Binding between the integrin \( \alpha X\beta 2 \) (CD11c/CD18) and heparin

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The interactions between cell surface receptors and sulfated glucosaminoglycans serve ubiquitous roles in cell adhesion and receptor signaling. Heparin, a highly sulfated polymer of uronic acids and glucosamine, binds strongly to the integrin receptor \( \alpha X\beta 2 \) (p150,95, CD11c/CD18). Here, we analyze the structural motifs within heparin that constitute high-affinity binding sites for the \( \alpha X\beta 2 \) integrin. Heparin oligomers with chain lengths of 10 saccharide residues or higher provide strong inhibition of the binding by the \( \alpha X I \) domain to the complement fragment iC3b. By contrast, smaller oligomers or the synthetic heparinoid fondaparinux were not able to block the binding. Semipurified heparin oligomers with 12 saccharides residues identified the fully sulfated species as the most potent antagonist of iC3b, with a 1.3 \( \mu M \) affinity for the \( \alpha X I \) domain. In studies of direct binding by the \( \alpha X I \) domain to immobilized heparin we found that the interaction is conformationally regulated and requires \( \text{Mg}^{2+} \). Furthermore, the fully sulfated heparin fragment induced conformational change in the ectodomain of the \( \alpha X\beta 2 \) receptor, also demonstrating allosteric linkage between heparin binding and integrin conformation.

Introduction

Increasing evidence points to an important function of heparin in the immune system. Heparin is exclusively synthesized by connective tissue mast cells and released from storage granula in the inflammatory responses mediated by these leukocytes. Furthermore, several receptors on leukocytes are able to bind with high affinity to heparin. These include the \( \beta 2 \) integrins \( \alpha \text{M}\beta 2 \) (Mac-1, CD11b/CD18) and \( \alpha \text{X}\beta 2 \) (p150,95, CD11c/CD18)(1,2), which play key roles in the adhesion, migration, and binding of complement fragments by myeloid leukocytes. \( \alpha \text{M}\beta 2 \) and \( \alpha \text{X}\beta 2 \) integrins, also referred to as complement receptor (CR)\(^1\) 3 and CR4, respectively, bind strongly to a proteolytic fragment of complement factor 3 (C3) designated iC3b, as shown both by cellular and biochemical assays. iC3b plays an important role in phagocytic uptake of microbes by leukocytes of the myeloid lineages. Diamond \textit{et al.}(2) reported the adhesion of neutrophil granulocytes to heparin-coated surfaces through the \( \alpha \text{M}\beta 2 \) integrin, which is abundantly expressed on these leukocytes. The \( \alpha \text{X}\beta 2 \) integrin, primarily expressed on monocytes, macrophages, and dendritic cells, was also demonstrated to support adhesion to heparin by use of cell line transfectants(2).

Integrin receptors contain multiple domains in their ectodomain. \( \beta 2 \) integrins, which in addition to \( \alpha \text{M}\beta 2 \) and \( \alpha \text{X}\beta 2 \) include the \( \alpha \text{L}\beta 2 \) integrin (LFA-1, CD11a/CD18), bind ligands through an inserted (I) domain in the alpha subunit. Previous studies have indicated a central role of the \( \alpha M \) and \( \alpha X I \) domains in binding to heparin(2) and shown that the affinity of the \( \alpha X I \) domain for heparin is significantly higher than the affinity of the \( \alpha M I \) domain(3). In the metal-ion dependent

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\(^1\) Abbreviations: complement receptor (CR); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); metal-dependent adhesion site (MIDAS); \( N \)-hydroxysuccinimide (NHS)
adhesion site (MIDAS) of the I domain, a
Mg²⁺ ion forms a crucial bond to an acidic
residue in protein ligands. However, the
requirement for Mg²⁺ in the binding between
integrin I domains and heparin is unclear. For
many protein ligands the binding to integrin I
domains is regulated through conformational
changes, where the open conformation of the I
domain binds these ligands with several
magnitudes stronger affinity than the closed
conformation(4). By contrast, conformational
regulation of the binding by I domains to
heparin or other non-proteinous ligands has
not been studied.

The structure of heparin has been
subject to considerable investigation. Heparin
is a sulfated, linear polysaccharide with a
repeating disaccharide residue of D-glucosamine and uronic acids. Each repeating
residue of glucosamine and uronic acid may
hold a maximum of three sulfo groups but
other, less sulfated disaccharides can also be
isolated from enzymatically degraded heparin.
This microheterogeneity and the polydisperse
length with an average $M_r$ of natural heparin
chains distributed between 10,000-12,000
constitute together a highly complex
structure(5-7). No studies on integrins have
addressed the character of the binding sites in
heparin for I domains. These properties are
important in understanding the ability to bind
pharmacologically important
glucosamineglycans such as fondaparinux
sodium, a synthetic heparinoid recently
marketed for antithrombotic treatment(8).

