Paratope and Epitope Mapping of the Antithrombotic Antibody 6B4 in Complex with Platelet Glycoprotein Ibα*‡¶

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The monoclonal antibody 6B4 has a potent antithrombotic effect in nonhuman primates by binding to the flexible loop, also known as the β-switch region (amino acids 230–242), of glycoprotein Ibα (GPIbα). This interaction blocks, in high shear stress conditions, the specific interaction between GPIbα and von Willebrand factor suppressing platelet deposition to the damaged vessel wall, a key event in the pathogenesis of arterial thrombosis. To understand the interactions between this antibody and its antigen at the amino acid level, we here report the identification of the paratope of 6B4 and the respective epitope. 6B4 and GPIbα were previously found to bind to the flexible loop (residues 230–242) within the N-terminal domain of GPIbα into a β-switch conformation, which changes upon binding of VWF into a β-hairpin conformation, extending the existing VWF antiparallel β-sheet (5). The GPIbα gain-of-function mutations G233V and M239V found in platelet-type von Willebrand disease stabilize the β-hairpin conformation and increase the affinity of GPIbα for VWF 5–6-fold (4, 6). The globular domain is presented well above the plasma membrane by the sialomucin core, which is connected by a flexible hinge domain: the anionic sequence. The cytoplasmic tail of GPIbα contains binding sites for filamin A and 14-3-3 ζ, which play an important role in intracellular signaling upon ligand binding (3, 7, 8).

Previously, we prepared and characterized a murine monoclonal antibody (mAb) targeting the human GPIbα, designated as 6B4 (9). This mAb inhibits platelet adhesion under high shear stress conditions, as was shown in flow chambers (10). Injection of 6B4-Fab fragments has a potent in vivo antithrombotic effect in baboons (9, 11) but also on inhibiting ex vivo ristocetin-induced platelet aggregation (9). Contrary to most antithrombotic drugs, 6B4-Fab administration did not induce a significant prolongation of the bleeding time. The epitope recognized by 6B4 was mapped previously, using human/canine GPIbα to von Willebrand factor (VWF), which is bound to the collagen matrix exposed to the flowing blood upon vessel damage.

The structure of GPIbα consists of a globular N-terminal region, a sialomucin core, an anionic sequence, a transmembrane region, and a cytoplasmic tail. The N-terminal region (residues 1–282) consists of eight leucine-rich repeats (LRRs) and contains the binding sites for VWF, α-thrombin, P-selectin, Mac-1, high molecular weight kininogen, and coagulation factors XI and XII (2–4). Under nonliganded conditions, platelets present the flexible loop (residues 230–242) within the N-terminal domain of GPIbα into a β-switch conformation, as seen upon binding of VWF, 5–6-fold (4, 6). The globular domain is presented well above the plasma membrane by the sialomucin core, which is connected by a flexible hinge domain: the anionic sequence. The cytoplasmic tail of GPIbα contains binding sites for filamin A and 14-3-3 ζ, which play an important role in intracellular signaling upon ligand binding (3, 7, 8).

Platelets are a key factor in hemostasis (1). However, in some pathological situations, such as stroke or myocardial infarction, shear rate increases, causing platelet activation and thrombus formation, leading to vessel occlusion. This process is dependent on the binding of the platelet glycoprotein Ibα (GPIbα)4 to

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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4 The abbreviations used are: GPIbα, glycoprotein Ibα; VWF, von Willebrand factor; LRR, leucine-rich repeat; CDR, complementarity determining region; Ab, antibody; mAb, monoclonal antibody; WT, wild type.

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### TABLE 1
Candidate residues for mutagenesis

