

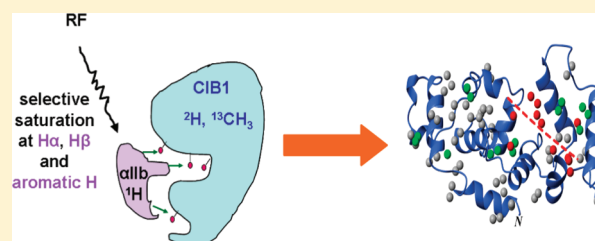
# Structural Basis for the Activation of Platelet Integrin $\alpha$ IIb $\beta$ 3 by Calcium- and Integrin-Binding Protein 1

Hao Huang<sup>†</sup> and Hans J. Vogel<sup>\*</sup>

Biochemistry Research Group, Department of Biological Sciences, University of Calgary, Calgary (AB), Canada, T2N 1N4

**S** Supporting Information

**ABSTRACT:** Calcium and integrin binding protein 1 (CIB1) is a specific binding partner for the cytoplasmic domain of the  $\alpha$ IIb subunit of the highly abundant platelet integrin  $\alpha$ IIb $\beta$ 3. This protein has been suggested to be involved in the regulation of the activation of  $\alpha$ IIb $\beta$ 3, a process leading to platelet aggregation and blood coagulation. In this work, the solution structure of the deuterated  $\text{Ca}^{2+}$ -CIB1 protein complexed with an  $\alpha$ IIb peptide was first determined through modern RDC-based NMR methods. Next, we generated a complex structure for CIB1 and the  $\alpha$ IIb domain ( $\text{Ca}^{2+}$ -CIB1/ $\alpha$ IIb) using the program Haddock, which is based on experimental restraints obtained for the protein–peptide interface from cross-saturation NMR experiments. In this data-driven complex structure, the N-terminal  $\alpha$ -helix of the cytoplasmic domain of  $\alpha$ IIb is buried in the hydrophobic pocket of the C-lobe of  $\text{Ca}^{2+}$ -CIB1. The C-terminal acidic tail of  $\alpha$ IIb remains unstructured and likely interacts with several positively charged residues in the N-lobe of  $\text{Ca}^{2+}$ -CIB1. A potential molecular mechanism for the CIB1-mediated activation of the platelet integrin could be proposed on the basis of the model structure of this protein complex. Another feature of this work is that, in the NMR cross-saturation experiments, we applied the selective radio frequency irradiation to the smaller binding partner (the  $\alpha$ IIb peptide), and successfully detected the binding interface on the larger binding partner  $\text{Ca}^{2+}$ -CIB1 through its selectively protonated methyl groups. This ‘reverse’ methodology has a broad potential to be employed to many other complexes where synthetic peptides and a suitably isotope-labeled medium- to large-sized protein are used to study protein–protein interactions.



## INTRODUCTION

Integrins are cell surface receptors that mediate cell adhesion processes, including cell–cell, cell–extracellular matrix, and cell–pathogen interactions.<sup>1,2</sup> In general, integrins are heterodimeric glycoproteins that are formed by a single  $\alpha$  and a single  $\beta$  subunit. Both the  $\alpha$  and  $\beta$  subunits are single-pass transmembrane proteins, and such proteins are often involved in signal transduction processes. Integrins are known to be involved in bidirectional signal transduction across the plasma membrane, commonly referred to as “inside-out” and “outside-in” signaling.<sup>1–3</sup> Both the  $\alpha$  and  $\beta$  subunits are made up of a large extracellular ectomembrane domain, a single transmembrane helix, and a small cytoplasmic domain.<sup>1,2</sup> It has been reported that the dissociation of the cytoplasmic domains of the  $\alpha$  and  $\beta$  subunits is critical for bidirectional transmembrane signaling events.<sup>4</sup>

The  $\alpha$ IIb $\beta$ 3 integrin heterodimer is exclusively expressed at very high levels in platelets. Because of its role in the formation of blood clots and maintaining hemostasis, the structure of this complex is of particular interest. These two subunits associate in the membrane and in the cytoplasm with a low affinity.<sup>2</sup> The interaction between these two parts of the dimeric integrin protein has been hard to detect in aqueous solution<sup>5</sup> or studies resulted in different structures, especially for the cytoplasmic domains of  $\alpha$ IIb $\beta$ 3,<sup>6–8</sup> probably reflecting the dynamic nature of the cytoplasmic domain of  $\alpha$ IIb $\beta$ 3. In the inside-out signaling

pathway, the cytoskeletal protein talin<sup>9</sup> has been reported to be able to bind specifically to the cytoplasmic domain of  $\beta$ 3 and in turn activate integrin  $\alpha$ IIb $\beta$ 3.

The calcium- and integrin-binding protein 1 (CIB1) was originally discovered as a binding partner for the cytoplasmic domain of  $\alpha$ IIb in a yeast two-hybrid screening study.<sup>10</sup> CIB1 is now known as a ubiquitous regulatory protein that is made up of four EF-hands<sup>11</sup> with a molecular weight of 22 kDa. CIB1 can carry a myristoyl group at its N-terminus, but the function of this myristoyl group in vivo is not yet fully understood since it has been found that CIB1 can interact with  $\alpha$ IIb in vitro without the myristoyl group.<sup>12–14</sup> The myristoyl group of CIB1 appears to act like a membrane anchor, similar to what has been found for many related calcium-binding proteins.<sup>15</sup> CIB1 has four helix–loop–helix EF-hand motifs, but only EF-III and EF-IV have the capacity to bind divalent metal ions.<sup>16</sup> In the resting state of most cells, magnesium is present in the cytoplasm at  $\sim$ 1–2 mM allowing the folded form of  $\text{Mg}^{2+}$ -CIB1 to be present.<sup>17</sup> When the cell is excited,  $\text{Ca}^{2+}$  enters the cytoplasm and its concentration rises to micromolar levels;  $\text{Ca}^{2+}$  displaces  $\text{Mg}^{2+}$  and forms  $\text{Ca}^{2+}$ -CIB1 in a well-folded conformation.<sup>16</sup> Interestingly, both  $\text{Ca}^{2+}$ -CIB1 and  $\text{Mg}^{2+}$ -CIB1 can interact with a synthetic peptide representing the  $\alpha$ IIb cytoplasmic domain