Here we analyze the binding between
heparin and the $\alpha X$ I domain and identify
oligomers with 10 saccharide residues or more
as potent ligands for the $\alpha X$ I domain. The
binding to heparin was dependent on Mg²⁺
and conformationally regulated similar to what has
been reported for protein ligands. Fully
sulfated oligomers bound the $\alpha X$ I domain
with the highest affinity and with sufficient
tensity to induce conformational change in
the ectodomain of $\alpha X\beta 2$ integrin.

Materials and Methods

Preparation and characterization of
bovine lung heparin oligosaccharides. The
heparin oligosaccharide mixture, prepared
from bovine lung heparin (Sigma, St. Louis,
MO) by controlled enzymatic
depolymerization with heparin lyase I (EC
4.2.2.7, IBEX, Montreal, Canada), was
fractionated by gel-permeation
chromatography on a P-10 column (BioRad,
Richmond, CA) to obtain size-uniform
oligosaccharides. The fraction consisting of
dodecasaccharides was further separated using
semi-preparative strong anion-exchange high-
performance liquid-chromatography on a 5 µm
Spherisorb™ column (Waters Corp., Milford,
MA) eluted with a linear gradient from 0.1 to
1.9 M NaCl (pH 3.5) over 180 min at a flow
rate of 4 ml/min(9). Six peaks, labeled
dp12(A), dp12(C), dp12(D), dp12(E), dp12(F),
and dp12(G), were collected, desalted, and
freeze-dried. The size and sulfatation of the
oligosaccharide samples were determined by
analysis on gels with a linear polyacrylamide
gradient from 12% to 22% (w/v), visualized
by Alcian Blue staining, and compared with a
banding ladder of heparin oligosaccharide
standards(10).

Recombinant I domains and SPR
assays for the interaction with heparin.
Expression and purification of recombinant
$\alpha M$ and $\alpha X$ I domains was described
earlier(3,11,12). In brief, the wild-type $\alpha X$ I
domain or open-conformation $\alpha M$ and $\alpha X$ I
domains carrying the mutations Ile314→Gly
or Ile-314→Gly, respectively, were expressed in $E. coli$
and purified from the soluble
dataction.

The affinity of the open-conformation
$\alpha M$ and $\alpha X$ I domains for heparin and heparin
fragments was measured by inhibition of I
domain binding to ligand. Experiments were
carried out by SPR with a BIACore 3000
instrument (Biacore, Upplsa, Sweden) in
CM-4™ chip flow cells coupled with 4,700-
5,300 arbitrary response units (RU; 1,000 RU
~ 1 ng protein per mm² of flow cell surface) of
iC3b (Calbiochem, San Diego, CA) and with a
reference cell coupled with ethanolamine in
parallel as described(12). The dissociation
constant ($K_D$) for the binding between the
open-conformation $\alpha M$ or $\alpha X$ I domain and
the immobilized iC3b was determined as
described(12) from fitting the Langmuir-Hill
Equation to the steady-state equilibrium
response levels ($R_{eq}$):

$$R_{eq} = (C_{free} \cdot R_{max})/(C_{free} + K_D)$$

In Equation (1) $C_{free}$ is the concentration of
free I domain and $R_{max}$ is the response level at
binding-saturating concentrations. For
The direct binding of I domains to heparin was analyzed by immobilizing heparin to CM-4 chip with a chip surface coated with carboxymethylated dextran. The surface was activated with a mixture 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) with Biacore’s kit (cat.no. BR-1006-33, Biacore, Uppsala, Sweden) and injection of 35 µl of 5 mM hydrazine over 7 min followed by blocking of unreacted sites by injection of 1 M ethanolamine hydrochloride, pH 8.0. Nitrous acid-depolymerized heparin with an average atomic mass of 5,314 Da – dp20 and a reducing terminal 2,5-anhydromannose (DH-03253, Celsus Laboratories Inc., Cincinnati, OH) was dissolved at 5 mg/ml in 10 mM Na-acetate, pH 4.5, and injected over the hydrazine-coupled surface in order to allow for aldehyde coupling through the reducing end of the heparin. The resulting hydrazone bond formed from the reaction between hydrazine and the aldehyde group is not stable in aqueous media and the bond was consequently reduced to a stable hydrazide bond by injection of 40 µl of cyanoborohydride coupling buffer (C4187, Sigma) over 7 min, followed by regeneration of the surface with injection of 5 µl 10 mM HCl. This procedure immobilized heparin at a level corresponding to 250 RU. The binding to the heparin-coupled surfaces was tested for the αM and αX I domains with the running buffers described above except that the MgCl₂ concentration was 1 mM.

**Exposure of activation-dependent epitopes in the β2 subunit of recombinant αXβ2.**

The ability of heparin and fragments of heparin to induce conformational change in the αXβ2 integrin was tested by use of the monoclonal antibody KIM127(13), which recognizes an activation-dependent epitope in the C-terminal region of the beta chain(14), in the setting of an ELISA experiment with soluble recombinant αXβ2.

Soluble, heterodimeric αLβ2 and αXβ2 integrins with a C-terminal, α-helical coiled-coil clasp were expressed in mammalian CHO cell lines and purified as described(15). The purified proteins were stored in 150 mM NaCl 20 mM Tris-HCl, pH
7.5 (TBS) containing 1 mM CaCl₂, and 1 mM MgCl₂.

