Inhibition of αIIbβ3 Ligand Binding by an αIIb Peptide that Clasps the Hybrid Domain to the βI Domain of β3

Wen Hwa Lee¹, Elisabeth Schaffner-Reckinger²a, Demokritos C. Tsoukatos³, Kelly Aylward⁴,⁵, Vassilios Moussis³, Vassilios Tsikaris³, Paraskevi Trypou³, Marion Egot⁴,⁵, Dominique Baruch⁴,⁵, Nelly Kieffer²,⁶, Christilla Bachelot-Loza¹,⁵*

¹ SGC, University of Oxford, Oxford, United Kingdom, ² Laboratoire de Biologie et Physiologie Intégrée, (CNRS/GDRE-ITI), University of Luxembourg, Luxembourg City, Luxembourg, ³ Department of Chemistry, Section of Organic Chemistry and Biochemistry, Ioannina, Greece, ⁴ Inserm UMR_S 1140, Faculté de pharmacie, Paris, France, ⁵ Université Paris Descartes, Sorbonne Paris Cité, Paris, France, ⁶ CNRS-LIA124, Sino-French Research Center for Life Sciences and Genomics, Rui Jin Hospital, Jiao Tong University School of Medicine, Shanghai, China

a Current address: Laboratory of Cytoskeleton and Cell Plasticity, LSRU, University of Luxembourg, Luxembourg City, Luxembourg
b Current address: MINT Europe NV, Sint-Martens-Latem, Belgium
* christilla.bachelot-loza@parisdescartes.fr

Abstract

Agonist-stimulated platelet activation triggers conformational changes of integrin αIIbβ3, allowing fibrinogen binding and platelet aggregation. We have previously shown that an octapeptide, P¹YMESRADR⁸, corresponding to amino acids 313–320 of the β-ribbon extending from the β-propeller domain of αIIb, acts as a potent inhibitor of platelet aggregation. Here we have performed in silico modelling analysis of the interaction of this peptide with αIIbβ3 in its bent and closed (not swing-out) conformation and show that the peptide is able to act as a substitute for the β-ribbon by forming a clasp restraining the β3 hybrid and βI domains in a closed conformation. The involvement of species-specific residues of the β3 hybrid domain (E356 and K384) and the β1 domain (E297) as well as an intrapeptide bond (pE315-pR317) were confirmed as important for this interaction by mutagenesis studies of αIIbβ3 expressed in CHO cells and native or substituted peptide inhibitory studies on platelet functions. Furthermore, NMR data corroborate the above results. Our findings provide insight into the important functional role of the αIIb β-ribbon in preventing integrin αIIbβ3 head piece opening, and highlight a potential new therapeutic approach to prevent integrin ligand binding.

Introduction

Integrin αIIbβ3, the platelet fibrinogen and von Willebrand factor (vWF) receptor, plays a key role in hemostasis and thrombosis, by promoting platelet aggregation and thrombus formation at sites of vascular injury. As circulating platelets in blood are constantly exposed to high...
concentrations of fibrinogen, αIIbβ3 ligand binding has to be tightly controlled to prevent inappropriate thrombus formation. Integrin αIIbβ3 is therefore maintained in a constitutive low-affinity state, and agonist-induced platelet stimulation is required to convert αIIbβ3 from a low- to a high-affinity state, able to bind ligands [1].

Unravelling the molecular mechanisms that regulate platelet integrin αIIbβ3 activation has been the focus of intense research. Initial structural information came from electron microscopy (EM) pictures of purified αIIbβ3 showing its particular shape with a globular head piece connected to two flexible stalks [2]. The 3.1Å crystal structure of the ectodomain of integrin αvβ3 [3], and later of αIIbβ3 [4, 5], revealed that the ligand-binding globular head piece is composed of the N-terminal part of both α and β subunits, comprising the β-propeller of the α subunit associated to the βI and hybrid domains of the β subunit. The flexible stalks or legs comprise multiple domains, corresponding to an Ig-like “thigh” and two β-sandwich “calf” domains for the α subunit, and one PSI, four EGF and one β-tail (βTD) domains for the β subunit. An unexpected initial finding however was the bent, V-shaped conformation of the αvβ3 receptor with a sharp knee-like kink in the middle section of both legs, bringing the headpiece into intimate contact with the lower leg domains.

