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Heparin Modulates the Conformation and Signaling of Platelet Integrin α Ib β 3

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Abstract

Introduction—The glycosaminoglycan heparin has been shown to bind to platelet integrin α Ib β 3 and induce platelet activation and aggregation, although the relationship between binding and activation is unclear. We analyzed the interaction of heparin and α Ib β 3 in detail, to obtain a better understanding of the mechanism by which heparin acts on platelets.

Methods—We assessed conformational changes in α Ib β 3 by flow cytometry of platelets exposed to unfractionated heparin. In human platelets and K562 cells engineered to express α Ib β 3, we assayed the effect of heparin on key steps in integrin signaling: phosphorylation of the β 3 chain cytoplasmic tail, and activation of src kinase. We measured the heparin binding affinity of purified α Ib β 3, and of recombinant fragments of α Ib and β 3, by surface plasmon resonance.

Results and Conclusions—Heparin binding results in conformational changes in α Ib β 3, similar to those observed upon ligand binding. Heparin binding alone is not sufficient to induce tyrosine phosphorylation of the integrin β 3 cytoplasmic domain, but the presence of heparin increased both β 3 phosphorylation and src kinase activation in response to ligand binding. Specific recombinant fragments derived from α Ib bound heparin, while recombinant β 3 did not bind. This pattern of heparin binding, compared to the crystal structure of α Ib β 3, suggests that heparin-binding sites are located in clusters of basic amino acids in the headpiece and/or leg domains of α Ib. Binding of heparin to these clusters may stabilize the transition of α Ib β 3 to an open conformation with enhanced affinity for ligand, facilitating outside-in signaling and platelet activation.

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Conflict of Interest

No potential conflict of interest exists for any of the study authors.

Keywords

heparin; integrin α IIb β 3 (platelet glycoprotein IIb/IIIa); platelet activation; src kinase; surface plasmon resonance; tyrosine phosphorylation

The glycosaminoglycan heparin is a potent anticoagulant that is extensively used in the prevention and treatment of cardiovascular disorders. However, the efficacy of heparin is limited in part by its effect on platelets. Patients receiving intravenous heparin commonly experience an immediate, transient but mild non-immune-mediated thrombocytopenia, associated with biochemical evidence of platelet activation [1–5]. Heparin-mediated platelet activation should be distinguished from the much rarer syndrome of heparin-induced thrombocytopenia, caused by the formation of antibodies to platelet factor 4 (PF4) in a complex with heparin [1]. Both in vivo and in vitro, unfractionated heparin appears to be a stronger stimulant of platelet activation than lower molecular weight heparins [6–10]. In previous work, we have characterized the heparin oligosaccharide that binds to platelets, and shown that heparin binds to platelet integrin α IIb β 3 (platelet glycoprotein IIb/IIIa) [11–15]. Thus, it seems likely that heparin binding to α IIb β 3 is directly related to its stimulatory effects. Understanding the interaction of heparin with platelets will help in the development of safer anticoagulants.

Integrin α IIb β 3 is the major platelet surface receptor for fibrinogen and other RGD-containing proteins. Like other integrins, α IIb β 3 is a bidirectional receptor that undergoes conformational changes and induces intracellular signaling upon ligand engagement (outside-in signaling), as well as upon cell activation by soluble such as thrombin or ADP (inside-out signaling). Both processes contribute to a signaling cascade that ultimately results in profound morphological and biochemical changes in the platelet [16,17]. One of the first steps in this cascade is tyrosine phosphorylation of the integrin β 3 cytoplasmic tail, which alters its association with the platelet cytoskeleton, leading to shape changes, and ultimately to aggregation and release of mediators contained in platelet α granules [18].