Plastic microtiter wells were treated with rabbit polyclonal antibodies against the ACID/BASE coiled-coil clasp as described by Takagi et al. (16). The wells were incubated overnight at 4°C with 50 μl of 5 μg/ml antibodies in 30 mM NaNO₃, 0.15 M Na₂CO₃, 0.35 M NaHCO₃, pH 9.6, followed by washing in TBS with 0.1% (v/v) Triton X-100 (TBS-T), and blocking with 1% (w/v) bovine serum albumin (Sigma) in TBS. After washing in TBS-T, 50 μl of recombinant αXβ2 or αLβ2, diluted to a final concentration of 0.5 μg/ml in TBS with 5 mM CaCl₂, were added to antibody-coated wells and incubated at room temperature for 1.5 h, followed by three washes in TBS-T. The wells with immobilized integrins were incubated with heparin, bovine heparan sulfate (H-7640, Sigma), porcine chondroitin sulfate type A (C-0914, Sigma), purified dp12(E) heparin, or fondaparinux sodium in concentrations between 5 and 500 μg/ml in TBS with 1 mM CaCl₂ and 1 mM MgCl₂. For comparison immobilized αXβ2 and αLβ2 integrins were also incubated with 100 μg/ml dp12(E) heparin in TBS with 1 mM CaCl₂ and 1 mM MgCl₂, or in TBS with 1 mM CaCl₂ and 1 mM MgCl₂, without glucosaminoglycans, or in TBS with 2 mM MnCl₂, or in TBS with 1 mM CaCl₂, 1 mM MgCl₂, and 5 μM XVA143 (17). The wells were incubated at room temperature for 15 min. Biotinylated mAb KIM127 diluted to 1 μg/ml or biotinylated mAb IB4 diluted to 5 μg/ml in TBS with 0.1% (w/v) BSA were incubated in the wells at room temperature for 15 min. The signals were read following incubation with streptavidin-labeled horseradish peroxidase and addition of 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Zymed, San Francisco, CA).

Results

The affinity of the αX I domain correlates with heparin oligosaccharide length. By controlling enzymatic depolymerization with heparin lyase I it is possible to derive from unfractionated heparin a set of oligomers with defined lengths. We employed oligomers ranging in lengths from a degree-of-polymerization (dp) of two saccharide residues (dp2) to 14 saccharide residues (dp14) with a Mₙ approximately between 500 and 3,500, respectively, as inhibitors of the open-conformation αX I domain binding to iC3b. The αX I314G I domain binds the iC3b fragment of complement factor C3 with a Kᵢ of 1.5 μM (Fig. 1A). When mixed with the αX I domain prior to application to SPR, heparin oligosaccharides dp8, dp10, dp12, and dp14 inhibited binding to iC3b, whereas the dp2, dp4, and dp6 oligomers had only a marginal influence on the response level (Fig. 1C). A range of oligomer concentrations was used to inhibit binding to two different iC3b sensor chip preparations, which in the absence of inhibition bound 1,610 RU and 1,760 RU of αX I domain when 1.4 μM was applied (Fig. 2). The concentration required to obtain a 50% reduction in the SPR response level was 45 μM, 5.3 μM, and 4.5 μM for the dp10, dp12, and dp14 oligomers, respectively (Fig. 2B and 3D). Unfractionated heparin is a potent inhibitor of binding by the open-conformation αX I domain to immobilized iC3b (Fig. 2A). With an average Mₙ of 11,000 and dp of 42, the concentration of heparin required to obtain 50% inhibition was 0.30 μM.

The αM I domain, mutated similarly to the αX I domain to favor the open conformation (11), bound iC3b with a Kᵢ of 1.5 μM (Fig. 1B) in agreement with earlier reports (3,11). No significant inhibition was observed from application of the size-sorted heparin oligomers to the binding by the open-conformation αM I domain to immobilized iC3b (Fig. 2D). The interaction between heparin oligomers and the αX and αM I domains was further analyzed by considering the influence of the oligomers on the initial on-rate as has been described for other inhibition assays with use of SPR (18). In order to check the validity of Equation (3) (see Materials and Methods) we titrated the 1 domain concentration for either construct from 0.28 μM to 3.14 μM and measured the initial on-rate Vᵢ at t = 4 s. As shown by the plot in Fig. 3A there was a linear relationship between the applied 1 domain concentration and Vᵢ with correlation coefficients close to 1 suggesting that the binding reaction was not limited by mass transport. Furthermore, from the slope of the line (αVₑ) of 33.10⁰ RU⋅M⁻¹⋅s⁻¹ and Rₘₐₓ of 3,160
RU as estimated from Equation (1), $k_{on}$ was calculated to be 10,400 M$^{-1}$s$^{-1}$, which is within 20\% of our earlier published value of 8,400 M$^{-1}$s$^{-1}$(12). Since mass transport effects are only pronounced when $k_{on}$ significantly exceeds 10,000 M$^{-1}$s$^{-1}$(19), and since our analysis confirmed the linear relationship between $V_i$ and the I domain concentration, we concluded that the kinetics of our binding assay are not limited by mass transport. We therefore analyzed the influence of the size-sorted heparin oligomers and native heparin on the initial on-rate. The heparin oligomer concentrations required to reduce the initial on-rate 50\% for dp8, dp10, dp12, and dp14 were 56 µM, 22 µM, 1.8 µM, and 1.1 µM, respectively (Fig. 3D).