Received: November 28, 2011

Published: January 27, 2012

and part of its transmembrane domain, with similar micromolar affinities.<sup>12</sup> Solution NMR studies of CIB1 require deuteration and methyl group labeling<sup>17,18</sup> because of its relatively large molecular weight (24 kDa including the purification-tag). It has been shown that Ca<sup>2+</sup>-CIB1 and Mg<sup>2+</sup>-CIB1 have a similar overall topology with Mg<sup>2+</sup>-CIB1 having a relatively more exposed hydrophobic pocket.<sup>17</sup> NMR chemical shift perturbation studies concerning the interaction of  $\alpha$ IIB with both Ca<sup>2+</sup>- and Mg<sup>2+</sup>-bound CIB1 suggest that both the Ca<sup>2+</sup>- and Mg<sup>2+</sup>-forms interact with  $\alpha$ IIB via a hydrophobic pocket in the C-terminal domain of CIB1.<sup>17</sup> It has been reported previously that CIB1 is capable of disrupting the association of  $\alpha$ IIB and  $\beta$ 3 by binding to  $\alpha$ IIB and in turn activate the integrin  $\alpha$ IIB $\beta$ 3.<sup>14</sup> Furthermore, blocking complex formation between the Wiskott-Aldrich syndrome protein (WASP) and CIB1 has been shown to affect the conformational change of  $\alpha$ IIB $\beta$ 3 and further affects its interaction with fibrinogen.<sup>19</sup> In contrast, CIB1 has also been suggested to negatively regulate the activation of integrin  $\alpha$ IIB $\beta$ 3 by competing with talin for binding to  $\alpha$ IIB $\beta$ 3.<sup>20</sup> Therefore, structural characterization of the CIB1/ $\alpha$ IIB complex is of considerable interest to provide further insight into the mechanism of the regulation of the activation of  $\alpha$ IIB $\beta$ 3 by CIB1 or to determine the role of CIB1 in collaborating with talin in the regulation of  $\alpha$ IIB $\beta$ 3 activation as suggested by Yuan et al.<sup>20</sup> Even though the CIB1/ $\alpha$ IIB interaction has been suggested to be similar to that of the homologous protein calcineurin B (CnB) with calcineurin A (CnA) in terms of the relative orientation of the proteins,<sup>13</sup> no direct experimental results have been reported to date to support this notion. By determining a structure for the Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB complex, we hope to obtain insight into the inside-out signaling of integrin as well as to establish the connections between the Ca<sup>2+</sup>-signaling pathway and the integrin-signaling pathways.

In this work, we report a structure for the Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB complex. An experimental approach of employing reverse cross-saturation NMR experiments using the labeled methyl groups of Ile/Leu/Val (I/L/V) in an otherwise deuterated CIB1 protein has been implemented to accurately identify the interface for the Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB complex. Moreover, residual dipolar couplings (RDCs) as determined in multiple alignment media were used to provide additional information about the structure of Ca<sup>2+</sup>-CIB1 in complex with  $\alpha$ IIB.

## MATERIALS AND METHODS

**Sample Preparation.** The CIB1 protein containing different stable isotope labels was prepared as previously described.<sup>17,21</sup> The [U-<sup>2</sup>H,<sup>15</sup>N,<sup>13</sup>C]-labeled sample was used to acquire backbone <sup>1</sup>D<sub>CN</sub> and <sup>1</sup>D<sub>NH</sub> residual dipolar couplings (RDCs). The {[U-<sup>15</sup>N, <sup>2</sup>H, <sup>12</sup>C]; Ile $\delta$ 1-[<sup>13</sup>CH<sub>3</sub>]; Leu,Val-[<sup>13</sup>CH<sub>3</sub>,<sup>12</sup>CD<sub>3</sub>]}-labeled sample was used for the methyl cross-saturation experiments to determine the interface for the interaction between CIB1 and the human platelet  $\alpha$ IIB subunit. The 26-residue  $\alpha$ IIB synthetic peptide (Ac-L<sub>983</sub>VLAMWVKVGFKRNRPPLLEEDDEEGQ<sub>1008</sub>-OH) as previously described<sup>18</sup> was used; this peptide corresponds to amino acids 983–1008 of the  $\alpha$ IIB cytoplasmic domain and part of the transmembrane domain (hereafter referred to as  $\alpha$ IIB-L, Figure 1). Another truncated 15-residue synthetic peptide (Ac-L<sub>983</sub>VLAMWVKVGFKRN<sub>997</sub>-NH<sub>2</sub>) ( $\alpha$ IIB-S, Figure 1) was also studied, which corresponds to amino acids 983–997 of the  $\alpha$ IIB subunit. These synthetic peptides were both purchased from GenScript Corp. and they were more than 95% pure as determined by mass spectrometry and HPLC. Protein and peptide concentrations were determined by using the extinction coefficients:  $\epsilon_{280}$  = 3040 for CIB1,  $\epsilon_{280}$  = 5500 for  $\alpha$ IIB-L, and  $\epsilon_{280}$  = 5500 for  $\alpha$ IIB-S.



**Figure 1.** The sequences of the  $\alpha$ IIB peptides used in this work.  $\alpha$ IIB-L represents the human integrin  $\alpha$ IIB 983–1008 region, in which the L983, W988, F992, and F993 have been previously identified to be critical for the interaction between Ca<sup>2+</sup>-CIB1 and  $\alpha$ IIB.<sup>13</sup>  $\alpha$ IIB-L contains part of the transmembrane domain (shaded in gray box) and the full cytoplasmic domain of integrin  $\alpha$ IIB.  $\alpha$ IIB-S represents  $\alpha$ IIB residues 983–997. The R995 (red) has been reported to form a salt bridge with the integrin  $\beta$ 3 subunit, which is critical for the association of the cytoplasmic domain of  $\alpha$ IIB and  $\beta$ 3.<sup>7,58,59</sup>

**NMR Experiments.** NMR spectra were recorded at 37 °C on a Bruker AVANCE 500 MHz or a Bruker AVANCE 700 MHz NMR spectrometer each equipped with a triple resonance inverse cryoprobe with a single axis z-gradient. Each Ca<sup>2+</sup>-CIB1 sample, with or without  $\alpha$ IIB peptides, contained 0.5–0.7 mM CIB1 in 50 mM HEPES, 100 mM KCl, 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS), 10 mM DTT, 10% or 99.9% D<sub>2</sub>O, pH 7.5 ± 0.05; 2 mM CaCl<sub>2</sub> was added for the Ca<sup>2+</sup>-bound sample and 3 mM MgCl<sub>2</sub> for Mg<sup>2+</sup>-bound sample. The [U-<sup>2</sup>H,<sup>15</sup>N,<sup>13</sup>C]-labeled Ca<sup>2+</sup>-CIB1 sample complexed with  $\alpha$ IIB-L was used to acquire the backbone RDCs (<sup>1</sup>D<sub>CN</sub> and <sup>1</sup>D<sub>NH</sub>) in different alignment media. One of the alignment media used was 14 mg/mL pf1 phage (Asla Lab) for partial alignment of protein molecules.<sup>22</sup> A second medium used was the polyethylene glycol ether (PEG) medium containing 5% C12E5/hexanol in 0.96/1 ratio.<sup>23</sup> The <sup>1</sup>D<sub>CN</sub> RDC were measured using the 3D IPAP-J-HNCO (CA) experiment,<sup>24</sup> with 1024 × 128 × 40 complex points. The digital resolution was 2.06 Hz/pt in F2 (<sup>13</sup>C). A scale factor of 4 was used in the measurement of the <sup>1</sup>D<sub>CN</sub> RDCs. The <sup>1</sup>D<sub>NH</sub> RDCs were measured using the 2D IPAP-HSQC experiment,<sup>25</sup> with 1024 × 512 complex points, a digital resolution of 1.1 Hz was obtained after linear prediction and zero filling.