On the basis of data obtained from crystal structures of αIIbβ3 [4, 5], epitope mapping with anti-αIIb, α-β3 or -αIIbβ3 antibodies [6, 7], as well as EM and cryo-electron microscopy (cryo-EM) pictures [8–11], it is now largely accepted that the bent-closed conformation represents the low-affinity, resting state of αIIbβ3. Integrin affinity upregulation relies on several major conformational changes, namely integrin extension at the knees, leg separation, and a β3-subunit swing-out motion at the interface between the βI and hybrid domains, converting the headpiece from a closed to an open conformation, thus facilitating ligand binding by the headpiece [12, 13].

Experiments based on mutant αIIbβ3 receptor function or LIBS antibody bindings have shown that the low-affinity state of β3 integrins is stabilized by several clasps, two of which exist at the transmembrane and cytoplasmic face of the receptor [14–16]. Additional contacts also exist in multiple points along the α and β extracellular legs [6, 15], which might be involved in modulating the transition through different conformations between the inactive and active states of integrins. More importantly however, a clasp in the headpiece, involving the β-ribbon of the αv β-propeller domain (residues 301–308) or the corresponding αIIb domain (segment 313–320) appear to play a major role in preventing the swing-out motion of the β3 hybrid domain [5, 17]. This is consistent with previous suggestions of the existence of such a clasp in integrin αIIbβ3 [4], together with results from targeted molecular dynamic (TMD) studies [18]. These results have been confirmed by Kamata et al. by engineering disulphide bonds between these regions resulting in inhibition of ligand binding [19], and further corroborated using new engineered disulphide bond mutant receptors [20]. Also, peptides corresponding to the αv or the related αIIb sequence as well as the β3 sequence of this clasp promoted integrin activation [17]. Intriguingly, the reported αIIb peptide (pMESRADRK) almost completely overlaps with an octapeptide pYMERSADR, previously shown by us to exert an inhibitory effect on αIIbβ3 integrin function by blocking fibrinogen binding to αIIbβ3 [21, 22]. Considering these apparently contradictory data, we have performed in silico structural modelling of our octapeptide with αIIbβ3 in its bent-closed conformation (PDB: 3FCS) and used in silico as well as experimental data to demonstrate that the αIIb octapeptide, initially thought to interact with fibrinogen [21], substitutes for the αIIb β-ribbon by establishing interactions with both the βI and hybrid domain of β3, thus preventing the swing-out motion of the hybrid domain necessary for head opening and high-affinity ligand binding.
Materials & Methods

Peptide Synthesis

Peptides were synthesized on a Wang resin (0.75 meq g⁻¹ resin) using the standard SPPS methodology [23, 24]. Five octapeptides were synthesized: native peptide pYMESRADR, 3 substituted peptides pYMESRAAR, pYMESAADR and pYMESRADA and the ”shifted” peptide pMESRADRK.

Structural Modelling and Analysis

Structural modelling and analysis was performed using the program ICM-Pro v.3.7-2a (www.molsoft.com). All the relevant and available structures were analysed. A combination of superimposition and modelling was performed to generate atomic models of the non-existent integrin conformations, assuming that similar motion ranges are shared by close members of the integrin family. Superimposition was performed (as implemented in ICM) for the domains forming the headpieces followed by tethering of corresponding atoms from homologous and/or identical domains. The tether energies were minimised whilst phi/psi torsion angles of residues found at interdomain hinge regions were made to rotate within allowed regions. Next, side-chain clashes were solved using local energy minimisation cycles. The remaining clashes were solved through Monte-Carlo searches followed by local energy minimisation cycles. The length of the Monte-Carlo searches was defined by the algorithm implemented in ICM.

Platelet Preparation

Venous blood from informed healthy donors was obtained from the French blood bank institute (EFS) according to the agreement between INSERM and EFS (CPSL C UNT—06/EFS/029). During the medical examination preceding blood donation, the medical doctor of EFS informed the healthy donors that part of their blood could be used for research. All blood samples used in the present study have been provided by healthy donors, which signed the agreement. The samples were anonymized at the EFS prior deliverance to the laboratory. According to the French law (L1211-2), this research is considered as a non-interventional research that does not require prior approval of the ethics committee. Blood was collected in Vacutainer tubes containing ACD-A (citric acid-citrate-glucose) (BD Vacutainer, Becton Dickinson, Le Pont de Claix, France) and was centrifuged for 12 min at 210g in presence of PGE1 2 x 10⁻⁷ M to obtain platelet-rich plasma (PRP). PRP was diluted with washing buffer (103 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose and 36 mM citric acid; pH 6.5) containing PGE1 2 x 10⁻⁷ M and 0.06 U apyrase/ml (Sigma Aldrich, St. Louis, MO, USA), then centrifuged for 12 min at 1240g to pellet the platelets. This washing step was repeated and the platelets were finally resuspended at 2.5 x 10⁸ per ml in reaction buffer (10 mM Hepes, 140 mM NaCl, 3 mM KCl, 5 mM NaHCO₃, 0.5 mM MgCl₂, 10 mM glucose and 2 mM CaCl₂; pH 7.4).

