A Mechanism for Localized Dynamics-driven Affinity Regulation of the Binding of von Willebrand Factor to Platelet Glycoprotein Ibα

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The abbreviations used are: vWF, von Willebrand factor; GPIbα, platelet glycoprotein Ibα (GPIbα) under the high flow conditions present in circulating blood (1). The intrinsic adhesive properties of vWF are tightly regulated such that plasma vWF and GPIbα coexist without interaction under normal conditions. However, vWF could be activated to bind platelets under pathophysiological conditions, causing such conditions as microthrombosis for patients with thrombotic thrombocytopenic purpura (2) and bleeding for patients with type 2B von Willebrand disease (3). Therefore, understanding the regulatory mechanism of vWF activity is important for the diagnosis, treatment, and prevention of vWF-related diseases.

In circulation, the vWF presents as multimers of different molecular masses. Each vWF monomer consists of five types of repeat domains in the order of D1-D2-D1-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK, of which the A1 domain contains the known GPIbα-binding site (4). The crystal structure of the A1 domain comprises one globular body region and the N- and C-terminal arms connected by a disulfide bridge between the residues Cys509 and Cys695 (5) (Fig. 1, A and B). The body region consists of a six-stranded hydrophobic β-sheet flanked by three amphipathic α-helices on each side. The N-terminal arm folds over the body region and contacts with the bottom face of the body region, whereas the C-terminal arm extends downwards from the body. Crystallographic studies reveal that the concave face of GPIbα wraps around the body region in a pincer-like grip with the β-switch and β-finger regions (Fig. 1C). Both arms are not involved in the interaction with GPIbα but approach the β-finger-binding site (6).

Several lines of evidence indicate that plasma vWF is autoinhibited by internal domain-domain interactions between A2 and A1 (7), D’D3 and A1 (8), as well as the N-terminal flanking region of A1 and A1A2A3 tridomain (9). An isolated recombinant A1 domain binds GPIbα spontaneously (10). Hence, the exposure of the GPIbα-binding site in the A1 domain through a conformational change of vWF serves as an underlying mechanism of activating plasma vWF multimers. This activation process can be induced by high fluid shear forces detected at a site of arterial stenosis (11, 12).

The bond strength for the interaction between the exposed A1 and GPIbα is essential for efficient platelet adherence because this physical bonding must withstand strong hydrodynamic forces acting on cells. It has been demonstrated that the

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Background: Gain of function (GOF) mutations enhance the vWF-GPIbα interaction.

Results: GOF mutations induce destabilization of the N-terminal arm and increase mobility of the α2-helix.

Conclusion: Dynamics-driven up-regulation of A1 affinity to GPIbα serves as a GOF mechanism of type 2B mutations.

Significance: These results are helpful in understanding the structural basis of GOF mutants and in developing allosteric drugs against the activated A1 domain.

Binding of the A1 domain of von Willebrand factor (vWF) to glycoprotein Ibα (GPIbα) results in platelet adhesion, activation, and aggregation that initiates primary hemostasis. Both the elevated shear stress and the mutations associated with type 2B von Willebrand disease enhance the interaction between A1 and GPIbα. Through molecular dynamics simulations for wild-type vWF-A1 and its eight gain of function mutants (R543Q, I546V, ΔSS, etc.), we found that the gain of function mutations destabilize the N-terminal arm, increase a clock pendulum-like movement of the α2-helix, and turn a closed A1 conformation into a partially open one favoring binding to GPIbα. The residue Arg578 at the α2-helix behaves as a pivot in the destabilization of the N-terminal arm and a consequent dynamic change of the α2-helix. These results suggest a localized dynamics-driven affinity regulation mechanism for vWF-GPIbα interaction. Allosteric drugs controlling this intrinsic protein dynamics may be effective in blocking the GPIb-vWF interaction.
A1-GPibα bond is a “flex bond” having two states with different lifetimes and force resistances (13); a tensile force changes the equilibrium as well as the switching rate between the two states (13). The gain-of-function (GOF) mutation R543Q associated with type 2B von Willebrand disease reduces the force that is required by the switch (14). In contrast, the WT A1 has been shown to form a catch bond with GPibα, where the bond lifetime is prolonged by increasing force (15). A type 2B mutation can result in a left shift or a complete loss of the catch bond characteristics by increasing the lifetime at low force (15, 16). In addition, the binding affinity of an isolated A1 domain is increased ~2.5-fold for the mutant R543Q (6) and 20-fold for the mutant R545A (17), independent of fluid shear stress. A similar increase in binding affinity can be induced in the isolated WT A1 domain by reduction and alkylation of the Cys509–Cys695 disulfide bond and/or by exposure to acidic pH (18, 19). These data suggest that the A1 domain itself may take either a low or a high affinity conformation, and tensile forces and GOF mutations favor the high affinity conformation.