**Reverse Methyl Cross-Saturation.** Reverse methyl cross-saturation experiments were performed using 500  $\mu$ M samples of {[U-<sup>15</sup>N, <sup>2</sup>H, <sup>12</sup>C]; Ile $\delta$ 1-[<sup>13</sup>CH<sub>3</sub>]; Leu,Val-[<sup>13</sup>CH<sub>3</sub>,<sup>12</sup>CD<sub>3</sub>]}-labeled CIB1 in 100 mM KCl, 99.9% D<sub>2</sub>O, 10 mM DTT, 50 mM d<sub>18</sub>-HEPES (98% pure) with and without 600  $\mu$ M  $\alpha$ IIB-L at pD 7.5 (not corrected for isotope effects). Four scans were used, resulting in a total measurement time of just 40 min. The methyl-based cross-saturation irradiation using an RF (radio frequency) field was applied as suggested,<sup>26</sup> which covers from 3.5 to 8.5 ppm with the irradiation centered at 6.0 ppm, a region including the residual water protons, amide protons, aromatic protons, most of the  $\alpha$ -protons, and some of the other aliphatic ( $\beta$  and  $\gamma$ ) protons of the  $\alpha$ IIB-L peptide. Selective irradiation was done by using the adiabatic WURST-2 band with a strength up to 0.17 kHz. The saturation time (T<sub>sat</sub>) was set at 1.5 s and the interscan delay was 2 s. On the basis of the spectra with (T<sub>sat</sub> = 1.5 s) and without irradiation (T<sub>sat</sub> = 0 s), the signal loss was calculated using the intensity (I) ratio for each methyl group: Signal Loss = 1 - [I<sub>(T<sub>sat</sub>=1.5)</sub>/I<sub>(T<sub>sat</sub>=0)</sub>]. Error bars were generated from duplicates of the cross-saturation experiments.

**Structure Calculation.** A two-stage simulated annealing approach<sup>27</sup> based on three sets of <sup>1</sup>D<sub>NH</sub>, <sup>1</sup>D<sub>CN</sub> RDCs measured in two alignment media (phage pf1 and organic solvent PEG) was implemented for the structure determination of Ca<sup>2+</sup>-CIB1 in complex with  $\alpha$ IIB-L using the program XPLOR-NIH 2.18. This calculation protocol is designed for studies of homologous proteins or the same protein under different conditions.<sup>27</sup> Briefly, for stage 1, the temperature was cooled from 200 to 20 K, and a strong unramped force constant at 300 kcal mol<sup>-1</sup> rad<sup>-2</sup> for the dihedral angle (derived from the chemical shifts of Ca<sup>2+</sup>-CIB1 in complex with  $\alpha$ IIB-L using the program Talos,<sup>28</sup> after correction for the deuterium isotope effect<sup>29</sup>) was used to ensure proper secondary structure of the resulting structure. Also in stage 1, the force constant of dipolar couplings was ramped from 0.05 to 5 kcal mol<sup>-1</sup> Hz<sup>2</sup>. The obtained structure still has a certain amount of RDC energy; in stage 2, the temperature was cooled from 20 to 2 K and the lowest RDC energy

structure obtained from stage 1 was used as a starting model. In stage 2, the force constant for the dihedral angle (derived from the lowest energy structure from stage 1) was ramped down from 300 to 50 kcal mol<sup>-1</sup> rad<sup>-2</sup> while the force constant for RDC was kept static at 1 kcal mol<sup>-1</sup> Hz<sup>-2</sup>. Other force constants used in the calculation using Xplor-NIH were the same as previously described.<sup>17,30</sup> The same approach has been used successfully to determine the structures of various other proteins.<sup>30–35</sup> The structure was deposited to the Protein Data Bank with accession code 2LMS. NMR data were deposited to the BMRB with the code 18099.

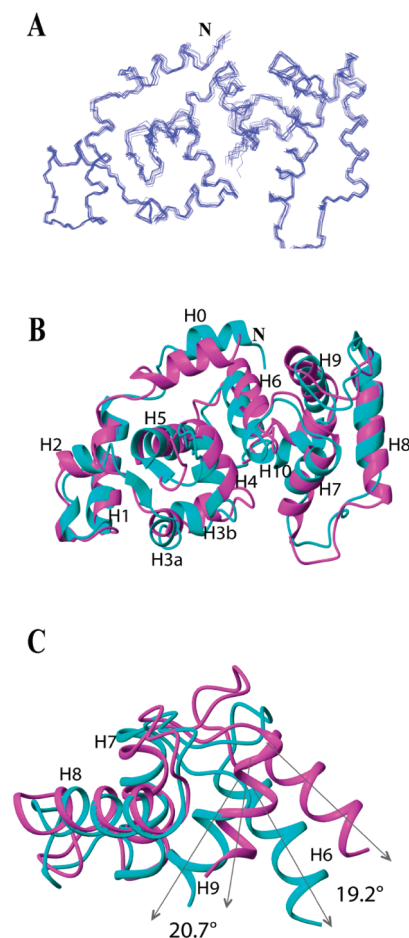
The structure of  $\alpha$ IIB-L was generated based on a recent NMR structure for the transmembrane and cytoplasmic domains of  $\alpha$ IIB/ $\beta$ 3 (PDB 2KNC).<sup>7</sup> In this study, the  $\alpha$ IIB subunit adopts an  $\alpha$ -helical structure from I966 until N996 with an extended C-terminal tail at R997–E1008. We used the homology modeling program Swiss-Model web-server<sup>36</sup> to generate the starting structure for  $\alpha$ IIB-L, which has a Q1008 residue<sup>13,18</sup> instead of E1008 at the C-terminal end. The structure of  $\alpha$ IIB-L (L983–Q1008) obtained from homology modeling is essentially the same as the published  $\alpha$ IIB structure in the  $\alpha$ IIB/ $\beta$ 3 complex.<sup>7</sup> The structure of  $\alpha$ IIB-L generated in this manner was used in the subsequent docking model studies of the Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB-L complex. In doing so, we assume that the tail of  $\alpha$ IIB does not undergo a large conformational change when it binds to Ca<sup>2+</sup>-CIB1.

