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Binding between the Integrin αXβ2 (CD11c/CD18) and Heparin*

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The interactions between cell surface receptors and sulfated glucosamineglycans 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 αXβ2 (p150,95, CD11c/CD18). Here, we analyze the structural motifs within heparin that constitute high affinity binding sites for the I domain of integrin αXβ2. Heparin oligomers with chain lengths of 10 saccharide residues or higher provide strong inhibition of the binding by the αX 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 saccharide residues identified the fully sulfated species as the most potent antagonist of iC3b, with a 1.3 μM affinity for the αX I domain. In studies of direct binding by the αX I domain to immobilized heparin, we found that the interaction is conformationally regulated and requires Mg^{2+}. Furthermore, the fully sulfated heparin fragment induced conformational change in the ectodomain of the αXβ2 receptor, also demonstrating allosteric linkage between heparin binding and integrin conformation.

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 β2 integrins αMβ2 (Mac-1, CD11b/CD18) and αXβ2 (p150,95, CD11c/CD18) (1, 2), which play key roles in the adhesion, migration, and binding of complement fragments by myeloid leukocytes. αMβ2 and αXβ2 integrins, also referred to as complement receptors 3 and 4, respectively, bind strongly to a proteolytic fragment of complement factor 3 designated iC3b, as shown by both cellular and biochemical assays. iC3b plays an important role in phagocytic uptake of microbes by leukocytes of the myeloid lineages. Diamond *et al.* (2) reported the adhesion of neutrophil granulocytes to heparin-coated surfaces through the αMβ2 integrin, which is abundantly expressed on these leukocytes. The αXβ2 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. β2 integrins, which in addition to αMβ2 and αXβ2 include the αLβ2 integrin (LFA-1, CD11a/CD18), bind ligands through an inserted (I) domain in the α subunit. Previous studies have indicated a central role for the αM and αX I domains in binding to heparin (2) and shown that the affinity of the αX I domain for heparin is significantly higher than the affinity of the αM I domain (3). In the metal ion-dependent adhesion site (MIDAS)[2] of the I domain, a Mg^{2+} ion forms a crucial bond to an acidic residue in protein ligands. However, the requirement for Mg^{2+} 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 nonproteinous 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 the average M_r of natural heparin chains distributed between 10,000 and 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 αX I domain and identify oligomers with 10 saccharide residues or

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[2] The abbreviations used are: MIDAS, metal ion-dependent adhesion site; RU, arbitrary response unit(s); MES, 4-morpholineethanesulfonic acid; TBS, Tris-buffered saline.

more as potent ligands for the αX I domain. The binding to heparin was dependent on Mg^{2+} and conformationally regulated similar to what has been reported for protein ligands. Fully sulfated oligomers bound the αX I domain with the highest affinity and with sufficient potency to induce conformational change in the ectodomain of αXβ2 integrin.

MATERIALS AND METHODS

Preparation and Characterization of Bovine Lung Heparin Oligosaccharides—The heparin oligosaccharide mixture, prepared from bovine lung heparin (Sigma) 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 (Bio-Rad) to obtain oligosaccharides uniform in size. 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 αM and αX I domains was described earlier (3, 11, 12). In brief, the wild-type αX I domain and open conformation αM and αX I domains carrying the mutations Ile-316 → Gly and Ile-314 → Gly, respectively, were expressed in *Escherichia coli* and purified from the soluble fraction.

The affinity of the open conformation αM and αX I domains for heparin and heparin fragments was measured by inhibition of I domain binding to ligand. The experiments were carried out by SPR with a BIAcore 3000 instrument (Biacore, Uppsala, Sweden) in CM-4™ chip flow cells coupled with 4,700–5,300 arbitrary response units (RU; 1,000 RU = ~1 ng of protein/mm^2 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 αM or αX 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}).