Recent evidence indicates that heparin augments the ligand-induced phosphorylation of platelet cytoplasmic proteins involved in multiple signal transduction pathways, including through integrin α IIb β 3 [19]. In the work presented here, we show that heparin directly amplifies signaling through integrin α IIb β 3. Our experiments show that heparin augments ligand-induced conformational changes in α IIb β 3, as well as increasing tyrosine phosphorylation of both the integrin β 3 cytoplasmic domain and associated src kinase. In addition, we map the binding sites of heparin on the α IIb β 3 molecule. Our results indicate that heparin-induced platelet activation is caused by the binding of heparin to α IIb β 3, with induction or stabilization of a more activated conformation. We find that heparin binds predominantly to the α IIb subunit, and suggest some potential heparin-binding sites within the receptor.

Materials and methods

Detailed procedures and additional figures are included in the Supporting Information for this paper. For all experiments, we used a standard unfractionated porcine mucosal heparin (Celsus, Inc., M.W. ~15,000 Da, 179 units/mg).

Flow cytometry of integrin activation

All blood samples were collected from normal volunteers after obtaining informed consent following protocols approved by the Institutional Review Board. Whole blood was collected into Vacutainers containing 3.2% buffered sodium citrate (Becton-Dickinson) and

immediately centrifuged to obtain platelet-rich plasma (PRP). PRP was incubated with PBS, heparin (Celsus, 5 μ g/ml final concentration), EDTA (5mM final), or eptifibatide (Millennium Pharmaceuticals/Schering, 300 μ M final) and saturating concentrations of fluorescent antibodies or isotype controls. Samples were fixed and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Both forward and side scatter detectors were set on logarithmic scales, and the analysis gate was set on the major population of platelets. The percentage of positive platelets and their mean fluorescent intensity were calculated from single-parameter histograms of a minimum of ten thousand events from the gated region

Western blot analysis of protein tyrosine phosphorylation

For analysis of integrin β 3 phosphorylation in human platelets, PRP was incubated with eptifibatide and/or heparin, washed, then lysed and immunoprecipitated with an anti- β 3 monoclonal antibody (mAb) (PM6/13, Millipore). Samples were analyzed by Western blotting (Western Breeze kit, Invitrogen) using a polyclonal rabbit antibody directed against a peptide from human integrin β 3 containing phosphotyrosine (pY)759 (Santa Cruz Biotechnology). After detection of phosphorylated β 3, the membranes were stripped (ReBlot Plus Strong, Millipore) and total β 3 was detected using a mouse mAb (clone 1, BD Transduction Laboratories). Scanned films were analyzed using ImageJ (<http://rsb.info.nih.gov/ij/index.html>), Microsoft Excel, and Prism5 (GraphPad) software.

K α I**IIb** β 3 cells, derived from K562 cells transfected with plasmids expressing integrins α I**IIb** and β 3, were cultured as previously described [20]. To assay integrin β 3 phosphorylation, cells were washed and spread on fibrinogen-coated Petri dishes. Nonadherent cells were removed, the adherent cells were lysed directly in sample buffer, and total protein analyzed by Western blotting. For detection of src activation, polyclonal anti-pY416 and monoclonal rabbit anti-src (Cell Signaling Technologies) were used as the primary antibodies.

Preparation, expression, and purification of α I**IIb** and β 3 proteins and fragments

Intact human α I**IIb** β 3 was purified from platelet concentrates as previously described [15]. Recombinant fragments of α I**IIb** and the extracellular domain of β 3 were generated by PCR cloning into His-fusion vectors and expressed in BL21 (DE3) bacteria (α I**IIb** fragments) or S2 insect cells (β 3 extracellular domain). The α I**IIb** fragments encompassed amino acids 1–269 (Region 1), 263–559 (Region 2), and 547–867 (Region 3) (Supplemental Figs 1 and 2). The β 3 extracellular domain included amino acids 28–716. For purification of α I**IIb** fragments, inclusion bodies were solubilized and purified on a cobalt affinity column following manufacturer's protocols (Talon, Clontech). The β 3 extracellular domain was isolated from supernatants of S2 cells by affinity chromatography on Talon resin.