However, the superposition of 12 A1 crystal structures (the WT A1 or its GOF mutants, unliganded or liganded with GPibα, activators, or inhibitors) in the Protein Data Bank shows no significant structural heterogeneity to interpret these affinity differences (supplemental Fig. S1) (5, 6, 20–26). Because a crystal structure at a moderate resolution only provides time- and crystal lattice-averaged conformation present for more than 20% of the time (27), it is unknown whether the high affinity conformation is dynamically transient or reflects one of the known crystallized structures (3). Recently, the A1-GPibα bond stability was correlated inversely with the intrinsic thermodynamic stability of the A1 domain (16). This suggests that apart from static structures, dynamic properties associated with the backbone and side chain mobilities responsible for the conformational stability may also play a key role in regulating the ligand-receptor interaction.

Molecular dynamics (MD) simulation has a unique ability to describe protein dynamics by tracking the precise position of each atom at any instant in time, and most importantly, can not only determine what is changing upon perturbations but can also tell us how and why the change happens (28). To better understand the molecular basis of GOF mutants, we herein examined the dynamic properties of the wild-type vWF A1 and its eight GOF mutants through MD simulations. The results depict a localized dynamics-driven affinity regulation mechanism for binding of vWF to GPibα, provide a novel insight into the structural basis of GOF mutants, and may assist in developing allosteric drugs against the activated A1 domain.

**EXPERIMENTAL PROCEDURES**

**System Setup**—Two groups of molecular systems of vWF A1 structures were set up for MD simulations. The first group has four structures, namely the unliganded WT vWF A1 (Protein Data Bank code 1AUQ) (5) (Fig. 1A), its two gain-of-function mutants I546V (Protein Data Bank code 1IJB) (21) and R543Q (Protein Data Bank code 1M10) (6), and a modeled mutant labeled with “ΔSS” (Fig. 1B). Among the 25 type 2B mutants, R543Q and I546V were selected, because the thermodynamics and kinetics properties of these two mutants were better demonstrated than others (14–16, 19, 29). The mutant ΔSS was modeled by reducing the Cys509–Cys695 disulfide bond in the WT A1 via the AUTOPSF plug-in of VMD (30) (Fig. 1B). Both R543Q and I546V were reconstructed by replacing their N-terminal tails (residues 498–508) with that (residues 498–508) of the WT A1, through aligning with the body region of the WT A1 and making the N-terminal tails have an initial conformation the same as that of the WT A1. Missing atoms (all hydrogen atoms, side chain of Lys560 in WT A1, and side chain of Arg771 in I546V A1) in the partially resolved residues were added via Swiss-Pdb Viewer (31).

The reconstructed models of both R543Q and I546V were minimized along the protocol such that all atoms except those in the junction residues Tyr508 and Cys509 were fixed in the first 1000 minimization steps, the followed 2000 minimization steps were run for the free N-terminal tails (residues 498–508) joined to the fixed body, and the last 5000 minimization steps were performed for all atoms unconstrained. In each of the minimized models of R543Q and I546V, the irrational bond length of the peptide bond 508–509 had reverted to a normal value of 1.33 Å, and the 508–509 peptide plane had recovered too. The key contacts between the N-terminal arm and the rest of A1...
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were examined to be preserved (supplemental Fig. S2 and Table S1).

The other group includes six structures, of which, one, labeled as “WT-NC,” was a WT A1 reconstructed through taking the body of A1 from the crystal structure of the complex WT-A1/GPIbα (Protein Data Bank code 1SQ0) (20) and adding the N-terminal arm with the same protocol used in R543Q and I546V, and others, including a GOF mutant E501A (32) and four type 2B mutants (R578Q, R578W, R578P, and R578L) (33–35), were modeled per homology by replacing the corresponding side chain in WT A1 and subsequently performing 1000 steps of minimization in vacuo while all atoms except the mutated residue were fixed. The WT-NC A1 was used for a negative test to verify whether the reconstructed R543Q and I546V mutants were rational or not, and each GOF mutation (E501A, R578Q, R578W, R578P, and R578L) was selected to further examine whether it would cause a change of the dynamic properties of A1.

The protonation state of each titratable protein residue at neutral pH was determined with the software PROPKA (36). The terminal patches ACE and CT3 were added to the N terminus and the C terminus, respectively, to mimic the continuation of the protein chain. The crystallographic water molecules were retained. Each structure was soaked with TIP3P water molecules in a rectangular box with walls at least 15 Å away from any protein atom. Na+ and Cl− counter ions at physiological concentration of 150 mM were added into the water boxes to achieve charge neutrality and to mimic the real physiological environment.