$$R_{eq} = (C_{free} \cdot R_{max})/(C_{free} + K_D) \quad \text{(Eq. 1)}$$

In Equation 1 C_{free} is the concentration of free I domain, and R_{max} is the response level at binding-saturating concentrations. For inhibition experiments with the I314G αX I domain, unfractionated bovine heparin (H-0777l Sigma), fondaparinux sodium (Arixtra™; GlaxoSmithKline, Philadelphia, PA), size-sorted heparins, and purified dp12 heparin oligosaccaharides were mixed at concentrations from 0.2 to 100 μg/ml with a fixed concentration of 0.63 or 1.4 μM of the αX I domain in running buffer with 150 mM NaCl, 10 mM $MgCl_2$, 10 mM MES, pH 6.0. Similarly, the I316G αM I domain at a concentration of 1.4 μM was mixed with the heparins in running buffer with 150 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4. The samples of I domain and heparin were injected over the reference and iC3b-coupled surfaces with a contact time of 240 s followed by a dissociation phase of 120 s and regeneration in 1.5 M NaCl, 50 mM EDTA, 100 mM MES, pH 6.0. The strength of the interaction between I domain and heparin in solution was approximated as the concentration inhibiting binding (measured in RU at injection stop) to iC3b by 50%.

As an alternative way of determining the affinity of the interaction between heparin oligomers and the αX I314G I domain, we analyzed the influence of the heparin oligomers on the initial on-rate (V_i) of binding between the I domain and immobilized iC3b. The rate constants for the association (k_{on}) and dissociation processes (k_{off}) of the I domain binding to iC3b are related to the response level (R) during the injection phase through Equation 1.

$$dR/dt = k_{on} \cdot C_{free} \cdot (R_{max} - R) - k_{off} \cdot R \quad \text{(Eq. 2)}$$

In Equation 2, t is the time point following injection start. Early during the injection phase where $R_{max} \gg R$, the contribution to the response level from release of material from chip surface can be neglected, and Equation 2 with α as the product of the constants k_{on} and R_{max} can thus be simplified to the following.

$$dR/dt = \alpha \cdot C_{free} = V_i \quad \text{(Eq. 3)}$$

The slope of the tangent at a time point shortly following injection start (here chosen as $t = 4$ s) approximates dR/dt, which equals the initial on rate V_i. Because of the linear relationship between V_i and C_{free}, the concentration of competitor required to reduce the concentration of free I domain with 50% was determined by plotting V_i as a function of concentration of competitor, *i.e.* heparin oligomers.

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 and *N*-hydroxysuccinimide with Biacore's kit (catalog number 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 sodium acetate, pH 4.5, and injected over the hydrazine-coupled surface 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 medium, 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 of 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