Surface plasmon resonance detection of heparin binding

A gold-coated sensor chip (Nippon Laser and Electronics Lab) was coated with glucosamine N-sulfate (6-O-sulfate)-iduronic acid (2-O-sulfate) (GlcNS6S-IdoA2S) and placed in the sensing channel of a 2-channel SPR instrument (SPR-670, Moritex, Yokohama, Japan) [21,22]. A control chip coated with D-maltose was placed in the reference channel. Proteins in 0.1% TritonX-100 in PBS were injected simultaneously into both channels. Binding was detected, and binding curves and kinetic parameters calculated, using the manufacturer's software.

Analysis of heparin binding sites and α IIB β 3 conformation

Analyses were performed on the crystal structure of α IIB β 3 reported by Zhu, et al (PDB ID: 3FCS) [23] using the program Cn3D (version 4.1; <http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>).

Results

Heparin induces conformational changes in platelet integrin α IIB β 3

We have previously shown that integrin α IIB β 3 is the major binding site for heparin on platelets [15]. To determine whether heparin interaction directly modulates integrin α IIB β 3 conformation, we used flow cytometry to measure the binding of a panel of antibodies to activation-dependent epitopes on human platelets. These epitopes are masked in the resting form of α IIB β 3, and are exposed by conformational activation of the receptor and high-affinity ligand binding. The antibodies LIBS-1 and -6 bind to ligand-associated epitopes on integrin β 3, while PMI-1 and PAC-1 bind to α IIB [24–26]. PRP from five different normal volunteers was incubated with the respective antibodies in PBS alone, or in the presence of 5 μ g/ml heparin. Figure 1A shows the change in the percentage of platelets positive for each epitope, expressed as the ratio (% with heparin/% without heparin). Heparin increased the binding of all the activation-dependent antibodies, from an average of 1.4- (LIBS-1) to 1.8-fold (PAC-1). The increases in binding of both LIBS-6 and PAC-1 in the presence of heparin were statistically significant ($p < .05$, t-test). The presence of heparin alone did not induce the expression of the activation-dependent marker p-selectin (Figure 1A), suggesting that heparin binding alone was not sufficient to induce platelet activation.

The effect of heparin on ligand-induced conformational changes was assessed in a second set of experiments. PRP was incubated with the integrin antagonist eptifibatide, and the binding of LIBS-1 and 6 was measured in the presence or absence of heparin. Although eptifibatide prevents α IIB β 3 signaling and platelet aggregation [18], biophysical measurements indicate that eptifibatide binding can induce partial activation of the receptor [27]. Because a large percentage of platelets were LIBS-positive in the presence of eptifibatide, further changes are expressed as the ratio of the mean fluorescence intensity in the presence or absence of heparin. Figure 1B illustrates that the addition of heparin increases the binding of LIBS-1 in the presence of eptifibatide, while that of LIBS-6 is largely unaffected. Total surface expression of α IIB β 3, as measured by the antibody AP3, showed no changes with the different treatments.

Heparin augments signal transduction through integrin α IIB β 3

Ligand binding to integrin α IIB β 3 triggers signaling that ultimately results in platelet activation [16–18]. Because heparin appears to augment the ligand-bound conformation of α IIB β 3, we assayed the effect of heparin on tyrosine phosphorylation of the β 3 cytoplasmic tail, one of the earliest signaling events of outside-in signaling. PRP was incubated with eptifibatide and varying amounts of heparin, and the phosphorylation of tyrosines in the β 3 cytoplasmic tail was measured by Western blotting. To account for differences in gel loading, band intensities in the phosphotyrosine blots were normalized with the intensities of the total β 3 integrin bands before assessing the effect of heparin addition. Figure 2A shows that, while neither heparin nor eptifibatide alone stimulate tyrosine phosphorylation of the β 3 cytoplasmic domain, a low concentration of heparin (5 μ g/ml) significantly increased phosphorylation in response to eptifibatide.