Flow Cytometry

Flow cytometry experiments were performed using a Becton Dickinson FACSSort. Platelets were preincubated 5 min with either vehicle (NaCl 0.9%), native or substituted octapeptides (1 mM). When indicated, a further incubation was performed in the presence of RGDS (1 mM). Platelets were activated 10 min with thrombin 2.5 U/ml (Sigma Aldrich, St. Louis, MO, USA). Then, the samples were diluted 1:10 in reaction buffer and incubated with both fluorescein isothiocyanate (FITC)-conjugated PAC1 antibody (PAC1-FITC from Becton Dickinson, San Jose, CA, USA) and phycoerythrin (PE)-conjugated anti-CD62P (from Beckman Coulter, Brea, CA, USA) to measure αIIbβ3 activation and P-selectin expression, respectively. Platelet activation
induces the secretion of granule contents, consecutively secreted fibrinogen can bind to activated integrin. Therefore, fibrinogen binding was measured by single-colour flow cytometry using FITC-conjugated rabbit polyclonal anti-human fibrinogen antibody. Exposure of LIBS was evaluated performing mAb AP5 binding (generous gift of Dr T. J. Kunicki, The Scripps Research Institute, La Jolla, CA, USA), and revealed with PE-labelled anti-mAb antibody. In each experiment, controls were run with the corresponding isotype control. Single intact platelet gate was identified on resting sample by their characteristic profile on a right angle scatter (SSC) and forward angle scatter (FSC) plot. A total of 10,000 platelet events were recorded. Results were expressed as the mean fluorescence intensity of resting or thrombin-activated platelets preincubated with or without RGDS and/or octapeptides relative to the basal condition corresponding to resting platelets preincubated with vehicle.

Aggregation
Washed platelet aggregation was measured with a PAP-8E Biodata optical aggregometer. Platelets were pre-incubated with octapeptide (pYMESRADR, pYMESRAAR or pMESRADRK) (75 to 500 μM) or vehicle for 5 min at 37°C with stirring (1200 rpm). Thrombin was then added and aggregation was monitored for 5 min as the change in light transmittance. Results are expressed as the percentage of maximum aggregation ± SEM.

cDNA Constructs, Transfection and Generation of Stable Cell Clones
The cDNAs encoding αIIbR317A/D319A/R320A (αIIb3M) and β3K384A (β31M) were generated using the Quikchange site-directed mutagenesis method (Agilent Technologies, Santa Clara, CA, USA) and the pcDNA3.1(-)Neo-αIIbwt and pcDNA3.1(-)Zeo-β3wt as templates. The nucleotide sequence of the generated mutants was verified by automated sequencing. Chinese hamster ovary (CHO) cell (CRL 9096, American Type Culture Collection, Rockville, MD) transfections were performed using lipofectAMINE (Life Technologies Europe B.V., Ghent, Belgium) as previously described [25], and positive transfectants were screened by flow cytometry for cell surface expression of the recombinant mutant αIIbβ3 complex using an αIIbβ3 complex-specific mAb, Pl2-73 (kind gift from Dr Cécile Kaplan, Institut National de la Transfusion Sanguine, Paris, France), and subcloned by limiting dilution to establish stable cell clones. Finally, we selected the cell clones having a surface expression of the mutant αIIbβ3 receptors comparable to the cell clone expressing the wild type receptor (S1 Fig).

