulant activation of antithrombin by heparin. *Proc Natl Acad Sci U S A*. 94:14683–14688.
- Kiryushko D, Novitskaya V, Soroka V, Klingelhofer J, Lukanidin E, Berezin V, Bock E. 2006. Molecular mechanisms of Ca^{2+} signaling in neurons induced by the S100A4 protein. *Mol Cell Biol*. 26:3625–3638.
- Launikonis BS, Barnes M, Stephenson DG. 2003. Identification of the coupling between skeletal muscle store-operated Ca^{2+} entry and the inositol trisphosphate receptor. *Proc Natl Acad Sci U S A*. 100:2941–2944.
- Lidholt K, Weinke JL, Kiser CS, Lugenma FN, Bame KJ, Cheifetz S, Masague J, Lindahl U, Esko JD. 1992. A single mutation affects both

- N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc Natl Acad Sci U S A*. 89:2267–2271.
- Lorenz E, Muhlebach MS, Tessier PA, Alexis NE, Duncan Hite R, Seeds MC, Peden DB, Meredith W. 2008. Different expression ratio of S100A8/A9 and S100A12 in acute and chronic lung diseases. *Respir Med*. 102:567–573.
- Moroz OV, Antson AA, Dodson EJ, Burrell HJ, Grist SJ, Lloyd RM, Maitland NJ, Dodson GG, Wilson KS, Lukanidin E *et al*. 2002. The structure of S100A12 in a hexameric form and its proposed role in receptor signalling. *Acta Crystallogr D Biol Crystallogr*. 58:407–413.
- Moroz OV, Antson AA, Murshudov GN, Maitland NJ, Dodson GG, Wilson KS, Skibshoj I, Lukanidin EM, Bronstein IB. 2001. The three-dimensional structure of human S100A12. *Acta Crystallogr D Biol Crystallogr*. 57:20–29.
- Moroz OV, Blagova EV, Wilkinson AJ, Wilson KS, Bronstein IB. 2009. The crystal structures of human S100A12 in apo form and in complex with zinc: new insights into S100A12 oligomerisation. *J Mol Biol*. 391:536–551.
- Moroz OV, Burkitt W, Wittkowski H, He W, Ianoul A, Novitskaya V, Xie J, Polyakova O, Lednev IK, Shekhtman A *et al*. 2009b. Both Ca²⁺ and Zn²⁺ are essential for S100A12 protein oligomerization and function. *BMC Biochem*. 10:11.
- Moroz OV, Dodson GG, Wilson KS, Lukanidin E, Bronstein IB. 2003. Multiple structural states of S100A12: a key to its functional diversity. *Microsc Res Tech*. 60:581–592.
- Nguyen KG, Gillam FB, Hopkins JJ, Jayanthi S, Gundampati RK, Su G, Bear J, Pilkington GR, Jalah R, Felber BK *et al*. 2019. Molecular mechanisms of heparin-induced modulation of human interleukin 12 bioactivity. *J Biol Chem*. 294:4412–4424.
- Pumain R, Heinemann U. 1985. Stimulus- and amino acid-induced calcium and potassium changes in rat neocortex. *J Neurophysiol*. 53:1–16.
- Richard B, Swanson R, Olson ST. 2009. The signature 3-O-sulfo group of the anticoagulant heparin sequence is critical for heparin binding to antithrombin but is not required for allosteric activation. *J Biol Chem*. 284:27054–27064.
- Robinson MJ, Tessier P, Poulosom R, Hogg N. 2002. The S100 family heterodimer, MRP-8/14, binds with high affinity to heparin and heparan sulfate glycosaminoglycans on endothelial cells. *J Biol Chem*. 277:3658–3665.
- Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, Rothe K, Schubert K, Schoneberg T, Schaefer M, Krugel U *et al*. 2012. Extracellular Ca²⁺ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nat Commun*. 3:1329.
- Rouleau P, Vandal K, Ryckman C, Poubelle PE, Boivin A, Talbot M, Tessier PA. 2003. The calcium-binding protein S100A12 induces neutrophil adhesion, migration, and release from bone marrow in mouse at concentrations similar to those found in human inflammatory arthritis. *Clin Immunol*. 107:46–54.
- Sidler MA, Leach ST, Day AS. 2008. Fecal S100A12 and fecal calprotectin as noninvasive markers for inflammatory bowel disease in children. *Inflamm Bowel Dis*. 14:359–366.
- Skinner R, Abrahams JP, Whisstock JC, Lesk AM, Carrell RW, Wardell MR. 1997. The 2.6 Å structure of antithrombin indicates a conformational change at the heparin binding site. *J Mol Biol*. 266:601–609.
- Vallee BL, Falchuk KH. 1993. The biochemical basis of zinc physiology. *Physiol Rev*. 73:79–118.
- Xu D, Esko JD. 2014. Demystifying heparan sulfate-protein interactions. *Annu Rev Biochem*. 83:129–157.
- Yang Z, Tao T, Raftery MJ, Youssef P, Di Girolamo N, Geczy CL. 2001. Proinflammatory properties of the human S100 protein S100A12. *J Leukoc Biol*. 69:986–994.
- Zheng L, Baumann U, Reymond JL. 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res*. 32:e115.