Molecular Dynamics Simulations—Two software packages, visual molecular dynamics (VMD) for visualization and modeling (30) and NAMD 2.9 program for free MD simulations (37), were used in the simulations. The CHARMM22 all-atom force field (38), along with cMAP correction for backbone, particle mesh Ewald algorithm for electrostatic interaction and a 12 Å cutoff for electrostatic and van der Waals interaction, was used to perform MD simulations with periodical boundary condition and time step of 2 fs. All systems were subjected to energy minimizations for 5,000 steps with heavy atoms fixed and for another 10,000 steps with all atoms free. The energy-minimized systems were heated gradually from 0 to 310 K in 0.1 ns first and then equilibrated for 5 ns with pressure and temperature control. The temperature was held at 310 K using Langevin dynamics, and the pressure was held at 1 atmosphere by the Langevin piston method. From each system, three different structures in equilibrium were chosen as the initial conformations for free dynamics simulations to better capture the dynamics of the protein (28). The free dynamics simulations were run three times on each equilibrated system over 100 ns in a microcanonical ensemble (constant number of atoms, constant volume, and constant energy). The atomic coordinates were recorded and analyzed every other picosecond. All simulations were run on the Dell PowerEdge M910 supercomputer at the School of Bioscience and Bioengineering of South China University of Technology.

Data Analysis—All analyses were performed with VMD tools (30). The time courses of Cα root mean square deviation (RMSD) and radius of gyration (Rgyr) illustrated the conformational changes and the stabilities of the structures. The Cα root mean square fluctuation (RMSF) patterns marked the local structural flexibilities. The interhelical angle between the α2- and α1-helix in A1 was quantified by the cross-angle of two straight lines, which connected the N termini (residue 575 for α2 and 528 for α1) of the helices to their respective C termini (residue 582 for α2 and 542 for α1) for simplicity. A hydrogen bond was defined if the donor-acceptor distance was <3.5 Å and the donor-hydrogen-acceptor angle was >150°. To define a salt bridge, the distance between any of the oxygen atoms of acidic residues (Asp or Glu) and the nitrogen atoms of basic residues (Lys or Arg) must be within 4 Å. A hydrogen bond or salt bridge occupancy was defined as the percentage of bond survival time, during which a hydrogen bond or salt bridge formed in the period of simulation. The residue hydration frequency was defined as the ratio of the hydration events, in which at least one water molecule appeared at a position less than 4 Å apart from a hydrophobic residue, to all events with or without hydration. Both the residue hydration frequency and the solvent-accessible surface area (SASA) with a 1.4 Å probe radius were measured to examine whether the hydrophobic core was exposed or not. All molecular images were generated using VMD (30).

Statistical Analysis—The p values of unpaired two-tailed Student’s t test were used to indicate the statistical difference significance of the data (p < 0.05) or lack thereof (p > 0.05).

RESULTS

GOF Mutation Triggers the Switch from a Stable Conformation to a Localized Unstable One for the A1 Domain—Thermodynamic experiments demonstrated that type 2B mutations destabilize A1 (16). To reveal the structural changes caused by the mutations, we first performed free dynamics simulations three times over 100 ns with a time step of 2 fs for each of the WT A1 and its three mutants (R543Q, I546V, and ASS) (see “Experimental Procedures”). We observed this mutation-induced destabilization from the time courses of the Cα root mean square deviation (RMSD) (Fig. 2A and supplemental Fig. S3) and the gyration radius Rgyr (supplemental Fig. S4) of global A1 (residues 498–700), regardless of the C-terminal arm for its very flexible residue composition beyond Ala701. The type 2B mutations did remarkably raise the levels of both Cα RMSD and Rgyr or induce the destabilization, which occurred more severely in ASS than in both R543Q and I546V and was contributed mainly by the N-terminal arm (residues 498–508) rather than by the body region (residues 509–695) (Fig. 2, B and C). The major structural change occurred in the N-terminal flanking peptide (Fig. 2C) and was reflected by the increasing distance between the mass center (DMC) of the N-terminal arm and the body region for each mutant in comparison with the WT A1 (Fig. 2D), but the minor structural change in the body region was observable too (Fig. 2B). These structural changes were intuitively illustrated by a superimposition of four structures (Fig. 3A), each of which was an average over all conformational snapshots simulated one by one in duration of 100 ns in three runs for each of the WT A1 and its mutants.

These results suggest that the GOF mutations trigger the switch from a stable conformation of A1 to a localized unstable...
extend toward the A1 may make the N-terminal arm either move away from or toward the C-terminal disulfide bond. This localized destabilization of the N-terminal arm and the body region (Fig. 2, A and B) would weaken the interaction between the N-terminal arm and the body region. It demonstrates that an extensive N-terminal arm can shield the binding site for GPIbα, but the type 2B mutations are most likely to induce a conformational change in this peptide and make the binding site accessible, as suggested previously (5, 6, 39).