CHO Cell Adhesion
Cell adhesion was performed using flow conditions and real-time video microscopy as previously described [26]. Briefly, glass coverslips were precoated with 10 μg/ml fibrinogen (from Dako, Glostrup, Denmark), purified and depleted of contaminant fibronectin and vWF, and mounted onto a flow chamber of 200 μm height filled with IMDM. CHO cells resuspended in IMDM at a concentration of 1.25x10^6/ml were perfused over a fibrinogen-coated coverslip at flow rates from 60–200 μl/min to produce wall shear rates from 30–100 s⁻¹. In some experiments, cells were preincubated 20 min with 250–500 μM YMESRADR or YMESRAAR peptides before perfusion at 30 s⁻¹. Cells were visualized using a 10x Hoffman Modulation Contrast objective together with an inverted microscope. Real-time images were recorded using a charged-coupled-device camera. The number of adherent cells was counted over a 12 min time course using Histolab and Archimed software (Microvision Instruments, Evry, France).
Statistical Analysis

Results are expressed as mean ± SEM. Differences between samples incubated with vehicle and with peptides were assessed with Wilcoxon test. Cell adhesion mediated by αIIb3, αIIb3M or αIIb3 WT was compared with Mann-Whitney test (Prism 4.0, GraphPad Software). Degrees of statistical significance are defined as *P<0.05, **P<0.01 and ***P<0.001.

Results

Analysis of integrin αIIb β-ribbon residues involved in the clasp preventing αIIb3 opening

The octapeptide pYMESRADR derived from integrin αIIb, previously shown to inhibit platelet aggregation by blocking fibrinogen binding to activated αIIb3 [27], corresponds to residues 313–320 of the insert loop (β-ribbon), that extends between the β2 and β3 strands of the W5 blade of the αIIb β-propeller domain [5]. The crystal structure of the bent-closed β3 subunit conformation tightly accommodates this loop in a cleft formed between the βI, hybrid and the third and fourth I-EGF domains of β3. Within the loop, three continuous charged residues establish salt-bridges: E315(αIIb)-R317(αIIb), R320(αIIb)-E297(β3), K321(αIIb)-E356(β3). Also, the carbonyl group of the peptide bond between A318 and D319 (αIIb) from the β-ribbon was seen interacting with K384(β3) (Fig 1).

It is worth mentioning that there are several additional inter-domain interactions between the two integrin subunits outside the region covered by the β-propeller W5 blade loop. One of such interactions, between Y353(αIIb) and E297(β3) positioned in close vicinity of the β-ribbon can be found in αIIb3 crystal structures (PDBs 3FCS, 3FCU, 2VDO, 2VDL, 2VDM, 2VDN, 2VDP, 2VDQ, 2VDR, 2VC2, 2VDK).

Alignment of αIIb from several species shows that the β-propeller W5 blade loop is highly conserved, except for R320, which is substituted by histidine in pig and rabbit. It is noteworthy that residues K321 and Y353 are absolutely conserved in all the species included in the analysis (Fig 2A upper alignment) and that the loop is one of the most conserved regions outside the core β-propeller domain, contrasting with the variability seen elsewhere in this domain, and underlining its putative role as a molecular latch.

Similarly, the bent-closed conformation adopted by β3 also revealed a highly conserved surface across 13 species (Fig 2B upper alignment), which coincides with the interfacing region of αIIb and including the β-propeller W5 blade loop. When analysed against homologues, the level of conservation is rather low for the residues implicated in the W5 blade loop (in αIIb) and their counterpart interacting residues found in β3 (Fig 2A–2B lower alignments). This can be interpreted as a result of integrin evolution to diversify the combination between different partnering α and β subunits acquiring novel roles and functions as a consequence.

Modeling of the interaction of the octapeptide with β3 subunit

In order to explain the inhibitory effect of the pY313MESRADR320 octapeptide on integrin αIIb3 activation, we have performed structural modelling to determine how the octapeptide could replace the β-ribbon in restraining the β3 chain in its bent-closed conformation. We have performed in silico searches to assess whether a feasible conformation can be achieved, whilst observing the anchoring points used by the octapeptide, derived from the native interactions made by the hairpin. We have inspected the possible solutions and identified the most plausible conformation. In the in silico model of the octapeptide, the aforementioned residues involved in salt bridges from β3 are maintained, however these are now formed between the peptide and the hybrid domain (namely pD319(octapeptide)-K384(β3) and pR320...
Interestingly the intrapeptide salt bridge between pE315-pR317 is maintained, despite changes in the backbone geometry. More importantly, this geometry now allows a new interaction to be formed between pY313 (octapeptide) and E297 (β3), completing the restraining of the βI + hybrid domains to the geometry found in the bent-closed conformation (Fig 3).