To confirm that this effect was integrin-dependent, we repeated the experiment using K562 cells transfected with α IIB β 3 (K α IIB β 3) [20,28]. These cells have been shown to bind fibrinogen, and this model has been useful in dissecting integrin signaling. By flow

cytometry, α IIb β 3 cells do not express significant levels of other integrins (not shown). Figure 2B illustrates that phosphorylation of integrin β 3 in α IIb β 3 cells adhering to immobilized fibrinogen is significantly increased in the presence of heparin. As in platelets, this effect is observed at low concentrations of heparin, and is not increased at higher concentrations.

Another early marker of outside-in signaling by integrin α IIb β 3 is activation of the tyrosine kinase src, which is constitutively associated with the β 3 cytoplasmic tail [29]. In response to ligand binding, src is dephosphorylated at tyrosine-529 and phosphorylated at tyrosine-418, activating the kinase and initiating platelet activation. Figure 2C shows that, in α IIb β 3 cells adhering to fibrinogen, heparin significantly increases phosphorylation of endogenous src at tyrosine-418. These results indicate that heparin binding can amplify ligand-induced signaling through integrin α IIb β 3.

Identification of heparin-binding regions of α IIb β 3

The binding sites of heparin on the α IIb β 3 molecule were mapped more precisely by expressing components of the integrin heterodimer, and independently measuring their affinity for heparin structures, using surface plasmon resonance (SPR). To avoid complications arising from the structural heterogeneity of naturally occurring heparins, we immobilized a structurally defined disaccharide previously shown to bind to α IIb β 3 (GlcNS6S-Ido2S) on the SPR chip. Immobilization of the purified disaccharide also effectively increases the concentration of the active structure [22]. Binding was first measured to intact integrin α IIb β 3 purified from human platelets, and then to isolated fragments of α IIb and β 3 individually. Increasing concentrations of protein in buffer were passed over the immobilized oligosaccharide to assess binding. Figure 3A illustrates the binding curve for purified platelet-derived α IIb β 3, where the K_D was calculated to be 440nM. Figure 3 (B–E) are the binding curves for individual fragments of α IIb and the extracellular domain of β 3, and Table 1 summarizes the dissociation constants derived from these experiments. Region 1 of α IIb (corresponding to the amino-terminal half of the head domain [30]) and Region 3 (containing part of the thigh and both calf domains) showed significant binding to the immobilized disaccharide. In contrast, the extracellular domain of β 3 and α IIb Region 2 (the carboxy-terminal half of the head and adjoining thigh domain) did not appear to bind. These assays were repeated using unfractionated heparin immobilized on the SPR chip, which confirmed that only Regions 1 and 3 from α IIb bound to heparin (not shown), with affinities similar to the disaccharide. The calculated K_D for Region 1 was 68.1nM for the disaccharide (Table 1) and 106.6nM for unfractionated heparin.

Discussion

We have previously shown that heparin binds to platelet integrin α IIb β 3, resulting in platelet activation and aggregation [12,15]; however, in those experiments it was unclear if heparin binding alone could activate signaling. These experiments were undertaken to address this question. The flow cytometry experiments indicate that heparin binding can induce conformational changes in α IIb β 3 similar to those resulting from ligand binding, and that heparin augments the conformational changes induced by ligand binding. The absence of increased p-selectin expression in response to heparin alone suggests that platelets are not fully activated by heparin binding. Analyses of β 3 tyrosine phosphorylation and src activation further confirm that heparin alone does not produce detectable outside-in signaling, but in the presence of ligand, heparin significantly enhances signaling through α IIb β 3, both in platelets and in transfected cells expressing the integrin. Finally, through analysis of heparin-binding to isolated fragments of α IIb β 3, we mapped putative heparin-binding sites to the headpiece and genu of α IIb.

Gao et al. have also recently reported that heparin potentiates signaling through platelet α IIB β 3 [19]. Their study demonstrated that heparin augments phosphorylation of FAK and Akt in response to ligand binding. This current report extends these findings by documenting that the earliest events of the outside-in signaling cascade are potentiated by heparin. By using the K562 model system, which is devoid of other platelet receptors, we were able to show that heparin binding increases phosphorylation of the integrin β 3 cytoplasmic domain and activation of the associated src kinase in response to ligand binding. Taken together, these results indicate that heparin binding results in platelet stimulation at least in part by the induction or stabilization of an activated conformation of α IIB β 3, thus facilitating ligand binding to the integrin, and amplifying outside-in signaling and platelet activation.