GOF Mutation Changes the Localized Dynamic Property of the A1 Domain—Because these GOF mutations do not lead to notable structural change in the GPIbα binding interface, an alternative explanation for the affinity modulation may lie in the mutation-induced change of the dynamic property of A1. To examine this possibility, we analyzed the root mean square deviation (RMSD) per residue in A1 and found that these GOF mutations enhanced the mobility of backbone in A1 (Fig. 4A and supplemental Fig. S5). The most significant fluctuation occurred consistently in the α2-helix (residues 574–583) and a part of the conjoint β3α2-loop (residues 569–573) rather than in the rest of A1 except the N-terminal arm, and the GOF mutations enhanced the fluctuation of the region spanning residues 569–583 (Fig. 4A). This mutation-induced change of the intrinsic dynamic property one, and the region largely responsible for the destabilization is the N-terminal arm rather than the body region (Fig. 2, B and C), which remains stable even without the protection of the N-C-terminal disulfide bond. This localized destabilization of A1 may make the N-terminal arm either move away from or extend toward the β-finger-binding site of GPIbα (Figs. 2C and 3A), showing a structural heterogeneity of the N-terminal arm. This structural heterogeneity may be reflected by the various conformations of the N-terminal arm in the 12 A1 crystal structures (supplemental Fig. S1). The significant displacements of the N-terminal arm away from the body region in the GOF mutants (Figs. 3A and 2D) would weaken the interaction between the N-terminal arm and the body region. It demonstrates that an extensive N-terminal arm can shield the binding site for GPIbα, but the type 2B mutations are most likely to induce a conformational change in this peptide and make the binding site accessible, as suggested previously (5, 6, 39).
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may regulate the binding of A1 to GPIbα, because both the α2-helix and the β3α2-loop make up part of the GPIbα binding interface (Fig. 1C).

In addition to the mutation-induced increase of local flexibility, we also observed a so-called clock pendulum-like movement of the α2-helix swinging inward and outward of the A1 body during simulations (Fig. 3B). This movement caused the variation of the α2–α1 interhelical angle, whose mean for the WT A1 was smaller than those for the GOF mutants (Fig. 4B and supplemental Fig. S6). The increased α2–α1 interhelical angle in each mutant demonstrated a localized dynamics-driven process such that a GOF mutation would turn the closed conformation of the WT A1 into an open one (Fig. 3, A and B). The distribution of the α2–α1 interhelical angle (Fig. 4C) showed that a GOF mutation distinctly widened the conformational space sampled in the thermodynamic equilibrium. The conformations with an α2–α1 interhelical angle over 30° accounted for 34.0% for R543Q, 35.6% for I546V, and 25.5% for ΔSS but only 2.3% for WT A1. The newly increased populations in the sampled space might include the “activated” A1 conformation with high affinity to GPIbα. As a result, a mutation may enhance the transition from a closed conformation to a partly open one in favor of binding to GPIbα, consistent with the reports that a partial unfolding conformation of A1 has high affinity to GPIbα (16, 29, 40), but a combination of two type 2B mutations in a single construct does nothing for the affinity of binding to platelets (41).

Mutations Induce the Exposure of the Hydrophobic Core in the A1 Domain—We found from free dynamics simulations that the above mentioned mutation-induced change of the localized dynamic property was accompanied with a partial exposure of the hydrophobic core in the A1 domain. The hydrophobic junction between the α1- and α2-helix is zipper-like, and the zipper teeth are constituted by the side chains of the hydrophobic residues (Phe530, Leu533, Val537, Val538, and Met541 in the α1-helix and Leu577, Ala581, and Val584 in the α2-helix) (Fig. 5A). As the α2-helix swung like a clock pendulum, the zipper opened and closed repeatedly with various frequencies and amplitudes, leading to partially exposing the hydrophobic residues (Val553, Ala554, Val555, and Val556) in the β2-strand beneath the zipper teeth to the bulk water. For example, the zipper was closed when the α2-α1 interhelical angle took a value of 17° (Fig. 5B) but partially opened when the angle increased to 37° (Fig. 5C) because of the swinging out of the α2-helix.

The hydrophobic core in the WT A1 should be less solvent-exposed than those in the mutants, because the GOF mutations could enhance the clock pendulum-like movement of the α2-helix relative to the α1-helix (Fig. 4C). To examine it, we calculated the average SASA of hydrophobic residues 553–556 (Fig. 6A) and the hydration frequency for each of residues 553–556 (Fig. 6B). As expected, a GOF mutation resulted in a marked increase of both the SASA in the hydrophobic region of the β2-strand and the hydration frequencies of the hydrophobic residues 553–556 in comparison with the WT A1 (Fig. 6, A and B) and thereby destabilized the hydrophobic core through weakening the hydrophobic contact between the α1- and α2-helices (Fig. 5), indicating that the mutation-induced weakening of the hydrophobic junction between the α2- and α1-helix increased the clock pendulum-like movement of the α2-helix.