Interaction of the octapeptide with the β3 subunit of platelet integrin αIIbβ3

To further test the hypothesis that the octapeptide inhibits platelet activation by direct interaction with the β3 subunit, we investigated its ability to induce the exposure of LIBS (ligand-induced binding site) neo-epitopes on β3. LIBS epitopes were initially identified on αIIbβ3 following binding of peptide antagonists, such as RGDS, or certain antibodies [28] and are considered as markers of multiple conformational changes of αIIbβ3 that occur during the transition of αIIbβ3 from its unliganded-closed to liganded-open conformation [7]. Binding of the β3-specific AP5 LIBS antibody [29] was monitored by flow cytometry. The native octapeptide (Fig 4A) promoted AP5 antibody binding on resting platelets although to a lesser extent than RGDS did, whereas it was as powerful as RGDS to induce AP5 binding on thrombin-activated platelets. Co-treatment with Py315YMESRADR320 and RGDS did not result in a synergistic effect to induce further AP5 binding. Similar results were also obtained with another β3-specific LIBS antibody, LIBS1, (S2 Fig). Moreover, similarly to RGDS, the octapeptide inhibited
fibrinogen binding (Fig 4B left), as well as the binding of PAC1 (Fig 4B middle and Fig 4D), an antibody that specifically recognizes activated \(\alpha_{IIb}\beta_3\). This binding did not significantly affect platelet secretion, as shown by the absence of any effect on CD62P expression on thrombin-activated platelets (Fig 4B right). These results suggest that the octapeptide does not interact with fibrinogen but inhibits the activation of integrin \(\alpha_{IIb}\beta_3\). The inhibitory effect of the peptide was also tested in light-transmission aggregometry under stirring conditions and the results show that \(pY^{313}\)MESRADR\(^{320}\) inhibited platelet aggregation in a concentration-dependent manner (Fig 4C black line).

Fig 2. Amino acid sequence alignments. (A) Sequence alignments of the region surrounding amino acids 320, 321 and 353 from the human integrin \(\alpha_{IIb}\) subunit and \(\alpha_{IIb}\) from other species (upper sequences) or with other human \(\alpha\) subunits (lower sequences); (B) Sequence alignments of the regions surrounding amino acids 297, 356 and 384 from the human integrin \(\beta_3\) subunit and \(\beta_3\) from other species (upper sequences) or with other human \(\beta\) subunits (lower sequences). Conserved residues between human \(\alpha\) or \(\beta\) and other sequences are shown with light shading.

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In order to further validate our model, experiments were designed using variants of the octapeptide in which putative residues interacting with \(\beta_3\) were substituted. The importance of residues \(^pR317\), corresponding to the \(\alpha\)IIb amino acid involved in intrapeptide salt bridge R317-E315 (Fig 3), and \(^pD319\), was demonstrated by the loss of activity of \(^pR317A\) or \(^pD319A\) substituted octapeptides when tested by measuring AP5 binding (Fig 4A), platelet aggregation (Fig 4C grey line) and PAC1 binding (Fig 4D). In contrast, \(^pR320A\) decreased only partially the ability of the peptide to induce AP5 binding (Fig 4A), and consequently, decreased only faintly the peptide inhibitory activity on PAC1 binding (Fig 4D). Similar results were obtained with thrombin-induced fibrinogen binding to platelets (S3 Fig). Finally, we performed aggregation with an octapeptide shifted by one amino acid (\(^pM314ESRADRK\))\(^{321}\), which, even at 500 \(\mu M\), did not inhibit platelet aggregation (Fig 4C dashed line). This result shows that amino acid \(^pY313\) is essential for the inhibitory activity of the octapeptide.

Fig 3. Model of the interaction of the \(^pYMESRADR\) peptide with the \(\beta_3\) subunit. Conformation adopted by the \(^pYMESRADR\) peptide to bridge across the \(\beta\)I and hybrid domains of \(\beta_3\). Note that residue \(^pY313\) from the peptide (corresponding to the Y313 from the native hairpin) now replaces Y353 (cf. Fig 1) in interacting with the \(\beta\)I domain. The \(^pE315\)-\(^pR317\) intrapeptide salt bridge is also depicted.

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The importance of PY313 for the inhibitory activity of the octapeptide is in line with our modelling results, where PY313 plays a crucial role in bridging the β-propeller domain across to the βI + hybrid domains. However this requires the octapeptide to adopt a stable conformation in solution similar to the one found in our in silico modelling.