Mapping the binding sites of heparin on the α IIB β 3 molecule suggests a model for this mechanism. Classical linear heparin-binding motifs composed of appropriately spaced cationic residues [31–33] can be found in the extracellular domain of β 3 and Region 2 of α IIB. However, the results of the binding studies demonstrated that neither β 3 nor Region 2 bound heparin, while Regions 1 and 3 of α IIB bound with significant affinity to heparin and its disaccharide. Additionally, the overall densities of arginine and lysine residues in Regions 1–3 do not correlate with their heparin-binding affinity (6.3% arginine/lysine residues in Region 1, 8.8% in Region 2, 5.7% in Region 3). Thus, it seems unlikely that heparin is binding to linear or random distributions of basic amino acids in the recombinant fragments, suggesting that the major heparin binding site(s) are formed by folding of the α IIB polypeptide. One caveat is that these recombinant proteins may not be folded in precisely the same conformation as the native protein.

The crystal structure of the α IIB β 3 extracellular domain [23] reveals that several clusters of arginine and lysine residues are located on the external face of the protein. Region 1 (which binds heparin) contains a large cluster on one face of the β -propeller (Figure 4A and B). In particular, the arginines at positions 73, 77, 139, 140, and 208, along with lysine 118, are located around the cap subdomain of α IIB, adjacent to the residues essential for fibrinogen binding [34]. The basic amino acids contained in Region 3 (which also binds heparin) also appear to fall in several clusters (Figure 4C and D). Two clusters are located on either side of the genu, in the thigh and calf-1 domains, and a third on the opposite face of calf-1.

This correlation between experimental binding studies and the crystallographic model of α IIB offers new hypotheses about how heparin modulates integrin function. Heparin may interact with the cap subdomain, thereby modifying the conformation of the ligand-binding site. In addition, binding of a charged polysaccharide chain to the clustered basic amino acids on either side of the genu could facilitate or stabilize the opening of the thigh and calf domains, extending the leg and exposing the ligand-binding site. Finally, the third cluster of basic amino acids on calf-1 appears to lie near binding sites for calcium in the β -propeller in the inactive conformation, so binding of heparin to this cluster could potentially modify interactions of α IIB with divalent cations [34]. Others have shown that opening of the leg alone can increase the affinity of α IIB β 3 for ligand. Mutation of residues surrounding the genu that constrain the opening of the leg inhibits activation-induced increases in binding affinity [35,36]. In contrast, introduction of a glycosylation site “behind” the genu, which prevents the leg from closing, results in constitutive high-affinity ligand binding [36]. Similarly, mutations of β 3 that destabilize interactions with the α IIB leg, therefore favoring a more extended resting conformation, also confer constitutive high-affinity ligand binding [37]. Thus, heparin, by binding to one or more sites on the α IIB polypeptide, may stabilize an open, partially activated, conformation of α IIB β 3.

The demonstration that heparin can modulate the behavior of integrin $\alpha\text{IIb}\beta 3$ may have implications beyond the regulation of platelet activation [14]. As an antithrombotic drug, unfractionated heparin may have theoretical drawbacks, particularly when administered to patients with systemic platelet activation, or in combination with integrin antagonists like eptifibatide that may synergistically stabilize activated conformations of $\alpha\text{IIb}\beta 3$. Heparin has also been shown to affect processes such as tumor metastasis and leukocyte extravasation [38], in which the interactions of various integrins with their ligands appear to play a role in regulating cell adhesion. Thus, further understanding of the role of heparin in modulation of cell adhesion may advance the use of oligosaccharides derived from heparin for therapeutic purposes in cancer and inflammatory disorders, as well as in regulating coagulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