**Docking Model of the Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB-L Complex.** Using the refined solution structure of Ca<sup>2+</sup>-CIB1 in the complex and the structure of  $\alpha$ IIB-L, a docking model for the Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB-L complex was generated using the Haddock web-server.<sup>37,38</sup> It is known that the C-terminal extension of Ca<sup>2+</sup>-CIB1 experiences on/off slow motions from the hydrophobic pocket in the C-lobe of Ca<sup>2+</sup>-CIB1 in solution, thereby blocking nonspecific interactions.<sup>17,18</sup> Hence, in our calculations, the structure of the complexed Ca<sup>2+</sup>-CIB1 protein was truncated in order to avoid blocking the access of  $\alpha$ IIB-L to the hydrophobic pocket of Ca<sup>2+</sup>-CIB1; the truncated version of Ca<sup>2+</sup>-CIB1 contains residues 8–178 with the C-terminal tail of the protein (residues 179–191) removed. In the setup, positive methyl cross-saturation results were implemented as interfacial restraints for docking. The significantly affected methyl groups of residues I73, I114, L131, L135, I153, I168, V176, and I177 were set as the active residues, while the slightly affected methyl-containing residues I27, L28, L61, V76, V97, V132, L152, and I156 were set as passive residues in the Haddock calculations. Residues L983, W988, F992, and F993 in  $\alpha$ IIB-L have been reported to be critical for the interaction between Ca<sup>2+</sup>-CIB1 and  $\alpha$ IIB-L by a combined approach of site-directed mutagenesis and in vitro fluorescence studies.<sup>13</sup> Hence, these four residues were set as the active residues for  $\alpha$ IIB-L. Also in the setup, since  $\alpha$ IIB-L residues L983–R995 have been determined to be helical and the remainder (N996–Q1008) to be an extended structure,<sup>7,39</sup> fragment 995–1008 of  $\alpha$ IIB-L was set as fully flexible for the docking model generation. Because Haddock can automatically detect semiflexible fragments,<sup>38</sup> CIB1 was not set as fully flexible. RDC restraints that were used for the structure calculation for the Ca<sup>2+</sup>-CIB1 in complex with  $\alpha$ IIB-L were also implemented in the docking model generation. In addition, dihedral angle restraints for CIB1 (8–178) and  $\alpha$ IIB-L (983–995) were created based on the lowest RDC energy structure determined for the Ca<sup>2+</sup>-CIB1 in protein complex with  $\alpha$ IIB-L as well as the available  $\alpha$ IIB structure (PDB 2KNC) in complex with  $\beta$ 3.<sup>7</sup> Other parameters were standard default values except that 3000 structures were calculated for the stage of rigid body docking, 400 structures for the subsequent structural analysis stage, and 20 structures were set as the minimal number for clustering.<sup>37,38</sup> The lowest interaction energy docking structure cluster was taken as the complex model for Ca<sup>2+</sup>-CIB1/ $\alpha$ IIB-L.

**Further Characterization of Calculated Structures.** The lowest RDC energy structures of Ca<sup>2+</sup>-CIB1 (residues 8–191) in complex with  $\alpha$ IIB-L were selected for further analysis. Procheck 3.5.4<sup>40</sup> was used to examine the structures determined for stereochemical quality. An in-house script was used to measure the interhelical angles for the helix pairs in each EF-hand.

## RESULTS

**The Solution Structure of Ca<sup>2+</sup>-CIB1 in Complex with  $\alpha$ IIB-L Reveals an Enlarged Hydrophobic Pocket Induced by  $\alpha$ IIB-L Binding.** The solution structure (Figure 2) of the Ca<sup>2+</sup>-CIB1 protein in complex with  $\alpha$ IIB-L was refined



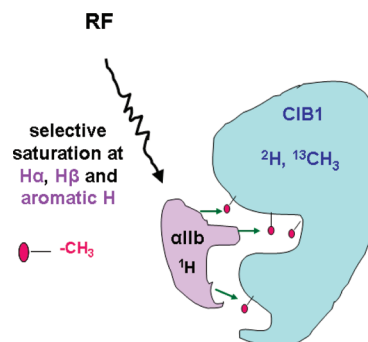
**Figure 2.** The structure of Ca<sup>2+</sup>-CIB1 in complex with  $\alpha$ IIB-L. (A) Superposition of the best 10 structures of the Ca<sup>2+</sup>-CIB1 protein in complex with  $\alpha$ IIB-L; (B) superposition of the solution structures of Ca<sup>2+</sup>-CIB1 alone (PDB 2L4H, cyan) and in complex with  $\alpha$ IIB-L (pink) (backbone rmsd = 3.7 Å). (C) The superimposed structures of the C-lobe of Ca<sup>2+</sup>-CIB1 alone (cyan) and in complex with  $\alpha$ IIB-L (pink) demonstrate the increased opening of helix pairs H6/H7 and H8/H9. The two structures were superimposed based on H7 and H8; thus, the opening is highlighted by the altered orientations of H6 and H9.

based on three sets of backbone RDCs obtained in two alignment media (NH and C'N RDCs in pf1, and NH RDCs in PEG) using the solution structure of Ca<sup>2+</sup>-CIB1 (PDB 2L4H) as the starting model and a two-stage low-temperature simulated annealing protocol.<sup>27</sup> The use of multiple sets of alignment media has been previously suggested because it can avoid the intrinsic degeneracy problem of using RDCs as well as avoid any preference for a minor conformation that could result from the use of a single alignment medium.<sup>41,42</sup> Some residues on the flexible loop of CIB1 (residues 137–145) and the C-terminal end (180–191) have reduced RDC values due to local mobility,<sup>43,44</sup> and these residues were excluded from the structure calculation.<sup>45</sup> A good structural precision has been obtained for the structure calculation; the backbone rmsd of the



10 lowest energy structures is 0.46 Å (Figure 2A). The statistics of the structure determination of the  $\text{Ca}^{2+}$ -CIB1 protein when complexed with  $\alpha\text{Ib-L}$  have been summarized in Supporting Information Table S1. The solution structure of the  $\text{Ca}^{2+}$ -CIB1 protein in the complex also shows a similar overall topology compared with the solution structure of  $\text{Ca}^{2+}$ -CIB1 alone, with a backbone rmsd of 3.7 Å (Figure 2B). Compared with the  $\text{Ca}^{2+}$ -CIB1 structure, the N-terminal  $\alpha$ -helix (Helix 0, H0) in the new structure has shifted to some extent. The N-terminal  $\alpha$ -helix is assumed to carry a myristoyl group *in vivo* which should associate with the cell membrane. Therefore, binding of  $\alpha\text{Ib-L}$  could affect the orientation of CIB1 relative to the cell membrane. Moreover, the interhelical angles measured using an in-house script suggest that the opening of the helix pairs in each EF hand changes upon binding  $\alpha\text{Ib-L}$  (Table S2). The two EF-hands in the C-lobe have a bigger interhelical opening than free  $\text{Ca}^{2+}$ -CIB1 by approximately 20° (Figure 2C). The resultant enlarged hydrophobic pocket in the C-lobe could readily adopt  $\alpha\text{Ib-L}$ . The Q factors<sup>46</sup> (Table S3 and Figure S1) before and after structural refinement denote a significant improvement in the results of fitting RDCs to the obtained structure of  $\text{Ca}^{2+}$ -CIB1 complexed with  $\alpha\text{Ib-L}$  compared with the starting model, that is, the free  $\text{Ca}^{2+}$ -CIB1 structure (PDB 2L4H).