**FIGURE 5.** The representative closed and open conformations of the hydrophobic core in A1 domain. A, the zipper-like hydrophobic junction between the α1- and α2-helices in Newcartoon representation. All involved hydrophobic residues (Phe530, Leu533, Val537, Val538, Met541, Leu577, Ala581, and Val584), functioned as the “zipper teeth,” in the α1- or α2-helix are represented as yellow licorices. B, a closed state of the hydrophobic core in the mutant R543Q with the α2-α1 interhelical angle of 17°. C, this closed conformation becomes an open one; as such, the partly open conformation with the α2-α1 interhelical angle of 37°. The solvent-accessible surface (yellow) is displayed around the hydrophobic residues with a probe radius of 1.4 Å (B and C). The partly exposed hydrophobic residues (green licorices) in the β2-strand beneath the hydrophobic junction between the α1- and α2-helices are shown in the open conformation too.

**FIGURE 6.** The mutation-mediated variation on the SASA and the residue hydration frequency. A and B, the SASA (A) and the residue hydration frequency (B), which are averaged over stimulation duration of 100 ns, of the hydrophobic core (residues 553–556) located at the β2-strand in the WT A1 and its three GOF mutants. The residue hydration frequency is the ratio of the hydration events, in which at least one water molecule appears at the position being less 4 Å apart from a hydrophobic residue, to all events with or without hydration. The data shown are means ± S.E. of three run results. The p values of the unpaired two-tailed Student’s t test are shown to indicate the statistical difference significance, or lack thereof.
Residue Arg<sup>578</sup> Serves as a Pivot in the Conformational Switch of the A1 Domain—A GOF mutation could change the dynamic properties of the N-terminal structure and the α2-helix, as mentioned above. However, it is unclear whether the localized structural change would correlate with the dynamic stabilization of the intradomain network of hydrogen bonds and/or salt bridges. We therefore examined the involved internal hydrogen bonds and/or salt bridges at the bottom of A1 via the free dynamics simulations and found that the dynamic change of the α2-helix is a consequent event after the N-terminal arm destabilization. In this process, residue Arg<sup>578</sup> at the α2-helix, together with its two partner residues Glu<sup>501</sup> at the N-terminal arm and Glu<sup>542</sup> at the C termini of the α1-helix, is indispensably involved (Fig. 7).

Arg<sup>578</sup> formed a firm salt bridge with Glu<sup>501</sup> but a very weak one with Glu<sup>542</sup> for the WT A1 (Fig. 7A). A mutation would make the firm salt bridge Arg<sup>578</sup>–Glu<sup>501</sup> (occupancy > 95%) very weak (occupancy < 10%) and the weak salt bridge Arg<sup>578</sup>–Glu<sup>542</sup> (occupancy < 5%) stronger (occupancy > 40%) (Fig. 7B). These results demonstrated that Arg<sup>578</sup> serves as a pivot in the conformational switch of A1 from a stable state to a localized unstable one. As a firm linker between the N-terminal loop and the α2-helix in the WT A1, the salt bridge Arg<sup>578</sup>–Glu<sup>501</sup> rather than the salt bridge Arg<sup>578</sup>–Glu<sup>542</sup> maintains the structural stability of the N-terminal arm and restrains the movement of the α2-helix relative to the α1-helix; contrarily, a GOF mutation makes the salt bridge Arg<sup>578</sup>–Glu<sup>501</sup> rarely formed so that the mutual restraint of the N-terminal loop and the α2-helix almost vanishes and subsequently reinforces the salt bridge Arg<sup>578</sup>–Glu<sup>542</sup> to form a considerable restraint on the movement of the α2-helix (Fig. 7 and supplemental Fig. S7). However, the salt bridge Arg<sup>578</sup>–Glu<sup>542</sup> (60% > occupancy > 40%) in the mutants should be a more flexible linker in comparison with the salt bridge Arg<sup>578</sup>–Glu<sup>501</sup> (occupancy > 95%) in the WT A1, meaning that enhancing the clock pendulum-like movement of the α2-helix ensues from loosing of the salt bridge Arg<sup>578</sup>–Glu<sup>501</sup>.

Also, we also observed another seven hydrogen bonds and three salt bridges, which are intimately involved in the intradomain interactions at the bottom of the A1 body region (Fig. 8). Of these interactions, the hydrogen bond Lys<sup>569</sup>–Gln<sup>548</sup> between the β3α2-loop and the β2α1-loop and the salt bridge Lys<sup>569</sup>–Asp<sup>610</sup> between the β3α2-loop and the β4α3-loop are unstable (occupancy < 30%), but others are not (occupancy ≥ 50%), for each of the WT A1 and its three mutants (supplemental Table S2). It demonstrated the insubstantial effects of the GOF mutations on the stability of the hydrogen bond and/or salt bridge network in the intradomain of A1 except both the α2-helix and the N-terminal loop. This should provide another insight into why the mutations cannot change the structural stability of the A1 body.