GlcNS6S-IdoA2S	glucosamine N-sulfate (6-O-sulfate)-iduronic acid (2-O-sulfate)
mAb	monoclonal antibody
PBS	phosphate-buffered saline
PRP	platelet-rich plasma
pY	phosphotyrosine
SPR	surface plasmon resonance

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Figure 1. Conformational changes in α IIb β 3 measured by flow cytometry

A, Relative binding of Pac-1, LIBS, and p-selectin antibodies to platelets in the absence of ligand. PRP was incubated with the indicated antibodies in the presence of 5 μ g/ml heparin. Data are expressed as the percentage positive platelets for heparin treatment divided by PBS control (means $\pm$ SEM of 5 different subjects). The addition of heparin increased LIBS-1 and PAC-1 binding significantly more than that of p-selectin, which was no different than PBS controls ($p < .05$, t-test). **B**, Increased binding of LIBS1 but not LIBS6 in the presence of heparin and ligand. PRP was incubated with EDTA (5mM) or eptifibatide (300 μ M) with and without heparin (5 μ g/ml). Separate aliquots were incubated with the conformation-

dependent antibodies LIBS-1, LIBS-6, or AP3(which recognizes all conformations of α .IIb β 3). The fold increase in mean fluorescent intensity of antibody binding over EDTA controls is shown. Values are the means $\pm$ SEM of 2 studies.

Figure 2. Phosphorylation of the integrin $\beta 3$ cytoplasmic tail and src in human platelets and KaIb $\beta 3$ cells

A, Human PRP was incubated with eptifibatide (300 μ Molar) and/or heparin (5 or 50 μ g/ml) and $\beta 3$ phosphorylation measured as described in the Methods. Values are the means $\pm$ SEM of 2 independent experiments, expressed as the fold change in $\beta 3$ phosphorylation compared to ligand alone. The Western blot of a representative experiment is depicted. **B**, Washed KaIb $\beta 3$ cells were adhered to immobilized fibrinogen in the absence or presence of heparin (means $\pm$ SEM of 3 studies). Asterisks indicate $p < 0.05$ (t-test) compared to the PBS control. **C**, Phosphorylation of src at tyr418 in KaIb $\beta 3$ cells adhering to fibrinogen with or without

heparin. Values are the means $\pm$ SEM of five experiments. Asterisks indicate $p<0.05$ (t-test) compared to control.

Figure 3. SPR binding analyses

A, $\alpha\text{IIb}\beta 3$. A typical binding experiment is illustrated, in which a range of concentrations of purified human $\alpha\text{IIb}\beta 3$ were perfused over the sugar chip immobilized with a structurally defined synthetic oligosaccharide of GcNS6S-Ido2S known to bind to the platelet. **B–E**, recombinant fragments of αIIb and $\beta 3$. For each fragment, binding to the defined heparin oligosaccharide is illustrated by the filled symbols, and binding to the control maltose chip by the open symbols. Values are the means $\pm$ SD from three independent experiments.

Figure 4. Clustering of basic amino acids in α IIb

Locations of basic amino acids in α IIb, based on the crystal structure of Zhu, et al [26].

Figures were generated using Cn3D (version 4.1,

<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>). **A & B**, two views of the α IIb headpiece. **B** is rotated approximately 90° from **A**. The α IIb protein backbone is in green.

The side chains of the Arg (R) and Lys (K) residues located in Region 1 (AA 1–262) are represented by blue sticks. **C**, an overview of the closed form of the α IIb chain. The Arg and Lys side chains in Regions 1 and 3 are depicted as blue sticks. **D**, the thigh and calf-1

domains of α IIb. The Arg and Lys residues within $15\text{\AA}$ of the genu are identified.

Table 1Dissociation Constants for purified α IIb β 3 and Recombinant Portions of α IIb β 3

	K_D (nMolar)
native α IIb β 3	440
Region 1 α IIb	68.1
Region 2 α IIb	no binding
Region 3 α IIb	169
recombinant β 3	no binding