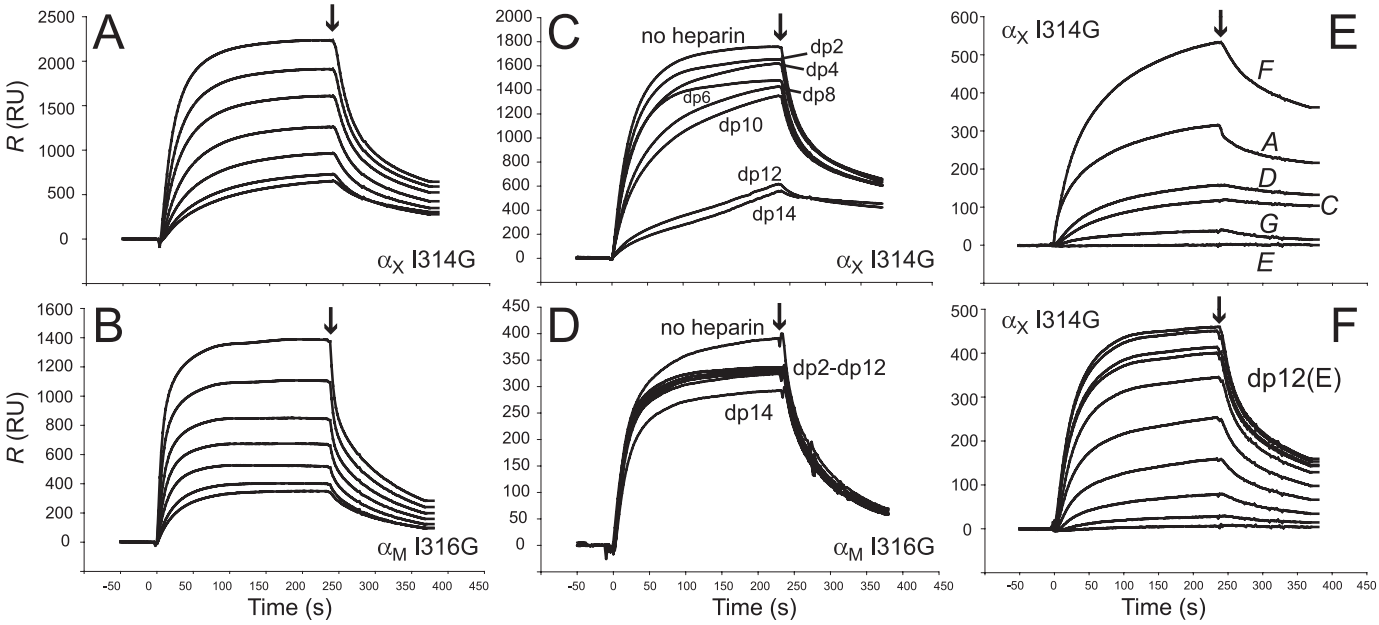


FIGURE 1. **Competitive inhibition with heparin fragments of the αM and αX I domain binding to iC3b monitored by SPR.** The ends of the injection phases are indicated with *arrows*. *A* and *B*, sensorgrams showing the binding of the αX I314G (*A*) or αM I316G (*B*) I domain to iC3b, when either I domain was injected in a series of dilutions at concentrations of 0.28, 0.41, 0.62, 0.93, 1.40, 2.09, 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 I314G and αM I316G, respectively, as determined from the steady-state equilibrium responses. The interaction with iC3b for the αX I314G (*C*, *E*, and *F*) or αM I316G (*D*) I domains was monitored in the presence of size-sorted heparin fragment (*C* and *D*) and chemically purified dp12 heparin oligomers (*E* and *F*). *C* and *D*, sensorgrams for injection of 1.4 μM αX I314G (*C*) or αM I316G (*D*) I domain either without any competitors or in the presence of 100 μg/ml of dp2, dp4, dp6, dp8, dp10, or dp12 size-sorted heparin oligomers. *E*, sensorgrams for injection of 0.69 μM αX I314G 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 I314G in the presence of 0.07, 0.13, 0.26, 0.52, 1.0, 2.1, 4.2, 8.3, 16.7, or 33.3 μM of the dp12(*E*) oligomer.

buffers described above except that the $MgCl_2$ 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 β chain (14), in the setting of an enzyme-linked immunosorbent experiment with soluble recombinant αXβ2.

Soluble, heterodimeric αLβ2 and αXβ2 integrins with a C-terminal, α-helical coiled-coil clasp were expressed in Chinese hamster ovary 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 $CaCl_2$ and 1 mM $MgCl_2$.

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 NaN_3, 0.15 M Na_2CO_3, 0.35 M $NaHCO_3$, 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 $CaCl_2$, 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 $CaCl_2$ and 1 mM $MgCl_2$. For comparison immobilized αXβ2 and αLβ2 integrins were also incubated with 100 μg/ml dp12(E) heparin in TBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, or in TBS with1 mM $CaCl_2$ and 1 mM $MgCl_2$, without glucosaminoglycans, or in TBS with 2 mM $MnCl_2$, or in TBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 μM XVA143 (17). The wells were incubated at room temperature for 15 min. Biotinylated monoclonal antibody KIM127 diluted to 1 μg/ml or biotinylated monoclonal antibody IB4 diluted to 5 μg/ml in TBS with 0.1% (w/v) bovine serum albumin were incubated in the wells at room temperature for 15 min. The signals were read following incubation with streptavidin-labeled horseradish peroxidase and the addition of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Zymed Laboratories Inc., 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 M_r 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 3 with a K_D of 1.5 μM (Fig. 1*A*). 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