**Reverse Cross-Saturation Detects the Interface of the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{Ib-L}$  Complex.** Cross-saturation NMR experiments employing backbone amide proton NH resonances were originally developed to detect the interface for protein–protein interactions.<sup>47</sup> Compared with the commonly used NMR Chemical Shift Perturbation (CSP) approach, which is widely used for determining the interface of protein/peptide complexes, the cross-saturation method avoids possible artifacts arising from induced conformational changes, where residues that experienced a large chemical shift change can in fact be far away from the actual interface.<sup>48</sup> A more recent version of the cross-saturation experiment uses isotope labeled methyl groups rather than backbone amides.<sup>26</sup> It has several advantages over the traditional cross-saturation backbone  $^{15}\text{N}$ -H method,<sup>47</sup> such as high sensitivity, higher efficiency of magnetization transfer, and suitability for studying hydrophobic interactions that are often involved in protein/protein complex formation.<sup>26</sup> Traditionally, the cross-saturation approach has been used to detect the binding interface of the smaller partner by irradiating the larger partner because the magnetization will be more efficiently transferred by spin diffusion<sup>49</sup> in a larger protein. In our work, we applied the selective irradiation in a reverse manner (referred to as reverse cross-saturation), in a way that is somewhat similar to previous studies.<sup>50,51</sup> We saturated the smaller binding partner ( $\alpha\text{Ib-L}$ , 3 kDa) to detect the interface on the larger binding partner ( $\text{Ca}^{2+}$ -CIB1, 24 kDa including the purification His-tag) (Figure 3). In our methyl-probe cross-saturation experiments, selective irradiation was employed to cover the spectral region from 3.5 to 8.5 ppm (Figure S2). To avoid artifacts, 99.9%  $\text{D}_2\text{O}$  was used as the solvent.<sup>26</sup> In the spectral area from 3.5 to 8.5 ppm (Figure S2), methyl labeled but otherwise perdeuterated  $\text{Ca}^{2+}$ -CIB1 has no signals except for some peaks due to the  $d_{18}$ -HEPES buffer (98% pure) used to dissolve the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{Ib-L}$  complex, while  $\alpha\text{Ib-L}$  contains aromatic protons, aliphatic protons ( $\text{H}\alpha$ ,  $\text{H}\beta$ ,  $\text{H}\gamma$ , etc.) and no amide protons (NH) because of the presence of the  $\text{D}_2\text{O}$  solvent (Figure S2). Therefore, the irradiation selectively targets  $\alpha\text{Ib-L}$  but not the deuterated CIB1. This saturation should then be transferred to the protonated methyl

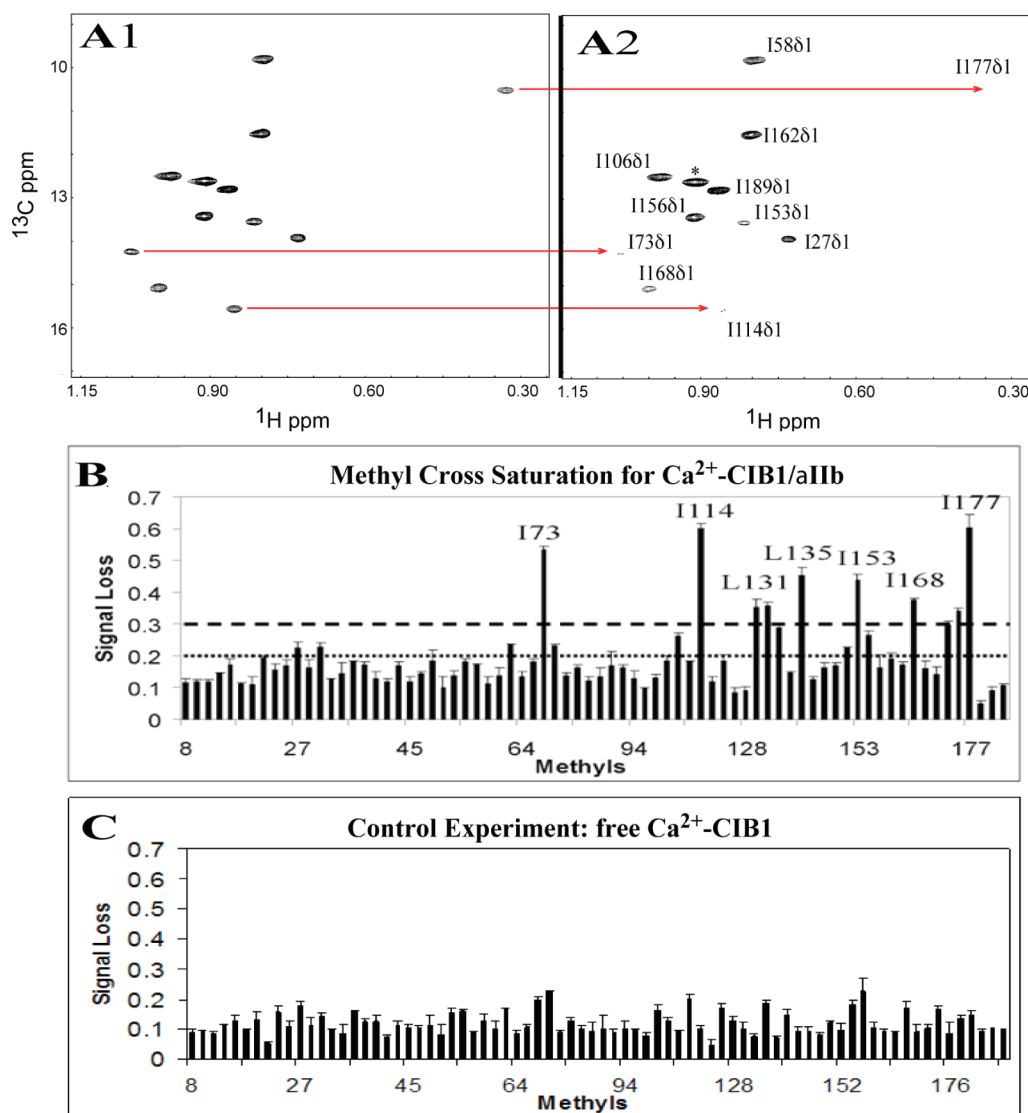


**Figure 3.** Schematic diagram illustrating the mechanism of the reverse methyl cross-saturation for the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{Ib-L}$  complex. Selective RF (Radio Frequency) irradiation was applied to the  $\text{H}\alpha$ ,  $\text{H}\beta$ , and aromatic proton spectral region of  $\alpha\text{Ib-L}$ , and the intensity change of the protonated methyl groups on deuterated  $\text{Ca}^{2+}$ -CIB1 was monitored.

groups involved in the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{Ib-L}$  interface by spin-diffusion (Figure 3). The interface of CIB1 in its complex with  $\alpha\text{Ib-L}$  can therefore be accurately mapped using cross-saturation experiments.<sup>48</sup> The results for these experiments are shown in Figure 4. In this case, control experiments were carried out on an NMR sample containing only  $\text{Ca}^{2+}$ -CIB1, and the results show that all the methyl groups were evenly affected to a minimum level (half of all methyls are below 0.1 and the other half are between 0.1 and 0.2 in terms of signal loss) (Figure 4C). Since the irradiation pulse was suggested to be adiabatic,<sup>26</sup> the signal loss in the control experiments is probably due to the presence of residual protons of the  $d_{18}$ -HEPES buffer (50 mM, 98% pure) (Figure S2; for further discussion see Shimada<sup>48,51</sup>).