We then revisited our data from a previous NMR structural and functional characterisation [27]. Upon examination of the solution conformational ensembles, it was clear that the backbone conformation of the native peptide in solution was being stabilized via an intra-molecular salt-bridge formed by pR317 and pD319 (Fig 5A). However, more interesting are the NMR data of the peptide with the Alanine substitution at position pD319. Indeed, this substitution induced a new backbone
conformation with a change of the intra-molecular salt-bridge from pR317-pD319 to pR317-pE315 (Fig 5B), almost identical to the conformation of the native octapeptide in our in silico model (Fig 3). This might be rationalised through the fact that most frequently a peptide will rearrange its solution conformation to a different allosteric conformation upon binding/interacting with another macromolecule. The NMR data thus suggest that when residue pD319 is engaged, possibly towards forming the first interaction between the octapeptide and K384
from the β3 subunit, it frees up the intramolecular interaction with R317, which then can engage E315 to compensate for the change (Fig 3) resulting in a more stable conformation of the peptide, in line with our in silico model (Fig 5B and Fig 3). This is furthermore reinforced by the fact that the inhibitory activity of the substituted pD319A octapeptide was only 9%.

Once in such stable conformation, pY313 ends up unexpectedly well-placed to form an interaction with the β3 headpiece E297 residue, effectively restricting to a certain degree both the swing-out and extension movements (Fig 3).

Mutations of either αIIb or β3 amino acids involved in the clasp result in an increased activity state of recombinant integrin αIIb3

We generated cell clones expressing αIIb3 with mutations in either αIIb or β3. In the cell clone αIIb3Mβ3, the αIIb subunit had a triple mutation R317A+D319A+R320A, which corresponds to the pRADR sequence considered to be the most crucial for the expression of the inhibitory activity [22]. The mutant αIIb was co-expressed with wild type β3 in CHO cells, while in the cell clone αIIb3M, the β3 subunit had a single K384A mutation and was co-expressed with wild type αIIb. Residue K384 in the αIIb3 crystal structure is indeed at a favorable distance to form salt bridges with αIIb (Fig 1).

It has been established that shear forces can increase the sensitivity of integrin αIIb3 adhesive interactions as demonstrated by the shear-dependent increase of adhesion of CHO cells expressing the P313K polymorphism of αIIb3 [30] and that αIIb3 activation is associated with a stabilization of integrin binding to fibrinogen under shear conditions between 30 s⁻¹ and 100 s⁻¹ [26]. In the present setting, we have demonstrated, as in previous work [26], that increasing shear rates results in decreased cell adhesion mediated by αIIb3. Interestingly, cells expressing the mutant integrins exhibited increased adhesion as compared to cells expressing wild type αIIb3. Notably, at 100 s⁻¹, only cell clone αIIb3M showed residual adhesion. As the receptors displayed similar density and accessibility on the cell surface (S1 Fig), the differences in cell adhesion under shear relate most likely to a different activation state of the αIIb3 receptor expressed on the cell surface (Fig 6).

In addition, CHO cell adhesion at the lowest shear rate was determined in the presence of the octapeptide. The pY313MESRADR320 native octapeptide strongly impaired adhesion of CHO cells expressing wild type αIIb3 or mutated αIIb3Mβ3 with a statistically significant effect observed at 500 μM, while the pY313MESRAAR320 substituted octapeptide had no effect (Fig 6B). In contrast, native pY313MESRADR320 was not able to inhibit αIIb3M CHO cell adhesion onto fibrinogen under shear stress. These results demonstrate that (i) the mutation of αIIb does not impact the binding of the peptide to the integrin in contrast to the β3K384A mutation (αIIb3M) and (ii) the residue pD319 in the octapeptide plays a crucial role in the binding of the octapeptide to integrin αIIb3.

Discussion

The aim of our study was to rationalise a model that explains the inhibitory effect on integrin αIIb3 activation by the pY313MESRADR320, an octapeptide which corresponds to αIIb β-ribbon sequence [4, 5, 18, 22]. Analysis of our in silico data have led us to propose a model in which the octapeptide substitutes the αIIb β-ribbon in restraining β3 in its closed conformation, by bridging the βI domain and the hybrid domain of the β3 subunit. This model was then used to guide the design of our experimental methodology.