Further Verification for the Type 2B GOF Mutation-induced Change of Dynamic Property of the A1 Domain—However, all of the analyses above were based on the assumption that the entire R543Q and I546V mutants, constructed by adding the N-terminal tail to the x-ray structures of the R543Q (Protein Data Bank code 1M10) and I546V (Protein Data Bank code 1JBJ), were reliable, because the key contacts between the N-terminal arm and the rest of A1 were preserved (supplemental Fig. S2 and Table S1). A negative test for this assumption was performed therefore by examining the dynamic property of a reconstructed A1 domain named WT-NC (see “Experimental Procedures”). Also, all the type 2B mutations locate at the lower base of A1 and/or at the same side of the β-sheet.
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TABLE 1
Comparison of dynamic properties of the two wild-type A1 with those of the eight GOF mutants

| Number | Name       | Body N-terminal arm | DMC* | θ° | Ratio of θ > 30° | SASA* | Occupancy | C<sub>RMSD</sub>* | A<sub>2</sub> | A | θ | A<sub>2</sub> |
|--------|------------|---------------------|------|----|-----------------|-------|-----------|-------------------|---------|----|-----|---------|
| 1      | WT         | 1.24 ± 0.16         | 3.18 ± 0.49 | 21.9 ± 0.2 | 22.6 ± 1.5 | 2.30 | 14.43 ± 0.95 | 95.7 ± 3.2 | 2.10 ± 3.55 |
| 2      | WT-NC      | 1.11 ± 0.16         | 4.04 ± 0.52 | 22.2 ± 0.6 | 21.5 ± 1.6 | 1.50 | 12.07 ± 1.26 | 96.9 ± 1.0 | 3.07 ± 1.24 |
| 3      | R543Q      | 1.47 ± 0.11         | 8.43 ± 2.28 | 23.9 ± 1.3 | 27.8 ± 3.4 | 3.40 | 16.85 ± 1.85 | 6.53 ± 4.2 | 45.9 ± 5.2  |
| 4      | I546V      | 1.42 ± 0.03         | 8.27 ± 4.65 | 24.2 ± 0.7 | 27.3 ± 3.2 | 35.6 | 15.76 ± 1.23 | 8.61 ± 6.6 | 48.5 ± 5.2  |
| 5      | ∆SS        | 1.61 ± 0.41         | 9.75 ± 5.23 | 24.2 ± 0.9 | 26.5 ± 1.9 | 25.5 | 18.88 ± 0.46 | 0.65 ± 0.7 | 38.3 ± 13.6 |
| 6      | E501A      | 1.34 ± 0.12         | 8.53 ± 3.08 | 22.8 ± 0.8 | 24.1 ± 2.4 | 10.0 | 14.60 ± 1.34 | N/A      | N/A          |
| 7      | R578Q      | 1.53 ± 0.05         | 9.86 ± 4.67 | 24.0 ± 0.9 | 27.6 ± 2.4 | 36.4 | 15.57 ± 2.50 | N/A      | N/A          |
| 8      | R578W      | 1.85 ± 0.33         | 7.25 ± 2.01 | 22.8 ± 0.2 | 30.1 ± 9.0 | 43.7 | 19.05 ± 1.16 | N/A      | N/A          |
| 9      | R578P      | 1.37 ± 0.03         | 11.4 ± 2.08 | 24.3 ± 0.9 | 23.8 ± 0.8 | 11.1 | 14.73 ± 0.71 | N/A      | N/A          |
| 10     | R578L      | 1.49 ± 0.08         | 6.29 ± 1.04 | 23.4 ± 1.3 | 24.0 ± 1.2 | 19.7 | 14.51 ± 0.45 | N/A      | N/A          |

* The data were averaged over the entire simulation duration of 100 ns for each of the three runs and shown as means ± S.D.

** The occupancy values were presented as means ± S.D. of three different occupancies measured from three independent simulations within simulation time of 100 ns for each A1, θ is the α2-α1 interhelical angle of A1, and SASA denotes the average solvent-accessible surface area of the hydrophobic residues 553–556 in the β2-strand.

(supplemental Fig. S8B), hinting that, as shown in R543Q, I546V, and ∆SS mutants (Figs. 2–4), the mutation-induced increase of flexibility in A1 may occur at other type 2B mutants too. To verify this hypothesis, we further examined the dynamic properties of other five mutants (E501A, R578Q, R578W, R578P, and R578L) via MD simulations (see “Experimental Procedures”). The mutant E501A has gain of function phenotype (32), and R578Q, R578W, R578P, and R578L are known as the type 2B mutations (33–35).