The affinity of the $\alpha$X I domain for anion-exchange purified heparin oligomers. To further characterize the interaction between heparin and the $\alpha$X I domain, we semi-purified the dodecasaccharide oligomers by strong anion-exchange high-performance liquid chromatography. The oligomers were separated in seven fractions (Fig. 4A), and six fractions contained semipurified species when analyzed by PAGE (Fig. 4B). Fractions D and E correspond to dodecasaccharides based on comparison to oligosaccharide standards(10). According to earlier analysis by 2D $^1$H-NMR spectroscopy(9) fraction E, of higher than 90\% purity (Fig. 4B), corresponds to a fully sulfated, i.e., with a maximum of 18 sulfo groups, dodecasaccharide. Fraction D contained undersulfated dodecasaccharides, i.e., with less than 18 sulfo groups. The fractions F and G contained oligomers with 10-14 saccharides, while fraction A in addition to dp12 oligomers contained highly sulfated decasaccharides (Fig. 4B).

Inhibition assays were carried out as for the size-sorted material (Fig. 1E and 2C) and the data were analyzed by determining the concentration required to lower the response level at the end of the injection phase by 50\%. The dp12(E) oligomers bound the $\alpha$X I domain with the highest affinity with an IC$_{50}$ of 1.3 µM (Fig. 1F). The less sulfated dp12(D) oligomers bound with an IC$_{50}$ of 3.3 µM. Both the dp12(A) and dp12(C) fractions showed a 5-fold lower affinity than the dp12(E) oligomers, consistent with the presence in this fraction of decasaccharides. The fraction dp12(F) had the lowest potency in inhibiting the binding between the $\alpha$X I domain and iC3b, while the dp12(G) showed an affinity close to the affinity of dp14 oligomers (Fig. 2C and 3D). These findings were further supported by the reduction by purified dp12 oligomers of the initial on-rate in binding by the $\alpha$X I domain (Fig. 3C-D). The rank among the six dp12 fractions of the IC$_{50}$ values was identical to that determined for the inhibition of the final response level, with dp12(E) the most potent ligand for the $\alpha$X I domain.

For comparison to the naturally derived heparin oligomers we included fondaparinux sodium, which is a synthetic pentasaccharide (dp5) with a $M_r$ of 1,728 and a total of 7 sulfo groups(8), i.e., 2 less than the maximum number of possible sulfo groups. At similar concentrations compared with those used for size-sorted heparin fragments or the chemically purified dp12 oligomers, fondaparinux was not able to affect the binding of the $\alpha$X I domain to iC3b (Fig. 2C).

Direct binding of the $\alpha$X and $\alpha$M I domain to surface-immobilized heparin. The direct binding of open and closed $\alpha$X and $\alpha$M I domains to surface-immobilized heparin was monitored by SPR. Nitrous acid depolymerization of native heparin generates oligomers containing terminal anhydromannose with a reducing end that allows for covalent coupling to hydrazine-coupled surfaces(20). Compared with native heparin the average $M_r$ was reduced approximately 2-fold from 10,000 (dp40) to 5,000 (dp20) with a ratio (mol/mol) of 0.8 aldehyde group per heparin oligomer (information provided by Celsus Inc., Cincinatti, OH). The open-conformation $\alpha$X I314G I domain at a concentration of 4.7 µM bound robustly to surface-immobilized heparin in the presence of 1 mM Mg$^{2+}$ (Fig. 5A). EDTA abolished binding by the open-conformation $\alpha$X I domain. The conformational regulation of the binding was studied by comparing the binding of the wild-type $\alpha$X I domain injected at a concentration of 10.6 µM. In this case only a minor response was observed, suggesting a weak affinity for heparin (Fig. 5A). Consistent with the observations that heparin in solution is a poor inhibitor of the binding by the open-conformation $\alpha$M I domain to iC3b, direct binding of this domain to immobilized heparin
was weak even when the domain was injected at a concentration of 10.6 μM in the presence of 1 mM Mg$^{2+}$ (Fig. 5B).