The proposed model is supported by following experimental data: (i) the octapeptide inhibits its platelet aggregation, fibrinogen and PAC1 binding to activated platelets; (ii) alanine replacement or deletion of the amino acids of the octapeptide underline the importance of pD319,
PR317, PR320, as well as PY313 for the inhibitory activity of the peptide. The unexpected involvement of PY313 may explain the apparent contradictory results with the shifted peptide P*M314*ESRADK321 which was suggested to activate platelets [17]; and finally, (iii) the octapeptide strongly impairs adhesion onto fibrinogen under flow of CHO cells expressing either the wild type receptor or the receptor with mutations in the \( \alpha IIb \beta 3 \)-ribbon. Additionally, the octapeptide is unable to inhibit adhesion of CHO cells expressing the \( \beta 3K384A \) mutant, demonstrating the role played by the salt bridge between pD319-K384(\( \beta 3 \)) in the octapeptide’s inhibitory activity.

It is also interesting to note that the heptapeptide LSARLAF, which was designed to bind to residues 315–321 of \( \alpha IIb \) activates \( \alpha IIb \beta 3 \) by directly inducing a conformational change in the receptor [31]. Again, this result agrees with our model, with the LSARLAF playing the opposite role of the PYMESRADR peptide. Indeed, by binding to the region in \( \alpha IIb \), which encompasses the \( \beta \)-ribbon, LSARLAF disrupts the interaction between the \( \beta \)-ribbon of \( \alpha IIb \) and the hybrid domain of the \( \beta 3 \) subunit, thus facilitating its extension. It is likely that full integrin activation by LSARLAF also requires an additional inside-out signalling pathway, as suggested by the study of Pearce et al. [32].

Taken together, our results indicate that the PY311 MESRADR320 octapeptide exerts its inhibitory effect on fibrinogen binding through (i) the establishment of ionic bonds with the \( \beta 3 \) subunit, involving residues K384 and E356 from the \( \beta 3 \) hybrid domain, and (ii) interaction with E297 from the \( \beta 3 \) I-like domain, thus bridging and stabilizing the \( \alpha IIb \beta 3 \) headpiece in the closed conformation (Fig 7). This is consistent with the multi-step activation model as proposed by Zhu et al., which depicts the process through eight distinct stages [33]. Transition from bent-closed to extended-closed steps occurs without major changes in the integrin \( \alpha IIb \beta 3 \) headpiece. This is followed by opening (swing-out) movement from extended-closed step to

Fig 6. CHO cell adhesion to fibrinogen in flow conditions. CHO cells expressing \( \alpha IIb \beta 3 \) wild type (WT \( \alpha IIb \beta 3 \)), mutated on \( \alpha IIb \) (\( \alpha IIb_{3M} \beta 3 \)) or on \( \beta 3 \) (\( \alpha IIb \beta 3_{1M} \)) were perfused over fibrinogen (A) at increasing shear rates (30 s\(^{-1}\), 50 s\(^{-1}\), 75 s\(^{-1}\) or 100 s\(^{-1}\)) or (B) were preincubated 20 min with vehicle (0), 250 μM or 500 μM PYMESRADR peptide, or substituted control peptide (PYMESRAAR) and then perfused at a shear rate of 30 s\(^{-1}\). Adherent cells were counted after 12 min exposure to shear. (A) Results are expressed as the number of adherent cells/field, obtained by counting 10 fields. Bars represent mean values and the interquartile range of at least 3 experiments. (B) Adhesion of WT (left panel), \( \alpha IIb_{3M} \beta 3 \) (middle panel) and \( \alpha IIb \beta 3_{1M} \) (right panel) cells in the presence of peptides or vehicle was quantified (as described in methods section). The results are expressed as percentage of adhesion of each clone in presence of peptides relatively to corresponding cells in presence of vehicle (0) normalized to 100%. (n = 6; mean ± SEM). *P<0.05, **P<0.01

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Fig 7. (A) Integrin extension model and structural rationalisation of inhibition of αIIbβ3 activation by octapeptide pYMESRADR. For clarity only selected domains of the αIIb and β3 subunits are shown. (I) αIIb3 in the initial bent-closed conformation, the β-ribbon is highlighted in the red circle. (II) initial release of the β-ribbon, to be followed by opening of integrin αIIb3 (IV). However, if the octapeptide is present (III), it will then substitute the β-ribbon in restraining βI (blue) and hybrid (green) domains, impeding opening and therefore preventing full activation of the integrin. (B) Schematic representation of αIIb3 clasp interactions and the binding positions of the pYMESRADR octapeptide. In the physiological clasp the β-ribbon and residue Y353 in the β-propeller domain (yellow) are linked to the β3 hybrid domain (green) and the βI domain (blue) concomitantly. When the physiological clasp is released, the octapeptide (red) can then engage with the available anchoring points to reestablish the restraint between the βI (blue) and the hybrid (green) domains. Note
extended-open step, which eventually leads to activation. In the swinging-out stage however, during the disengagement of the β-ribbon all the main interaction residues from β3—namely E297, E356 and K384 become available for binding by the octapeptide, leading to locking of the hybrid and β domains into an intermediate step resembling the extended-closed conformation (Fig 7)—thus precluding full activation of integrin αIIbβ3.