To identify the interface between  $\text{Ca}^{2+}$ -CIB1 and  $\alpha\text{Ib-L}$ , an identical setup as the one used for the control experiments was used for a sample of  $\text{Ca}^{2+}$ -CIB1 complexed with 1/1.2 ratio of  $\alpha\text{Ib-L}$ . The results obtained are illustrated for the superimposed HSQC spectra of the Ile region (Figure 4A1). With irradiation, the I73, I114, and I177 residues show significant signal loss compared to the reference spectrum ( $T_{\text{sat}} = 0$  s) (Figure 4A2 and B), whereas other methyl protonated isoleucines (e.g., I58, I106, I162, and I189) are relatively less affected by the cross-saturation. The significantly affected methyl-containing Ile, Leu, and Val residues with a signal loss ratio above 0.3 include I73, I114, L131, L135, I153, I168, V176, and I177 (Figure 4B), and these were classified as the active residues in the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{Ib-L}$  interface that make direct contact with the binding partner  $\alpha\text{Ib-L}$ . Other slightly affected methyl-containing residues with signal loss ratio between 0.2 and 0.3, which include I27, L28, L61, V76, V97, V132, L152, and I156 (Figure 4B), have been classified as passive residues that are close to the interface, but lack direct contact with  $\alpha\text{Ib-L}$ . The remaining peaks are the nonaffected or marginally affected methyl groups with a signal loss ratio less than 0.2 (Figure 4B), and these are considered not involved in the hydrophobic interactions between the protein and the peptide.

The cross-saturation effects were mapped on the newly determined structure of  $\text{Ca}^{2+}$ -CIB1 in complex with  $\alpha\text{Ib-L}$  (Figure 5B). Compared with methyl CSP (chemical shift perturbation) effects (Figure 5A), cross-saturation experiments provide a similar interface for the interactions between  $\text{Ca}^{2+}$ -CIB1 and  $\alpha\text{Ib-L}$ , suggesting that the C-domain of  $\text{Ca}^{2+}$ -CIB1 is the primary binding site for the cytoplasmic domain of  $\alpha\text{Ib-L}$ .



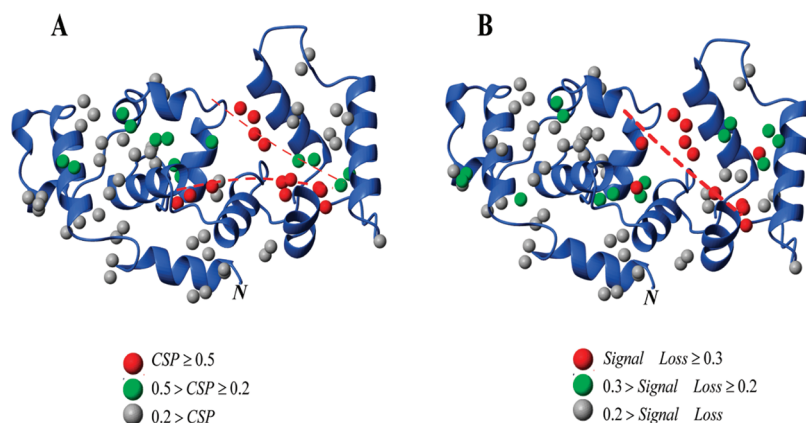
**Figure 4.** Reverse methyl cross-saturation experiment of the Ca<sup>2+</sup>-CIB1/αIIB-L complex. (A) HSQC spectra of Ca<sup>2+</sup>-CIB1/αIIB-L showing the effects of saturation on the Ile residues. The spectrum on the left is the <sup>1</sup>H, <sup>13</sup>C-HSQC with no saturation, and the spectrum on the right includes selective saturation. (B) Signal loss ratio of the intensities of the methyl groups in Ca<sup>2+</sup>-CIB1 complexed with αIIB-L, with and without the selective irradiation. The signal losses over 30% and 20% were highlighted with gridlines and these results were further utilized in the generation of docking complex structural model for Ca<sup>2+</sup>-CIB1 and αIIB-L using the program Haddock. (C) Control experiment of methyl cross-saturation with the free Ca<sup>2+</sup>-CIB1: only a minimum saturation effect was observed on nearly all methyl groups.

Small differences between CSP and cross-saturation were also observed; the distribution of active interfacial residues from the cross-saturation experiments (Figure 5B) seems to suggest one possible orientation for the αIIB-L peptide, whereas two possible orientations were suggested by CSP (Figure 5A). Clearly, a majority of the significantly affected methyl groups are found in the C-lobe of Ca<sup>2+</sup>-CIB1, except for residue I73, which is in the N-lobe.

The previously discussed C-terminal displacement mechanism for CIB1<sup>18</sup> allows us to interpret our methyl cross-saturation results. The C-terminal extension of CIB1 undergoes a conformational transition between free and bound states as suggested by our earlier backbone relaxation and relaxation dispersion measurements.<sup>17,18</sup> The three assigned methyl groups on the C-terminal extension of Ca<sup>2+</sup>-CIB1, that is, I189δ1, and V190γ1 and γ2, are only marginally affected by the saturation from αIIB-L, which suggests that these three residues are not in direct contact with αIIB-L in the complex. Thus, the

cross-saturation results are consistent with the backbone relaxation and relaxation dispersion results regarding the flexibility of the C-terminal extension; the extension remains flexible when the protein binds to αIIB-L.<sup>17,18</sup>

**αIIB-L Has an N-Terminal α-Helix and an Extended C-Terminal Tail.** Initial attempts of using isotope-filtered<sup>52</sup> or transferred NOESY experiments<sup>53</sup> to determine the structure of αIIB-L in complex with Ca<sup>2+</sup>-CIB1 failed to provide sufficient intermolecular NOEs between αIIB-L and Ca<sup>2+</sup>-CIB1. Even though NMR titration experiments (results not shown) suggest that the interaction between these two is in slow exchange regime, previous studies<sup>12</sup> suggested that αIIB interacts relatively weakly with Ca<sup>2+</sup>-CIB1 with a dissociation constant (*K<sub>d</sub>*) of 1.4 μM at 37 °C. This exchange behavior seems to have hindered the determination of the solution structure of αIIB-L in complex with Ca<sup>2+</sup>-CIB1. Isotope-labeled αIIB-L was then prepared through bacterial expression as a fusion protein but the solubility in aqueous solution became a problem, even