**Induction of activation epitopes in the αXβ2 ecto domain by anionic sugars.** The epitope recognized by the monoclonal β2 antibody KIM127(13) is a read-out for the large structural changes within the heterodimeric molecule that brings the β2 integrins from a resting, non-ligand binding conformation to the active, ligand binding conformation(14). Recombinant, soluble αXβ2 integrin was indirectly immobilized in microtiter wells and incubated with the dp12(E) oligomer. The binding of the KIM127 antibody showed a dp12(E) concentration-dependent increase with half-maximum saturation at 50 μg/ml corresponding to an oligomer concentration of 17 μM (Fig. 5C). Heparin, heparan sulfate, and fondaparinux produced a detectable increase in epitope exposure but were clearly less potent agents than the dp12(E) oligomers. Chondroitin sulfate did not change the epitope exposure even when applied at a concentration of 500 μg/ml (Fig. 5C). We also compared the dp12(E)-induced KIM127 epitope exposure with other conditions known to alter the conformation of β2 integrins. In buffer with Mg$^{2+}$ and Ca$^{2+}$, the αXβ2 integrin remains largely in a resting state with a low exposure of the KIM127 epitope (Fig. 5D). Addition of Mn$^{2+}$, a well-established inducer of conformational change and activator of ligand binding in integrins, clearly increased KIM127 epitope exposure (Fig. 5D). XVA is a small-molecule antagonist of β2 integrin ligand binding, which acts through allosteric regulation of the β2 chain(17,21). As reported for αLβ2 integrin(21) XVA induced KIM127 exposure in the αXβ2 integrin (Fig. 5D) consistent with the recent observation that XVA changes the conformation of αXβ2 integrin(15). Addition of 100 μg/ml dp12(E), i.e., at a binding-saturating concentration (Fig. 5C), produced a KIM127 exposure comparable to that observed for the application of Mn$^{2+}$ or XVA to the experiment (Fig. 5D). αLβ2 integrin showed no alterations in the KIM127 epitope exposure upon incubation with the dp12(E) oligomer (Fig. 5E) or with heparin, fondaparinux, heparin sulfate, or chondroitin sulfate (data not shown). Application of the IB4 mAb to the β2 chain showed that the amounts of αLβ2 and αXβ2 integrin immobilized in the wells were comparable (data not shown).

**Discussion**

In this study we analyze the binding between heparin and αXβ2 integrin and show that the αX I domain has high affinity for fully sulfated heparin oligomers with a length of 12 monosaccharide residues. Our data suggest a conformational regulation of the binding between heparin and the αXβ2 integrin, which demonstrates the role of conformational regulation in integrin binding to a natural, non-proteinous ligand. Furthermore, the strength of the binding between dp12 heparin oligomers and the intact receptor is sufficient to extend the αXβ2 integrin as shown by KIM127 epitope exposure.

The αXβ2 integrin contains two chains, each with several domains (Fig. 6). The I domain of the α chain is the major ligand binding for several protein ligands such as fibrinogen and iC3b. More recent work has identified the I domain as also a binding domain for heparin(3). The I domain may take two different conformations referred to as the "closed" or "open" conformation. As reported earlier the open-conformation αX I domain binds strongly to heparin(3), but a characterization of the binding motif in heparin has not been provided. In the present study we provide a more detailed analysis of the interaction between heparin and the αX I domain and estimate the strength of the interaction through the ability of the heparin oligomers to inhibit the binding of the open-conformation αX I domain to iC3b as monitored by SPR. The degree of inhibition was assessed by comparing the response level at the end of the injection phase in the absence or presence of heparin oligomers. However, as equilibrium was not reached for all samples, we also compared the influence of heparin oligomers on the initial on-rate in the binding between the αX I domain and iC3b; we found good agreement between the two approaches suggesting that the IC$_{50}$ values determined from these measurements are a reliable estimation of the strength of binding between the heparin oligomers and the I domain.

Enzymatic digestion and fractionation of heparin into low-molecular-weight
eparin oligomers showed that the αX I domain bound dp12 and dp14 oligomers with an affinity corresponding to a 50% inhibitory concentration of 1-5 μM. By contrast, dp10 oligomers bound the I domain with considerably weaker affinity corresponding to an IC₅₀ of 45 μM suggesting that a minimum of 12 saccharide residues is required to obtain high affinity binding between the αX I domain and heparin. Consistent with these data, fondaparinux, a short (dp5) synthetic heparin which is used in anticoagulant treatment as replacement for naturally derived heparins(8), showed no detectable interaction with the αX I domain. Further purification of the dp12 oligomers provided oligomers with varying degrees of sulfation. Comparison of the ability of these oligomers to inhibit the binding of the open-conformation αX I domain showed that the most sulfated of the oligomers, dp12(E), had the highest affinity for the αX I domain.

Earlier studies have shown that binding by αMβ2 and αXβ2 integrins to heparin is dependent on divalent cations(2). However, both receptors contain multiple Mg²⁺ and Ca²⁺ binding sites in their ecto domains, which precludes conclusions on which particular metal ions were required for the binding to heparin. The integrin I domain MIDAS contains one metal-ion binding site(4), which has a 100-fold stronger affinity for Mg²⁺ than for Ca²⁺ and thus, at physiological concentrations of these ions, primarily is occupied with Mg²⁺(22,23). Crystal structures show a coordination at the MIDAS typical for Mg²⁺ but not for Ca²⁺. In the open, ligand-binding conformation, the MIDAS Mg²⁺ ion directly coordinates the sidechains of two Ser and one Thr residue. The MIDAS metal ion is in the center of the ligand binding site, and directly coordinates a Glu sidechain in protein ligands(4).