All results are summarized in Table 1, and we found that the C<sub>RMSD</sub> RMSD and DMC of the N-terminal arm remained at higher levels for all mutants and so did the α2-α1 interhelical angle and the RMSF (in the α2-helix and a part of the conjoint β3α2-loop) (supplemental Figs. S9 and S10) in comparison with the WT A1. The fluctuation of the N-terminal loop in each of the five newly examined mutants was stronger than those in both WT A1 and WT-NC A1, as observed in R543Q, I546V, and ∆SS mutants (Table 1 and supplemental Fig. S9). This observation suggests that a gain of function through destabilizing the N-terminal arm and increasing flexibility of the α2-helix may serve as a general mechanism for a type 2B mutation. In addition, the mutation-induced loss of the salt bridge between the α2-helix and the N-terminal tail for each mutant (E501A, R578Q, R578W, R578P, or R578L) further exhibited the pivot role of the residue Arg<sup>578</sup> in maintaining stabilization of the N terminus.

The salt bridge Arg<sup>578</sup>–Glu<sup>501</sup> between the α2-helix and the N-terminal tail in the WT-NC A1 was strong, making the N-terminal tail in the WT-NC stable, as in the WT A1. As a result, the difference in the dynamic properties of the WT A1 and the WT-NC A1 was not significant (Table 1 and supplemental Figs. S9 and S10). This result indicated that the higher fluctuations in both the entire I546V and R543Q mutants came from the type 2B mutations rather than the manually added N-terminal arms, suggesting these computer models of the I546V and R543Q mutants were available.

**DISCUSSION**

Less conformational change occurs in the vWF A1 domain, not like the homologous von Willebrand factor A domain, the integrin I domain, whose affinity is regulated by the obvious structural changes caused by a piston and connecting rod-like movement of the C-terminal α7-helix (42). This is why the structural basis of the GOF variants of the native vWF A1 domain remains an open question. From observation via free dynamics simulations on nanosecond scale, we demonstrated that a GOF mutation triggers the switch of A1 from a stable state to a localized unstable one. This switch is relevant to a localized dynamics-driven process, including a destabilization of the N-terminal arm and a consequent increase in flexibility of the α2-helix, and serving as a possible mechanism for vWF A1 activation and affinity modulation upon GOF mutation. In comparison with the WT A1, the partly open conformation of a GOF mutant should have higher compliance with the binding site of GPIbα, based on the conformational selection theory of molecular recognition (43, 44).

This GOF mutation effect is similar to those demonstrated in previous reports, such that the destabilization of A1 reduces concomitantly the interactions among A1-A2-A3 domains and thereby enhances the binding to GPIbα (45); the amino acid sequence 475–497, which stabilizes A1A2A3 tridomain (9), detaches from the body region together with the posterior N-terminal arm (residues 498–508) and induces a conformational change in the A1 domain to up-regulate A1 affinity, and as a linker between the A1 domain and the D’D3 domains, the N-terminal arm probably plays an important role in orientating the A1 domain with the neighboring domains and in exposing the GPIbα-binding site in arterial bleeding (42, 46).

Proteins are not static building blocks. Experimental and computational data suggest that dynamics and motions of protein structure are indispensable to protein activity modulation (47–51). We showed that each type 2B GOF mutation involved herein made A1 partially open. This is consistent with reports that a partial unfolding conformation of A1 has high affinity to GPIbα (16, 29, 40), because an open conformation of A1 may be in favor of unfolding, which may first occur at such weak regions as the β3α2-loop and the α2-helix (52, 53) (Figs. 7 and 8). The similar effects of the GOF mutations on the structure and dynamics of the A1 domain in the present results are also in good agreement with the experimental results such that a combination of two type 2B mutations in a single construct had no additive effect on the affinity of binding to platelets (41), possibly coming from the flexible motifs, such as the N-terminal arm, the α1-helix, the β3α2-loop, the β2-strand, and the α2-helix (Fig. 4A), which govern the conformational transition of A1, may be inherently sensitive to mutational events (54).
It was demonstrated that the allosteric communications can be transmitted solely by a variation in protein motions, which do not cause a macroscopic structural change at the backbone level (55, 56). This argument suggests that the mutation-induced change of dynamic property of A1 transmits the allosteric signals to the active site first and then makes A1 favoring binding to GPIbα. In signaling, the elaborate salt bridge network wrapping around the lower rim of A1 (supplemental Fig. S8A) should serve as the intramolecular allosteric signal pathway(s), because the type 2B mutation sites locate at or near the rim (5). In this region sensitive to mutagenesis, the motif that illustrated the largest mutation-induced deviation was observed to be around the hydrophobic cluster, which is comprised of the N-terminal arm, α1-helix, α1β2-loop, β2-strand, and α2-helix (Fig. 4A). Of the 16 type 2B mutation sites, 13 locate at and others are close to this cluster (supplemental Fig. S8B), which almost completely overlaps with the salt bridge network, showing that the hydrophobic interaction also participates in the allosteric signal transmission. Also, water molecules may be deeply involved in the allosteric signaling too, because water functions as a lubricant to ease rearrangements of necessary hydrogen bonds during conformational change (57). As the hydrophobic junction between the α1- and α2-helices is partly unzipped through the GOF mutations, a water channel toward the buried hydrophobic core forms (Fig. 5), and hydration ensues (Fig. 6). As a result, the local mobility of A1 increases (Fig. 4B) (58). The phenomenon of water molecules participating in the intramolecular interaction of A1 was observed with the x-ray crystallography (23). Therefore, water molecules should act as an effective medium for transmitting the allosteric signal at the mutation sites to the action site.