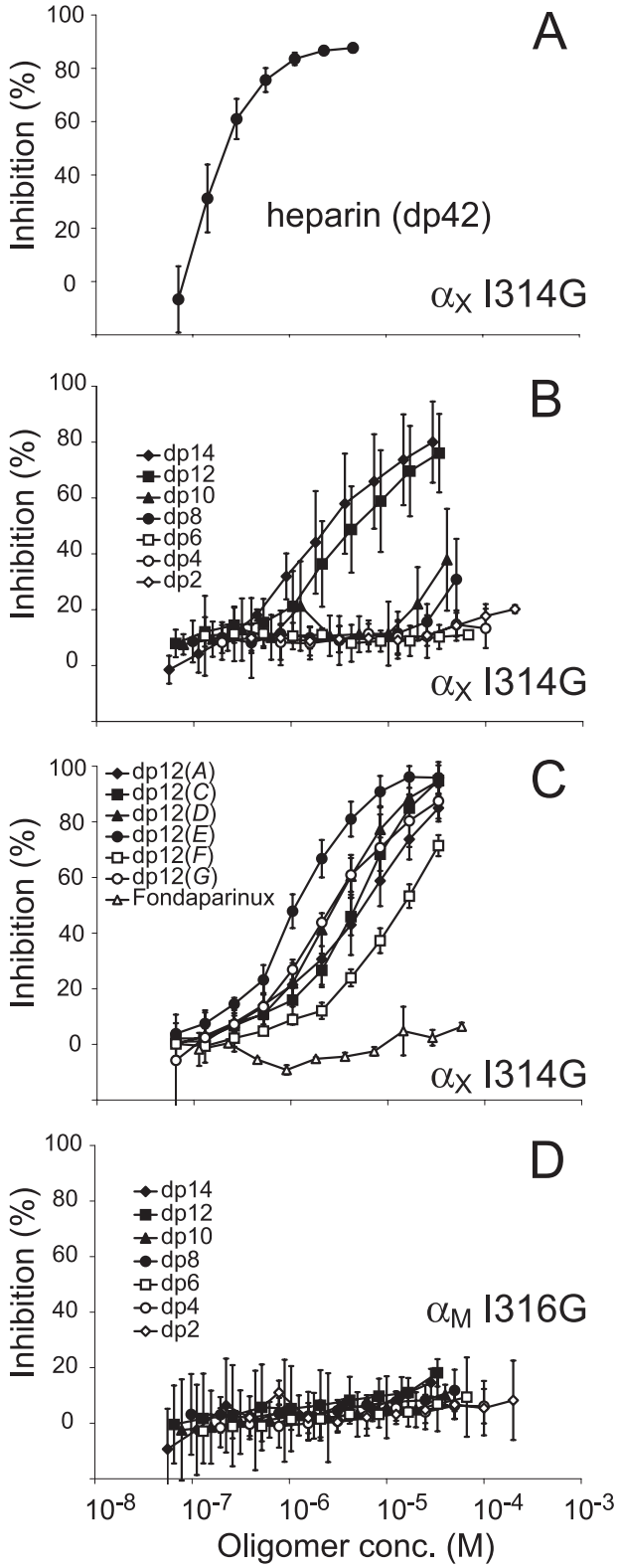


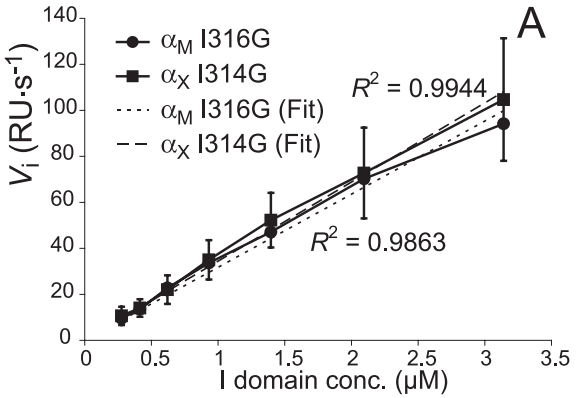
FIGURE 2. **Competitive inhibition of the binding by I domains to immobilized iC3b with heparin and heparin fragments.** In the calculation of oligomer concentrations, the average M_r of heparin was taken as 11,000, whereas the M_r of smaller fragments was estimated from a M_r of 500/disaccharide. *A*, inhibition of the binding by the open conformation αX I314G 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 was 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 I314G to iC3b in the presence of size-sorted heparins in oligomer concentrations from 0.13 to 66 μM. *C*, inhibition of the binding by the αX I314G I domain to immobilized iC3b with the fractions from ion exchange chromatography of the dp12 heparin oligomers. Fractions A, C, D, E, F, and G were applied at oligomer concentrations from 0.07 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 I316G to iC3b in the presence of size-sorted heparins is shown for oligomer concentrations from 0.13 to 66 μM.