We investigated whether the MIDAS site in the αX I domain was involved in heparin binding by coupling heparin to a SPR chip surface and flowing in the open-conformation I domain in the presence of Mg²⁺ or EDTA. Binding of the I domain to immobilized heparin clearly required Mg²⁺. While Ca²⁺ ions have been observed in several cases to contribute to the binding between heparin and metalloproteins, Mg²⁺-dependent protein binding to heparin has been less frequently observed, one example being heparin cofactor II(24). Thus, it appears that the heparin binding by the αX I domain is a rare case of a Mg²⁺ ion contributing to protein-heparin interaction.

The end-to-end length of heparin either determined from hydrodynamic measurements(25) or direct measurements on heparin in complex with thrombin resolved by X-ray crystallography(26) corresponds to 0.5 nm per monosaccharide unit. dp12 oligomers would thus be assumed to take a length of 6 nm, which is comparable to the diameter of the I domain at 5 nm(12). Consequently, our data suggest that topologically dispersed interactions on the αX I domain surface and heparin contribute to the binding. Similar findings were reported for other heparin-binding proteins(27), where the binding of heparin to diverse protein surfaces is facilitated by the induced fit made possible by the steric freedom of the heparin sulfo groups. In this context it is of interest that chemical fractionation of the dp12 heparin oligomers identified the maximally sulfated oligomer dp12(E) as the strongest binder of the αX I domain and that binding of the I domain to heparin required Mg²⁺. Structural studies on the αX I domain identified a stretch of positively charged or polar residues of a length of approximately 3 nm, uninterrupted by negatively charged residues and crossing the MIDAS with the positively charged Mg²⁺ ion(12). Binding of the αX I domain to protein ligands is Mg²⁺ dependent, just as we find here for binding to heparin. This finding suggests that the MIDAS proximal region constitutes the binding interface for heparin, as shown by crystal structures for other I domains bound to protein ligands(4). Indeed, the spatial organization of positively charged residues in an uninterrupted stretch through the MIDAS constitutes a structural feature that would seem ideal for binding polyanions such as heparin and our observation that a minimal length of 10-12 saccharide units is required to obtain a maximal affinity is in good agreement with the dimensions of the stretch of positively charged residues. While the heparin binding site would appear to be fully loaded with the dp12 oligomers, we find that native heparin, corresponding on average to a dp42 oligomer, has a higher affinity for the αX I domain than even the dp12(E) oligomers. One explanation
for this finding is likely to relate to the possibility of multiple binding sites within an oligomer the size of native heparin. We have calculated the 50% inhibitory concentration for heparin based on the molar concentration of the full length molecule but if the molecule contains multiple, and possibly overlapping, binding sites the correct concentration of binding sites is underestimated by our approach. However, as a stringent correction for this effect is complicated we have reported the strength of the interactions in terms of the molar concentration of oligomers.

The αM I domain does not have the spatial organization of positively charged residues around the MIDAS as is found on the αX I domain surface; perhaps in consequence of this, heparin bound with approximately 10-fold lower affinity to the open-conformation αM I domain than to the αX I domain. The present study shows that this property is not altered by shortening the length of the heparin.

Recent studies on integrin receptors have emphasized the importance of affinity regulation in the ligand binding by these receptors through large conformational changes in the receptor ecto domain(28). In the I domain, the conformational regulation is tightly linked with the ability of the MIDAS-chelated Mg\(^{2+}\) ion to coordinate acidic side chains of protein ligands. We probed the binding to immobilized heparin by the αX I wild-type domain, which we have shown takes the closed conformation with a weak affinity for protein ligands(12). Compared with the open-conformation αX I domain, binding by the wild-type domain was weak.

Further evidence of the importance of integrin conformation was provided from monitoring the exposure of an activation-dependent epitope in the β2 chain of immobilized αXβ2 integrin. Ligand binding by integrins is associated with a large change in the conformation of ecto domain of the receptor; in the resting state the receptor takes a bend conformation while a change to the unbent conformation makes the receptor competent for ligand binding (Fig.6). In the case of β2 integrins an epitope in the β chain recognized by the monoclonal antibody KIM127 is exposed when the receptor is found in the extended conformation(15). By incubating the αXβ2 integrin immobilized in ELISA microtiter wells with the dp12(E) oligomers we were able to strongly induce the exposure of the KIM127 epitope with half-maximum exposure at 17 µM. Integrin receptors exchange between the non-ligand and ligand-binding conformations(29), and hence application of an excess of a strong ligand like the dp12(E) heparin oligomer would be expected to stabilize the exposure of integrin epitopes characteristic of the ligand-binding conformation. Thus, our study demonstrates that the conformation of the αX I domain regulates binding by heparin, and conversely, that binding of heparin regulates the overall conformation of αXβ2.