In fact, this intermediate conformation captured by the binding of the octapeptide might explain our AP5 antibody binding results. Indeed, AP5 has been reported to only bind to αIIbβ3 in its activated state. In our scenario, αIIbβ3 already exposes epitopes recognised by AP5, albeit it is still in an inactive conformation. This is further reinforced by a similar observation made by Cheng et al. where a disulphide bond, which was engineered to perform a similar structural role as the one we believe the octapeptide is playing, does not completely inhibit the binding of AP5 induced by Kistrin (a small RGD-containing protein) [20].

However, and given that the octapeptide contains a RAD sequence, one might assume that this sequence could compete with the binding site of the integrin for the RGD domain of fibrinogen. On the other hand, our previous evidence that the octapeptide inhibited rabbit platelet aggregation ex vivo and in vivo, in fact strongly supports a non-RGD like mechanism of inhibition [34]. Indeed, rabbit platelets have previously been used as a suitable thrombosis model for testing the efficacy of peptides unrelated to RGD, since RGD is inactive on rabbit platelet aggregation and fails to displace fibrinogen from their surface [35, 36]. Moreover, it has been previously demonstrated that RAD peptides do not exhibit any activity and are used as negative controls [37–39]. These are strong arguments, although not definitive, that the octapeptide is probably not interacting with the RGD binding site of the integrin.

In conclusion, our data and those of the literature highlight the important role of the αIIb β-ribbon in maintaining the closed inactive conformation of αIIbβ3 by securing the hinge angle between the β and the hybrid domain. This precludes conformational changes necessary for the extension of integrin αIIbβ3 to its high-affinity ligand-binding state. The results of the present study lend further support to the above assumption by presenting detailed interactions between residues, and especially by emphasizing the role of Y353(αIIb) in bridging the hybrid domain of β3 with the β domains of β3. Finally, an allosteric inhibition of αIIbβ3, induced by the octapeptide, may provide an alternative pharmacological approach to the αIIbβ3 RGD-like antagonists. Such allosteric antagonists could reduce the risk of paradoxical receptor activation observed with the cooperative fibrinogen inhibitors [40, 41].

Supporting Information

S1 Fig. Flow cytometry analysis of αIIbβ3 expression in transfected CHO cells. CHO cells expressing the αIIbβ3 wild-type receptor (blue line), the αIIbβ3M mutant (αIIbR317A/D319A/R320A-β3) (green line) or the αIIbβ3M1 (αIIb-β3K384A) (red line) were labeled with saturating amounts of anti-αIIb-PE antibody. The isotype control is shown as the thin black line.

(PDF)

S2 Fig. Binding of LIBS1 antibody to platelets. LIBS1 or control antibody (NI) binding on resting or thrombin-activated platelets pre-incubated with vehicle, RGDS or δYMESRADR peptides. Bound LIBS1 was revealed with PE- anti-mAb antibody and quantified on Becton Dickinson FACSsort cytometer.

(PDF)
S3 Fig. Effect of octapeptides on fibrinogen-binding to platelets. Washed platelets (2.5x10^8 platelets/ml) were preincubated with vehicle (NaCl 0.9%), RGDS, pYMESRADR or with 317-substituted octapeptide (pYMESAADR), 319-substituted octapeptide (pYMESRAAR) or 320-substituted octapeptide (pYMESRADA) (500 μM) for 5 min. Platelets were then treated with 0.1 U/ml thrombin and fibrinogen-binding was measured by single-colour flow cytometry using a FITC-labeled rabbit anti-fibrinogen antibody. Results are expressed as mean fluorescence intensity of at least 3 experiments +/- SEM.

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Author Contributions

Conceived and designed the experiments: WHL DCT DB NK CBL. Performed the experiments: WHL ESR KA ME DCT VM VT PT CBL. Analyzed the data: WHL ESR KA DCT DB NK CBL. Wrote the paper: WHL ESR DCT NK CBL.

References