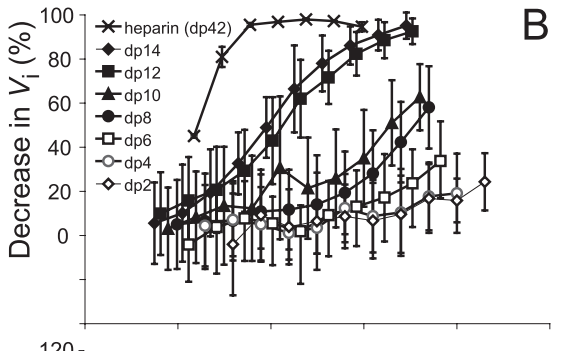
only a marginal influence on the response level (Fig. 1*C*). A range of oligomer concentrations was used to inhibit binding to two different iC3b sensor chip preparations, which in the absence of inhibition bound 1,610 and 1,760 RU of αX I domain when 1.4 μM was applied (Fig. 2). The concentration required to obtain a 50% reduction in the SPR response level was 45, 5.3, and 4.5 μM for the dp10, dp12, and dp14 oligomers, respectively (Figs. 2*B* and 3*D*). Unfractionated heparin is a potent inhibitor of binding by the open conformation αX I domain to immobilized iC3b (Fig. 2*A*). With an average M_r of 11,000 and dp of 42, the concentration of heparin required to obtain 50% inhibition was 0.30 μM.

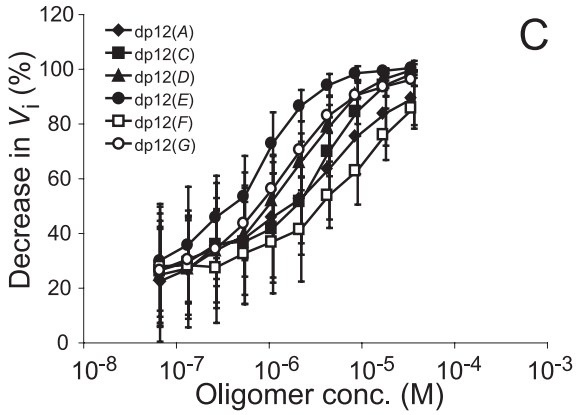
The αM I domain, mutated similarly to the αX I domain to favor the open conformation (11), bound iC3b with a K_D of 1.5 μM (Fig. 1*B*) in agreement with earlier reports (3, 11). No significant inhibition was observed from application of the size-sorted heparin oligomers to the binding by the open conformation αM I domain to immobilized iC3b (Figs. 1*D* and 2*D*).

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). To check the validity of Equation 3 (see "Materials and Methods"), we titrated the I domain concentration for either construct from 0.28 to 3.14 μM and measured the initial on-rate V_i at t = 4 s. As shown by the plot in Fig. 3*A*, there was a linear relationship between the applied I domain concentration and V_i with correlation coefficients close to 1, suggesting that the binding reaction was not limited by mass transport. Furthermore, from the slope of the line (α) of 33×10^6 RU M^{-1} s^{-1} and R_{max} 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). Because mass transport effects are only pronounced when k_{on} significantly exceeds 10,000 M^{-1} s^{-1} (19) and because 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, 22, 1.8, and 1.1 μM, respectively (Fig. 3*D*).

The Affinity of the αX I Domain for Anion Exchange-Purified Heparin Oligomers—To further characterize the interaction between heparin and the αX I domain, we semi-purified the dodecasaccharide oligomers by strong anion exchange high performance liquid chromatography. The oligomers were separated in seven fractions (Fig. 4*A*), and six fractions contained semipurified species when analyzed by PAGE (Fig. 4*B*). Frac-