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Figure legends

Figure 1. Competitive inhibition with heparin fragments of the αM and αX I domain binding to iC3b monitored by SPR. In panels A-F the end of the injection phases are indicated with arrows. (A-B) Sensorgrams showing the binding of the αX 1314G (A) or αM 1316G (B) I domain to iC3b, when either I domain was injected in a series of dilutions at concentrations of 0.28 µM, 0.41 µM, 0.62 µM, 0.93 µM, 1.40 µM, 2.09 µM, and 3.14 µM (corresponding to the ascending order of sensorgrams in A and B). Affinities for the I domain binding to iC3b corresponded to $K_D = 1.5$ µM and $K_D = 2.1$ µM for αX 1314G and αM 1316G, respectively, as determined from the steady-state equilibrium responses. The interaction with iC3b for the αX 1314G (C, E-F) or αM 1316G (D) I domains was monitored in the presence of size-sorted heparin fragment (C-D) and chemically purified dp12 heparin oligomers (E-F). (C-D) Sensorgrams for injection of 1.4 µM αX 1314G (C) or αM 1316G (D) I domain either without any competitors or in the presence of 100 µg/ml (33 µM) of dp2, dp4, dp6, dp8, dp10, or dp12 size-sorted heparin oligomers. (E) Sensorgrams for injection of 0.69 µM αX 1314G in the presence of 100 µg/ml dp12(A), dp12(C), dp12(D), and dp12(E), dp12(F), or dp12(G). (F) Sensorgrams for injection of 0.69 µM αX 1314G in the presence of 0.07 µM, 0.13 µM, 0.26 µM, 0.52 µM, 1.0 µM, 2.1 µM, 4.2 µM, 8.3 µM, 16.7 µM, or 33.3 µM of the dp12(E) oligomer.

Figure 2. Competitive inhibition of the binding by I domains to immobilized iC3b with heparin and heparin fragments. In the calculation of oligomer concentrations the average $M_i$ of heparin was taken as 11,000, while the $M_i$ of smaller fragments was estimated from a $M_i$ of 500 per disaccharide. (A) Inhibition of the binding by the open-conformation αX 1314G I domain to immobilized iC3b as a function of the concentration of native heparin oligomers. Samples with a fixed concentration of I domain at 1.4 µM were mixed with heparin and the SPR response level recorded at the end of the injection phase. The percentage of inhibition was calculated relative to the response level in the absence of heparin. (B) The binding by the αX 1314G to iC3b in the presence of size-sorted heparins in oligomer concentrations from 0.13 µM to 66 µM. (C) Inhibition of the binding by the αX 1314G I domain to immobilized iC3b with the fractions from ion exchange chromatography of the dp12 heparin oligomers. The fractions A, C, D, E, F, and G were applied at oligomer concentrations from 0.07 µM to 33 µM. For comparison, the influence of fondaparinux on the binding to iC3b is indicated. The inhibition, calculated as in A, is expressed as a mean value of two independent experiments ± difference from the mean. (D) Similar to the experiments in B inhibition of the binding by the αM 1316G to iC3b in the presence of size-sorted heparins is shown for oligomer concentrations from 0.13 µM to 66 µM.

Figure 3. Influence on the initial on-rate of the binding by αX 1314G I domain to iC3b by heparin and heparin oligomers. (A) The initial on-rate ($V_1$), measured 4 s after injection start as the slope of the tangent to the sensorgram, for the binding of the αM 1316G and αX 1314G I domains to iC3b (as shown in Fig. 1A-B) plotted as function of the applied I domain concentration. Linearity was confirmed from fitting a straight line (indicated with hatched lines) to the data. (B) The decrease in initial on-rate plotted as function of the heparin oligomer concentration for dp2, dp4, dp6, dp8, dp10, dp12, and unfractionated bovine heparin applied in a concentration range. (C) The decrease in initial on-rate plotted as a function of the concentration of dp12 oligomers applied in a concentration range from 0.07 µM to 33 µM. (D) The IC$_{50\%}$ values obtained for the inhibition of I domain binding to iC3b with heparin in solution. The IC$_{50\%}$ values were estimated either from the concentration of oligosaccharides decreasing the response level at the end of the injection phase by 50% or by a 50% decrease in initial on-rate. The IC$_{50\%}$ values included for the open-conformation αM and αX I domain for low molecular weight heparin ($M_i$ ~ 6,000 ~ dp21) were determined in an earlier report(3) from competition assays with immobilized fibrinogen.

Figure 4. Subfractionation of dp12 heparin oligomers. (A) Strong anion-exchange high-performance liquid-chromatography fractionation profile from purification of the dp12 heparin oligomers as monitored by absorbance at $\lambda$ = 232 nm. Fractions are indicated with capital letters.
(B) PAGE analysis of size-sorted dp10, dp12, and dp14 heparin oligomers and the charge-fractionated oligomers indicated with A, C, D, E, F, and G from the purification of the dp12 oligomers. The lane indicated with “M” shows a ladder of heparin oligosaccharide standards prepared from bovine lung heparin as described(10).