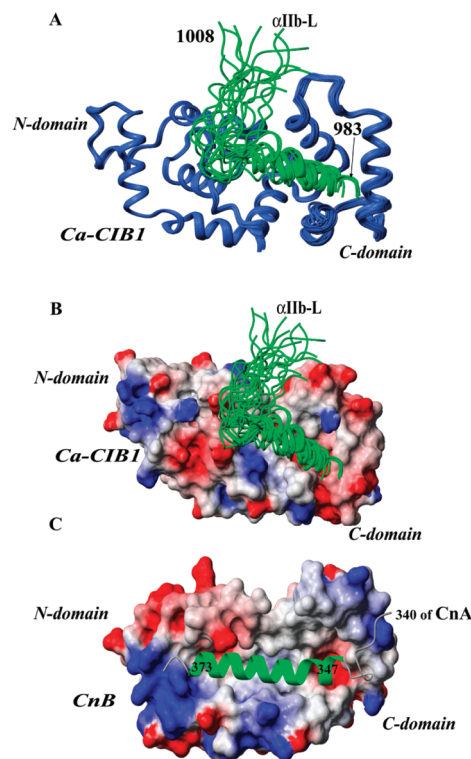


**Figure 5.** Mapping the reverse cross-saturation effect on the structure. (A) Mapping the methyl Chemical Shift Perturbation (CSP) on the structure of  $\text{Ca}^{2+}$ -CIB1 in complex with  $\alpha\text{IIb}$ ; the methyl CSP values were previously reported;<sup>17</sup> (B) mapping the signal loss values from reverse cross-saturation experiments on the structure of  $\text{Ca}^{2+}$ -CIB1 in complex with  $\alpha\text{IIb}$ . In both panels, the dotted lines (red) resemble possible orientations of  $\alpha\text{IIb-L}$  in its complex structure with  $\text{Ca}^{2+}$ -CIB1.

though the N-terminal acetylated synthetic  $\alpha\text{IIb-L}$  peptide was quite soluble. Fortunately, a structure for the  $\alpha\text{IIb}$  subunit (including the transmembrane and cytoplasmic domains) (PDB 2KNC), in complex with  $\beta 3$ , has recently been reported.<sup>7</sup> This structure of  $\alpha\text{IIb}$  (PDB 2KNC) covers the fragment of  $\alpha\text{IIb-L}$  used in our work (residues 983–1008). It is strikingly similar to a structure previously determined in a 45% trifluoroethanol aqueous solution for the synthetic  $\alpha\text{IIb-L}$  peptide,<sup>39</sup> containing a typical  $\alpha$ -helical structure (L983–N996) followed by an extended tail (R997–Q1008).

In addition, it should be noted that the C-terminal extension of CIB1 in the crystal structure of  $\text{Ca}^{2+}$ -CIB1 (1XO5) is folded back into the protein and adopts an  $\alpha$ -helical conformation (Figure S3). This implies that the hydrophobic pocket in the C-domain of  $\text{Ca}^{2+}$ -CIB1 can readily accommodate a binding partner in an  $\alpha$ -helical conformation. Therefore, we suggest that it is reasonable to assume that the fragment L983–R997 adopts an  $\alpha$ -helical conformation when interacting with  $\text{Ca}^{2+}$ -CIB1.

**The Structure of the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{IIb-L}$  Complex Reveals a Relative Orientation Similar to CnB/CnA.** The solution structure of the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{IIb-L}$  complex has been determined by data-driven docking. The 20 lowest energy structures of the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{IIb-L}$  complex are displayed in Figure 6 in both ribbon and surface charge forms. In Figure 6A, the N-terminal helical part of  $\alpha\text{IIb-L}$  interacts with the C-domain of  $\text{Ca}^{2+}$ -CIB1, while the negatively charged C-terminal tail of  $\alpha\text{IIb-L}$  remains unstructured (Figure 6A), possibly interacting with some positively charged residues, for example, R33 and K65 as was suggested by backbone CSP measurements.<sup>18</sup> In our docking experiment setup, we solely relied on the methyl cross-saturation results for  $\text{Ca}^{2+}$ -CIB1 and on previously reported critical residues in  $\alpha\text{IIb-L}$ ;<sup>13</sup> thus, these restraints do not reflect any potential electrostatic interactions. Therefore, the relative orientation of these two partners was mostly driven by the enthalpy of the hydrophobic interactions between the  $\alpha\text{IIb-L}$  helix and the C-domain of  $\text{Ca}^{2+}$ -CIB1. A related situation is found for the structure of the homologous protein calcineurin B (CnB) complexed with calcineurin A (CnA) (PDB 1TCO) (Figure 6C), in which the C-terminal domain of CnB interacts with the N-terminal of the CnB binding domain of CnA, with the CnB binding domain of CnA adopting an  $\alpha$ -helical conformation. Regarding the structure of the acidic tail (P<sub>998</sub>PLEEDDEEGQ<sub>1008</sub>) of  $\alpha\text{IIb}$ , we attempted

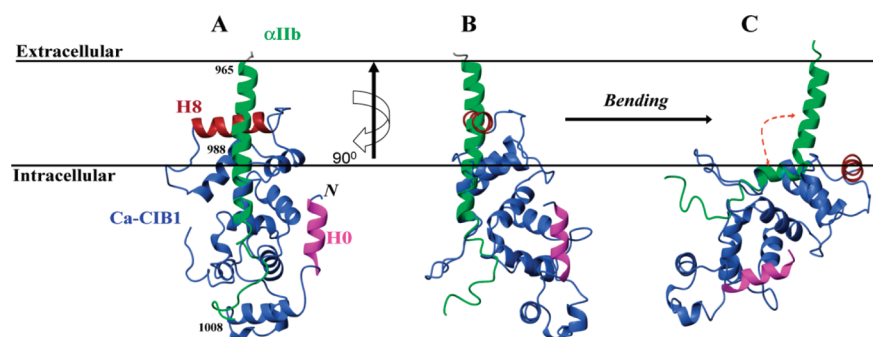


**Figure 6.** Structural model for the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{IIb-L}$  complex. (A) The superposition of the 20 best structures of the  $\text{Ca}^{2+}$ -CIB1/ $\alpha\text{IIb-L}$  complex generated using Haddock. (B) The  $\text{Ca}^{2+}$ -CIB1 (8–178) protein is represented with a surface structure, while  $\alpha\text{IIb-L}$  is represented as a ribbon structure. This figure was generated with the program MolMol.<sup>63</sup> The surface polarity is scaled with colors, using blue for the positively charged surface and red for the negatively charged surface, while a white color indicates nonpolar patches. (C) The surface charge model for the complex of CnB/CnA (PDB 1TCO), in which CnB is presented with a surface structure and CnA (residues 340–373) is presented as a ribbon structure.

to dock this portion better by adding R33 and K65 (residues identified by backbone CSP) of CIB1 as heavily affected residues but the tail still remained dynamic probably because no restraints were available for this portion of the  $\alpha\text{IIb}$  tail.