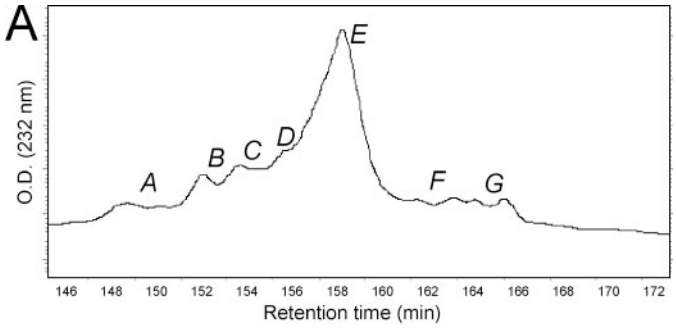




D

I domain	Ligand	Competitor	$IC_{50\%}$ (μM) Response level	$IC_{50\%}$ (μM) Initial on-rate (V_i)
αX	iC3b	dp8	*N.D.*	56.3±43
		dp10	45.0±7.07	21.9±18
		dp12	5.25±4.60	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	heparin	~ 0.10	
αM	fibrinogen	heparin	~ 1.50	

FIGURE 3. **Influence on the initial on-rate of the binding by αX I314G I domain to iC3b by heparin and heparin oligomers.** *A*, the initial on-rate (V_i), measured 4 s after injection start as the slope of the tangent to the sensorgram, for the binding of the αM I316G and αX I314G I domains to iC3b (as shown in Fig. 1, *A* and *B*) plotted as function of the applied I domain concentration (*conc.*). Linearity was confirmed from fitting a *straight line* (indicated with *dotted 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, dp14, 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 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_r = ~6,000 = ~dp21) were determined in an earlier report (3) from competition assays with immobilized fibrinogen.



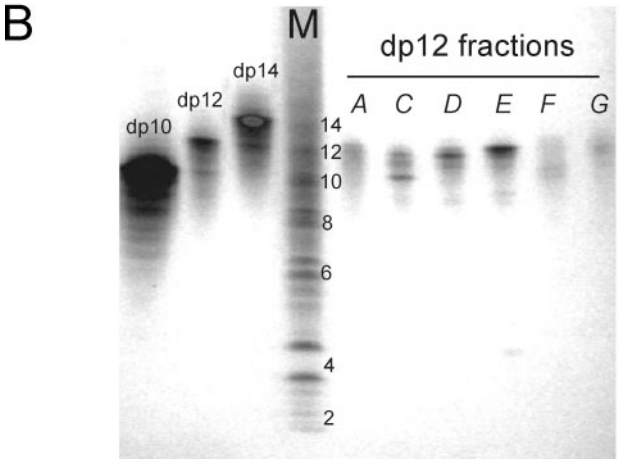


FIGURE 4. **Subfractionation of dp12 heparin oligomers.** *A*, strong anion exchange high performance liquid chromatography fractionation profile from purification of the dp12 heparin oligomers as monitored by absorbance at λ = 232 nm. The fractions are indicated with *capital letters*. *B*, PAGE analysis 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. *Lane M* shows a ladder of heparin oligosaccharide standards prepared from bovine lung heparin as described (10).

tions D and E correspond to dodecasaccharides based on comparison with oligosaccharide standards (10). According to earlier analysis by two-dimensional ^{1}H NMR spectroscopy (9), fraction *E*, of higher than 90% purity (Fig. 4*B*), 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, whereas fraction A, in addition to dp12 oligomers, contained highly sulfated decasaccharides (Fig. 4*B*).

Inhibition assays were carried out as for the size-sorted material (Figs. 1*E* and 2*C*), and the data were analyzed by determining the concentration required to lower the response level at the