| Mutanta | Mutation | Rationale for mutation | Primerb |
|---------|----------|------------------------|----------|
| VL      | Y27DA    | Tyr → Ala              | 5′-AGGCCATTTGTAAGATTACGGCCTATG-3′ |
|         | K27EA    | Lys → Ala              | 5′-CTAAGTACATCTATTCTAGGGATTGGAAGACATACTTTG-3′ |
|         | K27EE    | Lys → Glu              | 5′-CTAAGTACATCTATTCTAGGGATTGGAAGACATACTTTG-3′ |
|         | D28A     | Asp → Ala              | 5′-GTAGAACTTACCTATGAGATCGCATATAATTCGGCTCTCATG-3′ |
|         | D28R     | Asp → Arg              | 5′-GTAGAACTTACCTATGAGATCGCATATAATTCGGCTCTCATG-3′ |
|         | V92A     | Val → Ala              | 5′-ATTACGTACACACTTCCAGTATCTGCGTCAGCCCATAG-3′ |
|         | E93A     | Glu → Ala              | 5′-ATTACGTACACACTTCCAGTATCTGCGTCAGCCCATAG-3′ |
|         | Y94A     | Tyr → Ala              | 5′-GTGAACATTTGCAAGCTTCTGCTGCTGCTAGTG-3′ |
|         | S56A     | Ser → Ala              | 5′-GGGAGATTATAGCAGCTGAGCAAGACATTATATTCTCTCTGATG-3′ |
|         | N58A     | Asn → Ala              | 5′-ATGOGATGCTTGACACAGATCTATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
against purified human GPIIbα (9), purified by affinity chromatography with protein A-Sepharose CL-4B, and dialyzed overnight at 4 °C against PBS. Antibody purity was checked by SDS-PAGE under nonreducing conditions, followed by Coomassie Brilliant Blue staining. Concentrations were evaluated by optical density at 280 nm, and antibodies were kept at −20 °C before use.

Construction and Expression of 6B4 WT and Mutants—Recombinant 6B4 WT and mutants were prepared as chimeric human/murine IgG4 as previously described (17). Construction of 6B4 mutants was performed with the QuickChange XL site-directed mutagenesis kit according to the manufacturer’s instructions using pKaneo-CM30-L_var and pKaneo-50-dhfr-Hleuvar vectors, coding for the respective chimeric light and heavy chain of 6B4, and the appropriate primer couple (Table 1). After DpnI digestion and bacteria transformation, clones positive for the presence of plasmid DNA were selected, and their purified DNA was sequenced. All 6B4 antibodies were expressed in a transient expression system using human embryonic kidney cell line 293T/17 and Lipofectamine 2000™ as described before (17).

Purification and Characterization of 6B4 WT and Mutants—The different expressed antibodies were purified on a protein A-Sepharose CL-4B column and dialyzed against phosphate-buffered saline overnight at 4 °C. Quality control was performed by SDS-PAGE and Western blot analysis using a monoclonal anti-human IgG4-Fc-specific Ab, followed by goat anti-mouse horseradish peroxidase-labeled Ab before revelation using ECL™. Antibody concentration was estimated, in comparison with an IgG4 reference, by sandwich ELISA using anti-human IgG4-Fc-specific Ab for capture and an anti-human IgG horseradish peroxidase-labeled Ab for detection. mAb concentrations were adjusted to 1 μg/ml and kept at −20 °C before use.

Production and Characterization of rGPIIbα Mutants—WT and mutant rGPIIbα were produced in a transient expression system using 293T/17 cells and Lipofectamine 2000™. After 48 h, rGPIIbα secreted in the medium was concentrated using Centriprep-30 and Centricon-100 devices. The concentration of each mutant was determined by a two-step ELISA as described before (21).

Binding of 6B4 WT and Mutants to Human Platelets—The capacity of WT and mutant 6B4 to bind to platelets was tested in an ELISA system where the antibody was added, in a dilution series of 1:2, into wells precoated with fixed intact human platelets (17). Revelation was done by incubating with a monoclonal anti-human-IgG4 antibody (1:4000), followed by a goat anti-mouse horseradish peroxidase-labeled Ab (1:5000), before the addition of H₂O₂ and orthophenylenediamine, stop with H₂SO₄, and optical density determination (490–630 nm) on a microplate reader. Binding of 6B4 WT at saturation was set as 100%.

Binding of rGPIIbα WT and Mutants to Monoclonal Anti-GPIIbα Antibodies—Recombinant GPIIbα WT and mutants were tested for

| TABLE 3 Hydrogen bonds and salt bridges between 6B4 and GPIIbα in model 21 |
|----------------|----------------|----------------|----------------|----------------|
| Number | Amino acid | Position* | Atom | Amino acid | Position | Atom | Hydrogen bond energy kcal/mol |
| 1 | Ser | 67 | HG | Glu | 14 | OE1 | −2.5 |
| 2 | Asp | 28 | OD1 | Arg | 64 | HH2 | −1.7 |
| 3 | Lys | 27E | HZ3 | Asp | 83 | OD2 | −2.1 |
| 4 | Asp | 28 | OD1 | His | 86 | HE2 | −3.6 |
| 5 | Lys | 27E | HZ2 | Asp | 106 | OD2 | −2.2 |
| 6 | Thr | 53 | O | Lys | 231 | HZ1 | −2.5 |
| 7 | Tyr | 27D | HH | Asp | 235 | OD1 | −2.5 |
| 8 | Glu | 93 | OE1 | Lys | 237 | HZ3 | −2.5 |