Figure 5. Direct binding of αMβ2 or αXβ2 integrin I domains or the αXβ2 ectodomain to heparin oligomers. (A-B) Conformational requirements and dependence on Mg$^{2+}$-ion in the binding by αM and αX I domains to SPR surface-immobilized heparin. Sensorgrams are shown for the binding by 4.7 μM open-conformation αX I314G I domain (A) or 10.6 μM αM I316G I domain (B) in the presence of 1 mM MgCl$_2$ to heparin immobilized through covalent coupling to a chemically-introduced reducing terminus. For comparison, sensorgrams are shown for the binding by the open-conformation αX I domain in the presence of 1 mM EDTA or 10 μM wild-type αX I domain in the presence of 1 mM MgCl$_2$ (A). (C) Exposure of the KIM127 epitope in the integrin β2 chain in αXβ2 following incubation with heparin or heparin oligomers. The purified dp12(E) oligomers, heparin, fondaparinux, heparan sulfate, or chondroitin sulfate were incubated in a concentration range from 0 to 500 μg/ml with the αXβ2 immobilized in microtiter wells. Epitope exposure was monitored by ELISA. (D) KIM127 exposure monitored following incubation of αXβ2 in buffer with CaCl$_2$ and MgCl$_2$, dp12(E) heparin oligomers, MnCl$_2$, or the β2 integrin-binding compound XVA-143. (E) KIM127 exposure in αLβ2 in buffer with CaCl$_2$ and MgCl$_2$, dp12(E) heparin oligomers, MnCl$_2$, or XVA-143.

Figure 6. Domain structure of the β2 integrins. The α chain has five domains which are described in order from the N-terminus to the C-terminus. A 7-bladed β propeller domain contains an "inserted" I domain that constitutes the major ligand binding domain in β2 integrins. The thigh domain is joined through a flexible "genu" segment to the calf-1 and calf-2 domain. The β chain contains a domain structurally similar to the α chain I domain and hence referred to as the I-like domain followed by the hybrid domain, the plexin-semaphorin-integrin (PSI) domain, 4 integrin epidermal growth factor-like domains (indicated with E1-E4), and the β tail domain (βTD). Both the α and β chains have C-terminal transmembrane domains and short cytoplasmic tails. The figure illustrates the conformational change in the receptor ecto domain from a bent conformation to the extended conformation, which is competent for ligand binding. In the extended conformation an epitope in the C-terminal part of E2 domain is recognized by the KIM127 monoclonal antibody(13-15).
Fig. 1; Vorup-Jensen et al.
Fig. 2; Vorup-Jensen et al.

A. Heparin (dp42) inhibition of αx I314G.

B. Oligomer concentration (M) inhibition of αx I314G.

C. Dp12 inhibition of αx I314G.

D. Oligomer concentration (M) inhibition of αM I316G.
Fig. 3; Vorup-Jensen et al.

**A**

\[ V_I (RU\cdot s^{-1}) \]

\[ R^2 = 0.9944 \]

\[ R^2 = 0.9863 \]

**B**

Decrease in \( V_I \) (%)

**C**

Decrease in \( V_I \) (%)

**D**

| I domain Ligand Competitor | \( [C_{50\%}] (\mu M) \) | Response level | Initial on-rate (\( V_i \)) |
|---------------------------|-------------------------|----------------|---------------------------|
| dp8                       | N.D.                    | 56.3±43        |                            |
| dp10                      | 45.0±7.07               | 21.9±18        |                            |
| dp12                      | 5.25±4.80               | 1.76±1.24      |                            |
| dp14                      | 4.46±3.80               | 1.12±0.72      |                            |
| dp12(A)                   | 6.25±2.95               | 2.22±1.96      |                            |
| dp12(C)                   | 5.18±1.44               | 1.76±1.24      |                            |
| dp12(D)                   | 3.25±1.06               | 0.66±0.14      |                            |
| dp12(E)                   | 1.27±0.33               | 0.46±0.33      |                            |
| dp12(F)                   | 14.0±2.83               | 4.50±3.50      |                            |
| dp12(G)                   | 3.25±1.06               | 0.70±0.35      |                            |
| heparin                   | ~ 0.30                  | ~ 1.5          |                            |
| fibrinogen                | ~ 0.10                  |                |                            |
| heparin                   | ~ 1.50                  |                |                            |
Fig. 4; Vorup-Jensen et al.
Fig. 5; Vorup-Jensen et al.

A

\[ \alpha_x \text{I314G/Mg}^{2+} \]

\[ \alpha_x \text{Wt} \]

\[ \alpha_x \text{I314G/EDTA} \]

B

\[ \alpha_m \text{I316G/Mg}^{2+} \]

C

[Graph showing O.D. (405 nm) vs. Time (s) for various GAG concentrations]

D

[Bar chart showing O.D. (405 nm) for Mg/Ca, dp12(E), Mn/Mg/Ca, XVA]

E

[Bar chart showing O.D. (405 nm) for Mg/Ca, dp12(E), Mn/Mg/Ca, XVA]
Fig. 6; Vorup-Jensen et al.
Binding between the integrin αXβ2 (CD11c/CD18) and heparin
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