Unlike a gain of function through the type 2B mutation-induced increase of A1 flexibility, the fluid shear stress triggers the wild-type vWF activation in physiological conditions. Shear stress could greatly increase the oxidation of residues Met540 and Met541 at the α1-helix that are deeply buried by the adjacent α2-helix in the static structures (59). It suggests that shear stress may enhance A1 affinity through a similar mechanism as the GOF mutations, because A1 itself exposes the buried methionines through its conformational change under shear stress. Mechanically, in flows, stretching breaks the firm linker (the salt bridge Arg578–Glu501) between the N-terminal loop and the α2-helix first and then gradually unzips the hydrophobic junction of the α1- and α2-helices until the liganded A1 is partly unfolded. In this process, the liganded A1 may be more and more in favor of high affinity to GPIbα, suggesting another explanation for catch bond mechanism (15, 16). This is because a looser conjunction between the α1- and α2-helices in a GOF mutant up-regulates the affinity of A1 to GPIbα, as mentioned above, and the A1 structure at partly unfolding state takes a high affinity conformation in the urea unfolding process, as previously reported (16, 29, 40). Nevertheless, association of A1 with GPIbα should favor partly rather than overly unfolding of A1 (29). Thus, there should be a shear stress threshold, across which increasing shear stress will make the liganded A1 far away from its partial unfolding conformation ensemble in favor of association with its partner. Also, detachment of the N-terminal loop from the α2-helix requires stretching to be not too weak, meaning there is another shear stress threshold below which the phenomenon of the force-enhanced affinity does not occur. It follows that the dissociation of a liganded A1 from GPIbα may be tri- rather than bi-phase force-dependent, similar to the dissociation of E-selectin from its ligand (60, 61).

In the previous MD studies for the GOF phenomenon, one argued that conformational changes in the binding site of A1 are important for enhancing affinity. The present results showed that the mutation-induced GOF is relevant to the local mobility of A1. These two different arguments are complementary. The type 2B mutation R543Q was demonstrated to result in formation of an intramolecular salt bridge between Arg577 and the β3α2-loop of A1 and Glu14 at the β-finger of GPIbα (15). This could be demonstrated by the fact that the increased freedom of the α2-helix and the β3α2-loop in unliganded A1 caused by a GOF mutation would provide more chance for both Arg571 and Glu14 to meet and couple with each other. Through MD simulations for wild-type and mutant A1/GPIbα complexes, the bound A1 was observed to rotate away from the C terminus of GPIbα in the high force unbinding pathway but close to the GPIbα C terminus in thermal unbinding (62). In this process, the local mobility of A1 may be enhanced. This local mobility should be related to the reconstruction of the contact area between the two proteins under tensile force. The lower occupancies of the salt bridges in the mutant A1/GPIbα complex (62) might be related deeply to the mutation-enhanced fluctuations of the flexible α2-helix and β3α2-loop.

Indeed, the present work is limited not only by the small number of runs within the simulation time of 100 ns for each system, but also by the lack of experimental data and the reconstructed models of the mutants R543Q and I546V. However, the GOF phenotype of mutation E501A in an A1 variant had been obtained through the clustered charged to alanine scanning mutagenesis (32); the preservation of the key contacts between the N-terminal arm and the rest of A1 in the reconstructed models of R543Q and I546V (supplemental Fig. S2 and Table S1) should rationalize the present results; so did the negative test with the reconstructed model of the WT A1 (Table I and supplemental Figs. S9 and S10). Also, the flexibility in the flexible regions increases with each of the eight examined mutations (Table 1). Therefore, the present interpretation for the gain of function via the type 2B mutation-induced change of dynamic property of A1 should be persuasive, even without direct structural verification. This interpretation could be further tested through mutagenesis experiments such as introducing a disulfide bond between the N-terminal arm and the body of A1 for a GOF mutant or truncating the N-terminal loop of the WT A1. The present results provided a novel insight into the structural basis on the GOF variants of the native vWF A1 domain. The dynamics-driven regulation of function may serve as a mechanism not only for A1-GPIbα interaction but also for other receptor-ligand interactions.

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