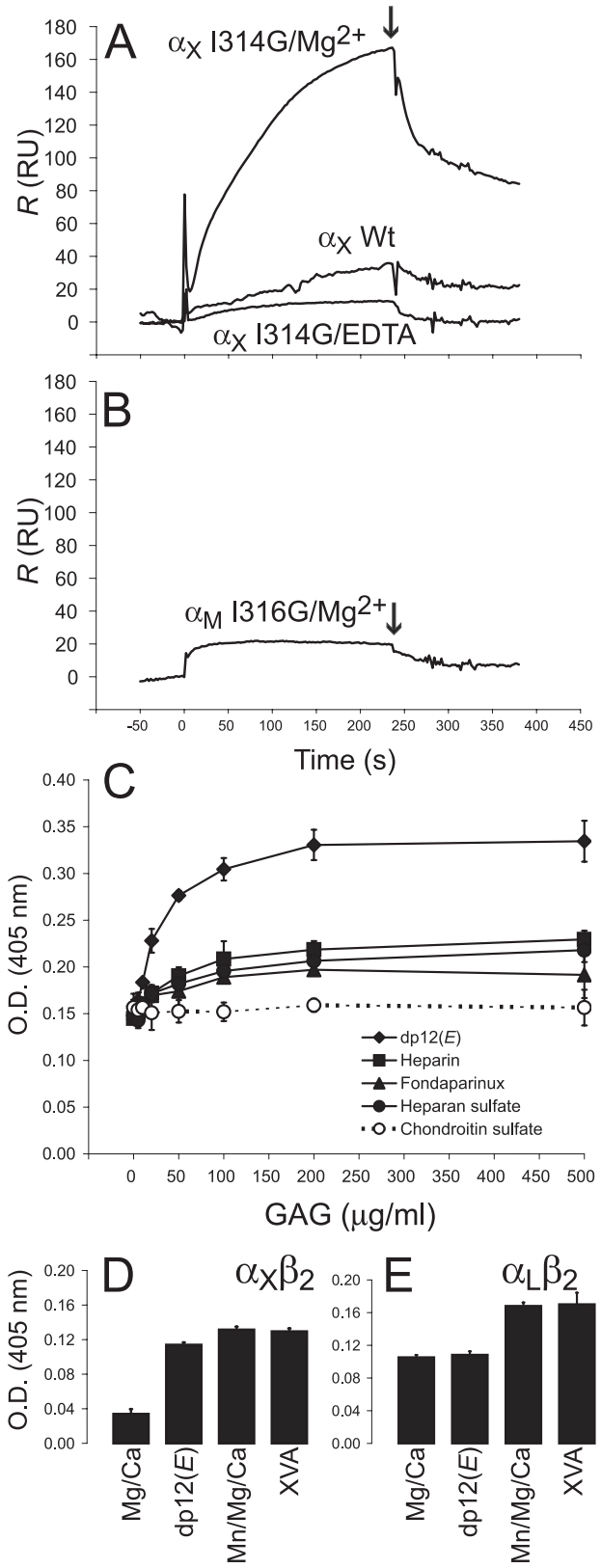


FIGURE 5. **Direct binding of αMβ2 or αXβ2 integrin I domains or the αXβ2 ectodomain to heparin oligomers.** *A* and *B*, conformational requirements and dependence on Mg^{2+} ion in the binding by αM and αX I domains to SPR surface-immobilized heparin. Sensorgrams are shown for the binding by 4.7 μM 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 enzyme-linked immunosorbent assay. *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.

end of the injection phase by 50%. The dp12(E) oligomers bound the αX I domain with the highest affinity with an IC_{50} of 1.3 μM (Fig. 1*F*). 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 αX I domain and iC3b, whereas the dp12(G) showed an affinity close to the affinity of dp14 oligomers (Figs. 2*C* and 3*D*). These findings were further supported by the reduction by purified dp12 oligomers of the initial on-rate in binding by the αX I domain (Fig. 3, *C* and *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 αX I domain.

For comparison with 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 αX I domain to iC3b (Fig. 2*C*).

Direct Binding of the αX and αM I Domain to Surface-immobilized Heparin—The direct binding of open and closed αX and αM 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 ~2-fold from 10,000 (dp40) to 5,000 (dp20) with a ratio (mol/mol) of 0.8 aldehyde group/heparin oligomer (information provided by Celsus Inc., Cincinnati, OH). The open conformation αX 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. 5*A*). EDTA abolished binding by the open conformation αX I domain. The conformational regulation of the binding was studied by comparing the binding of the wild-type αX 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. 5*A*). Consistent with the observations that heparin in solution is a poor inhibitor of the binding by the open conformation αM 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. 5*B*).