* Positions of the residues in 6B4 are noted in Kabat numbering.
their capacity to bind to coated monoclonal 6B4, 27A10, and 24G10 in an ELISA set up as described previously (21).

Statistical Analysis—The binding capacity of 6B4 and its mutants to GPIbα as well as the binding of GPIbα and its mutants to monoclonal anti-GPIbα antibodies were compared by Student’s t test. The differences were considered statistically significant when \( p < 0.05 \).

RESULTS

First Model Using ZDOCK—The 6B4 paratope was tentatively determined by constructing computer models of the variable regions of 6B4 bound to different crystal structures of GPIbα. In a first approach, a 6B4 computer model (17) was docked to the ligand-free conformation of GPIbα (1M0Z.pdb) using the ZDOCK program (12). The resulting model was characterized by one major binding site in which the mAb binds to the flexible loop of GPIbα. The model suggested that both the light and heavy chain of 6B4 contribute to the binding to GPIbα.

Based on this model, seven 6B4 residues were selected for mutation to Ala: four on the light chain (Y27D, K27E, Val92, and Glu93) and three on the heavy chain (Ser56, Asn58, and Ile100). Mutations of Val92 and Ser56 to Ala were included as negative control, since no major effect was expected. Only Y27DA and E93A were found to affect the binding of 6B4 to GPIbα (Fig. 2, A and B).

Second Model Using HADDOCK—Since the rigid body docking method ZDOCK only allow the prediction of two interacting residues, we, in a second approach, used the results from the first round as input to construct a new docking model using HADDOCK1.3 that allows for flexible docking in both 6B4 and GPIbα. We used the four available structures of the mAb plus the ligand-free and ligand-bound structures of GPIbα simultaneously in the docking experiment. The docking of 6B4 to the ligand-bound conformation of GPIbα (1SQ0.pdb) did not produce any acceptable model, since hardly any hydrogen bridges between the two proteins were found that furthermore mainly occurred between main chain elements (data not shown).

The docking results with the ligand-free GPIbα structure were grouped into clusters, which are defined as an ensemble of at least two conformations displaying a backbone root mean square deviation at the interface smaller than 1.0 Å (14). Of the energetically best models in each of the three clusters thus obtained (Table 2), docking model 21 in cluster 1 was selected, because both Y27D and Glu93 are predicted to bind GPIbα, which is in agreement with our previous experimental data (Fig. 1). Based on this docking model, light chain K27E, Asp28, and Tyr94 and heavy chain Ser77 and Y100C were selected for mutagenesis (Table 1).

In this model, Asp28 forms four interactions, two of which are salt bridges, with the side chains of Arg64 and His66 located in the GPIbα LHR2 and LRR3, respectively. To disrupt these interactions, Asp28 was not only mutated to Ala but also to the positively charged Arg (Table 1). Furthermore, also in this model (and in most generated models) K27E is predicted to play an important role in binding, since it forms two ionic interactions with Asp83 and Asp100 of GPIbα (Table 3, lines 3 and 5). In the first round, however, somewhat unexpectedly, mutation of K27E to Ala did not inhibit the binding of 6B4 to GPIbα (Fig. 2A), so also here we made a second mutant in which the negatively charged Asp is introduced instead. All 6B4 mutants, except for Y94A, which only was expressed in very low quantities, were tested for their capacity to bind to immobilized human platelets (Fig. 2A). Of the five new mutants tested, only S97T still bound normally to GPIbα on platelets. Fig. 2B clearly shows that six mutants (Y27DA and E93A from round 1 and
K27EE, D28A, D28R, and Y100CA from round 2) at 0.25 and 0.5 μg/ml maximally allowed 25% binding of 6B4 to GPIbα, whereas the other mutants (K27EA, V92A, S56A, N58A, and I100A from round 1 and S97T from round 2) show nearly normal binding. Based on the mutagenesis data, we can conclude that residues Tyr27D, Lys27E, and Asp28A of CDR L1, Glu93A of CDR L3, and Tyr100C of CDR H3 are part of the paratope of 6B4 (Fig. 2C). The other three CDRs do not seem to be involved in the binding.