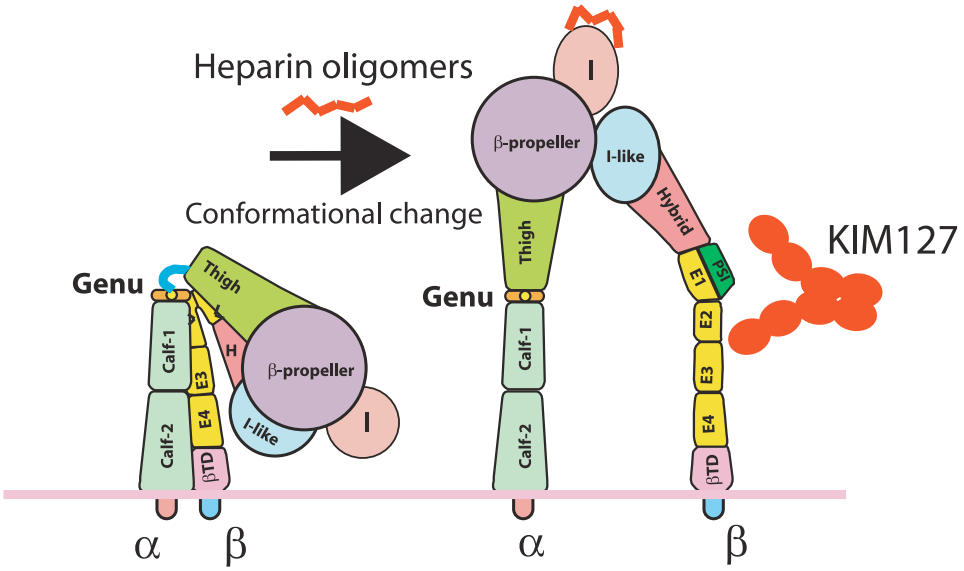


FIGURE 6. **Domain structure of the β2 integrins.** The α chain has five domains which are described in order from the N terminus to the C terminus. A seven-bladed β propeller domain contains an inserted I domain that constitutes the major ligand-binding domain in β2 integrins. The thigh domain is joined 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, four 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 ectodomain 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).

Induction of Activation Epitopes in the αXβ2 Ectodomain 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. 5*C*). 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. 5*C*). 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. 5*D*). The addition of Mn^{2+}, a well established inducer of conformational change and activator of ligand binding in integrins, clearly increased KIM127 epitope exposure (Fig. 5*D*). XVA is a small molecule antagonist of β2 integrin ligand binding that 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. 5*D*) consistent with the recent observation that XVA changes the conformation of αXβ2 integrin (15). The addition of 100 μg/ml dp12(E), *i.e.* at a binding saturating concentration (Fig. 5*C*), produced a KIM127 exposure comparable with that observed for the application of Mn^{2+} or XVA to the experiment (Fig. 5*D*). αLβ2 integrin showed no alterations in the KIM127 epitope exposure upon incubation with the dp12(E) oligomer (Fig. 5*E*) or with heparin, fondaparinux, heparin sulfate, or chondroitin sulfate (data not shown). Application of the IB4 monoclonal antibody 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, nonproteinaceous 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" and "open" conformations. 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 or heparin oligomers. However, because 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 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 affin-

ity corresponding to an IC_{50} 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 that 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 Mg^{2+}- and Ca^{2+}-binding sites in their ectodomains, 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 Mg^{2+} than for Ca^{2+} and thus, at physiological concentrations of these ions, primarily is occupied with Mg^{2+} (22).[3] Crystal structures show a coordination at the MIDAS typical for Mg^{2+} but not for Ca^{2+}. In the open, ligand-binding conformation, the MIDAS Mg^{2+} ion directly coordinates the side chains 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 side chain 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 Mg^{2+} or EDTA. Binding of the I domain to immobilized heparin clearly required Mg^{2+}. Although Ca^{2+} ions have been observed in several cases to contribute to the binding between heparin and metalloproteins, Mg^{2+}-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 Mg^{2+} 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/monosaccharide unit. dp12 oligomers would thus be assumed to take a length of 6 nm, which is comparable with 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 Mg^{2+}. Structural studies on the αX I domain identified a stretch of positively charged or polar residues of a length of ~3 nm, uninterrupted by negatively charged residues and crossing the MIDAS with the positively charged Mg^{2+} ion (12). Binding of the αX I domain to protein ligands is Mg^{2+}-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. Although 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 ~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 ectodomain (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 by 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 ectodomain of the receptor; in the resting state the receptor takes a bent conformation, whereas 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 enzyme-linked immunosorbent assay microtiter wells with the

[3] Vorup-Jensen, T., Waldron, T. T., Astrof, N., Shimaoka, M., and Springer, T. A., (2007) *Biochim. Biophys. Acta* **1774,** 1148–1155.

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 nonligand and ligand-binding conformations (23), 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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