**Epitope Determination on GPIbα**—Based on the docking model of 6B4 bound to GPIbα, a number of residues were predicted to be part of the epitope. All residues present at the antigen surface and forming hydrogen bonds or ionic interactions with residues of 6B4 could be putative residues of the epitope (Table 3). Several of these residues are located in the LRR: Arg64A, Asp83A, His86A, and Asp106A. Some other residues involved (Lys231A, Asp235A, and Lys237A) are part of the β-switch region, the key element that changes its conformation during ligand binding (5).

To validate our model, a set of 38 single to triple GPIbα mutants (21) containing 62 charged residues mutated to Ala (Fig. 3) was tested for the binding to wild type 6B4. As an additional control, two other inhibitory anti-GPIbα mAbs were tested, namely 24G10, which competes with 6B4 for the binding to human platelets (10), and 27A10 (22), which does not compete (Fig. 4A).

A marked impairment of the binding of mAb 6B4 was seen to GPIbα mutants D83A/H86A (Fig. 4A, lines 3 and 4), D106A (line 5), K149A/E151A/K152A (line 8), K288A/R290A (line 9), and D235A/K237A (lines 6 and 7), which contain all of the predicted residues except for Arg64A (line 2) and Lys231A (line 10), mutation of which did not affect binding. However, since the binding of all three antibodies (and others) to the first four mutants was similarly decreased, it is possible that these induce a conformational change in GPIbα, as previously hypothesized (21) and hence might have an indirect effect on the antibody binding. These residues, therefore, are being treated with caution in the description of the epitope, which on the other hand clearly involves Asp235A and Lys237A of the β-switch region (Fig. 4B).

**Refined Docking Model**—All of the results from the mutagenesis experiments on both 6B4 and GPIbα were combined and used for another docking round with HADDOCK1.3, using again the four 6B4 models and the ligand-free and ligand-bound GPIbα conformation. Results were ranked by interface area and energy contribution to the complex formation and visually analyzed. Also, here the only acceptable docking models could be made with the ligand-free GPIbα conformation, of which the best model, number 61 (Fig. 5 and supplemental material), is very close to the previous model 21. In model 61, 6B4 is rotated and translated into the N-terminal direction, resulting in an increased interaction area from 917.2 to 1164.8 Å². On the GPIbα side, only the β-switch region has a different conformation, leading to a difference in root mean square deviation of 3.4 Å between the mAbs and the β-switch region. The root mean square deviation between the two docking models, 21 and 61, is 8.2 Å when we consider the CDRs but only 7.7 Å when we restrain to the residues that are common in the interface.

A close comparison of the two docking models reveals that some of the amino acids of 6B4 that were involved in the interaction area of the docking model 21 no longer are (i.e. L27C). On the other hand, new residues are now situated in the interaction surface of 6B4, and these residues are not involved in the binding of 6B4. In model 61, CDR L2 with residues Met51 and Phe71 is part of the interacti

**DISCUSSION**

We have developed a monoclonal antibody, 6B4, targeting the human GPIbα, which is responsible for the binding of platelets to the exposed collagen in damaged vessels via von Willebrand factor under high shear stresses. After promising results in animal models of arterial thrombosis, we have developed a
FIGURE 4. Epitope of 6B4, 24G10, and 27A10 on GPIbα. Red, involved in 6B4 binding; green, not involved; blue, inconclusive due to likely misfolding of the mutant; orange, gain-of-function mutations that disrupt 6B4 binding. A, binding of monoclonal antibodies to mutant GPIbα. After expression and purification, each GPIbα was tested for the binding to coated mAb 6B4, 24G10, or 27A10 as described under "Experimental Procedures." Each bar represents the mean with S.E. value obtained for at least two independent duplicate assays. *, statistically different, with \( p < 0.05 \). Results with line numbers in boldface type are discussed under "Results," and 1–7 corresponds to the numbering in Table 3. B, surface representation of the N-terminal part of GPIbα with predicted residues involved or not in 6B4 binding. Gly\(^{233}\) and Met\(^{239}\), for which the gain-of-function mutation to Val disrupts the binding of 6B4, are in orange. The images of the three-dimensional models with the surface representation were generated with PyMOL (available on the World Wide Web).
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recombinant and humanized Fab fragment of 6B4. Importantly, at effective antithrombotic doses, 6B4-Fab does not prolong the bleeding time; nor does it induce thrombocytopenia (9, 11). To take full advantage of the in vivo effects of blocking GPIbα, a compound fit for oral administration is a prerequisite for a broad and prophylactic use. As a first step toward that goal, we here identified the paratope of 6B4 that confers the inhibitory properties of the molecule. In this study, we mapped both the paratope and the epitope of 6B4 by combining computer docking models with mutagenesis studies on both 6B4 and GPIbα.

Our first ZDOCK-based docking approach using 6B4 and GPIbα in its ligand-free conformation (1M0Z.pdb) identified only light chain residues Y27D and Glu95, of the seven selected residues, to be critical for the binding. This relatively poor result might be due to the fact that ZDOCK is an algorithm developed for rigid body docking. GPIbα, however, is a molecule with several known conformations, and also antibodies rearrange their residue side and/or main chains to improve the affinity for their targets. One way to overcome this problem would be to perform molecular dynamics simulations followed by calculation of relative free binding energies with the molecular mechanics Poisson-Boltzmann surface area as, for example, Wu et al. (23) did to resolve the interaction between the scorpion toxin ScyTx and the small conductance calcium-activated potassium channel Rsk2. An alternative strategy, which we followed, is using a docking strategy method that allows flexibility in both ligand (6B4) and target (GPIbα), such as HADDOCK1.3. Docking tasks were submitted, including both the ligand-free and ligand-bound conformation of GPIbα in combination with 6B4 models constructed with the Web Antibody Modeling program, next to the model that we previously used to prepare a humanized 6B4-Fab fragment (17). After ranking the hits and visual inspection, no good candidate models were identified with the ligand-bound conformation of GPIbα. This finding is in total agreement with our previous result, where the binding of 6B4 was some 6-fold lower to the gain of function (G233V and M239V) GPIbα, as compared with the wild type (10). These gains of function are found in platelet-type von Willebrand disease and enhance the affinity of GPIbα for VWF (6, 24). Furthermore, the structure of GPIbα carrying either one of these mutations is similar to its conformation in complex with the VWF A1 domain (19).

Next, 6B4 residues involved in the binding to ligand-free GPIbα, as deduced from the docking model 21, were expressed as single mutants. The chimeric human/mouse 6B4-IgG4 was chosen because it has the same characteristics (17) as the parental IgG and is easy to manipulate and to produce. Indeed, production of the mutants in quantities sufficient for the binding studies was possible for all 13 mutants except for Y94A. Binding experiments of 6B4 WT and its mutants were performed on whole fixed platelets of healthy volunteers, thereby presenting GPIbα in the GPIbα-IX-V complex. All together we positively identified three paratope residues in CDR L1 and one in CDR L3 and CDR H3 each, which all together are in close spatial proximity on the antibody surface.

To further validate docking model 21, we next explored the role of every charged residue in GPIbα by using the Ala scan library previously described (21). Mutation of most of the predicted interacting residues caused deficient binding. However, since a number of these mutations are suspected to induce a conformational change in GPIbα (21), which also here resulted in a decreased binding of two other anti-GPIbα antibodies with different epitopes (10), we cannot make a definitive statement on these, in contrast to residues Asp235 and Lys237 located in the β-switch region, which specifically affected binding of 6B4. These results are furthermore in perfect agreement with the study of Cauwenberghs et al. (10), where we used human/canine chimeric GPIbα to map the epitope.

Finally, we performed a new docking experiment, including all known experimental data, which yielded a final model in total agreement with all of the mutagenesis results.

In conclusion, by identifying the crucial residues involved in the paratope-epitope interaction of 6B4 with GPIbα, a detailed model of the complex at the atomic level is proposed. This information allows for a better understanding of the antithrombotic action of 6B4 and will be helpful in the design of improved molecules.

Furthermore, and in a broader perspective, the iterative method we used here to identify the interacting amino acid residues by alternating docking and mutagenesis experiments allows for a more rational identification of relevant residues than what a classical laborious mutagenesis scan can provide and can be readily applied to other protein-protein interactions for which sufficient structural information is available.

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