**The Model Is Supported by NMR Studies of the Interactions of  $\text{Ca}^{2+}$ -CIB1 with  $\alpha\text{IIb-L}$  and  $\alpha\text{IIb-S}$ .** A shorter





**Figure 7.** Possible conformational changes of  $\alpha$ IIb upon binding of  $\text{Ca}^{2+}$ -CIB1. (A) Superposition of the ordered structural fragment (983–995) of the averaged structure of  $\alpha$ IIb-L complexed with  $\text{Ca}^{2+}$ -CIB1 with the corresponding fragment of 2KNC. A steric clash was observed between 2KNC and the 8th helix of  $\text{Ca}^{2+}$ -CIB1 (H8, brown color); The N-terminal extension of  $\text{Ca}^{2+}$ -CIB1 (H0, pink color) points toward the cell membrane, which is consistent with its role of linking the myristoyl group and the  $\text{Ca}^{2+}$ -CIB1 to a membrane. (B) The side view of (A); (C) a possible bending mechanism was proposed for the conformational change of  $\alpha$ IIb upon binding of  $\text{Ca}^{2+}$ -CIB1, as indicated by the red dashed line with arrows. The letter N in italics indicates the N-terminal of  $\text{Ca}^{2+}$ -CIB1.

version of the  $\alpha$ IIb peptide ( $\alpha$ IIb-S, Ac-L<sub>983</sub>VLAMWVKVGFKRN<sub>997</sub>-NH<sub>2</sub>) was synthesized to examine the role of the acidic tail of  $\alpha$ IIb in its interaction with  $\text{Ca}^{2+}$ -CIB1. Specifically, the difference between the  $\alpha$ IIb-L and  $\alpha$ IIb-S peptides resides in the C-terminal fragment P<sub>998</sub>PLEEDDEEGQ<sub>1008</sub>, which bears multiple negative charges, making this region of  $\alpha$ IIb extremely acidic. In Figure S4A, the superimposed HSQC spectra of  $\text{Ca}^{2+}$ -CIB1/ $\alpha$ IIb-L and  $\text{Ca}^{2+}$ -CIB1/ $\alpha$ IIb-S show small differences with a few methyl groups experiencing minor chemical shift changes. This suggests that the C-terminal fragment P<sub>998</sub>PLEEDDEEGQ<sub>1008</sub> was not involved in specific interactions with  $\text{Ca}^{2+}$ -CIB1, which could affect the interactions between  $\text{Ca}^{2+}$ -CIB1 and  $\alpha$ IIb-L. The residues (L8, V45, V76, L92, L94, V97, I 106, and L170) with minor chemical shift changes are mainly located in the N-lobe of CIB1. On the other hand, these shifted methyl groups do not center at a specific location in the structure, implying a dynamic interaction between the N-lobe of CIB1 and the acidic tail (P<sub>998</sub>PLEEDDEEGQ<sub>1008</sub>). Perhaps this region of  $\alpha$ IIb can associate with other protein binding partners. A recent study<sup>54</sup> shows that this part of the  $\alpha$ IIb cytoplasmic domain can in fact play a role in platelet activation, but it only does so when it is anchored to the inner surface of the membrane.

**The Potential Roles of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  in the Regulation of CIB1/ $\alpha$ IIb Interaction.** Since  $\text{Mg}^{2+}$  ions are present in the cytoplasm with an almost invariant concentration of around 1–2 mM and the structure of  $\text{Mg}^{2+}$ -CIB1 is overall similar to that of  $\text{Ca}^{2+}$ -CIB1,<sup>16,17</sup> we were interested in the structural differences between the complexes of CIB1/ $\alpha$ IIb-L when bound to  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , in order to further understand the role of  $\text{Ca}^{2+}$  in the regulation of the CIB1/ $\alpha$ IIb interaction. Therefore, <sup>1</sup>H,<sup>13</sup>C-HSQC spectra for the methyl groups of CIB1 complexed with  $\alpha$ IIb-L were recorded in the presence of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  (Figure S4B). Similar to the backbone NMR studies,<sup>55</sup> methyl side chain <sup>1</sup>H,<sup>13</sup>C-HSQC spectra for  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -bound CIB1/ $\alpha$ IIb-L show nearly the same pattern with minor deviations except for two residues in the calcium/magnesium binding loop, I168 and L123 (Figure S4B). These results suggest that  $\text{Ca}^{2+}$ -CIB1 and  $\text{Mg}^{2+}$ -CIB1 interact with  $\alpha$ IIb in nearly the same manner.

## DISCUSSION

In this study, we have obtained a solution structure for the isotope-labeled  $\text{Ca}^{2+}$ -CIB1 protein, complexed with the

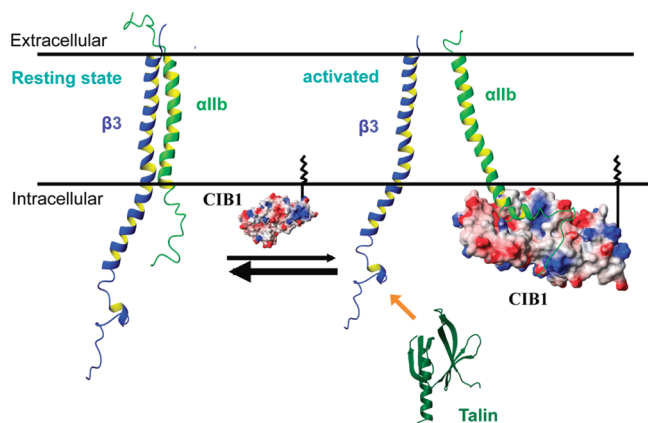
synthetic  $\alpha$ IIb-L peptide that encompasses the cytoplasmic domain and part of the transmembrane domain of the  $\alpha$ IIb subunit of platelet integrin. The structure for the complexed protein could be determined based on RDC restraints. With the use of the available structure of  $\alpha$ IIb (PDB 2KNC), a structural model for the  $\text{Ca}^{2+}$ -CIB1/ $\alpha$ IIb-L complex was obtained using data-driven docking based on reverse cross-saturation NMR experiments. The two binding partners in this complex structure adopt a relative orientation with the  $\alpha$ -helical N-terminal portion of  $\alpha$ IIb-L buried into the C-lobe hydrophobic pocket of  $\text{Ca}^{2+}$ -CIB1 and the negatively charged and extended C-terminal tail of  $\alpha$ IIb-L pointing toward the N-lobe of  $\text{Ca}^{2+}$ -CIB1 but being relatively dynamic. The superposition of the ordered structural fragment (residues 983–995) and the corresponding fragment of 2KNC provides the orientation of the  $\text{Ca}^{2+}$ -CIB1/ $\alpha$ IIb-L complex relative to the cell membrane (Figure 7A). As expected, the N-terminal extension of  $\text{Ca}^{2+}$ -CIB1 (helix 0, H0) points toward the cell membrane, which is consistent with its role in linking to a membrane-bound myristoyl group (Figure 7A). However, when modeled together with a membrane, a steric clash was observed between the transmembrane helix of  $\alpha$ IIb and the eighth helix of  $\text{Ca}^{2+}$ -CIB1 (H8) (Figure 7A). Moreover, CIB1 would become associated directly with the membrane to a large extent if the  $\alpha$ IIb subunit interacts with  $\text{Ca}^{2+}$ -CIB1 with both of its transmembrane and cytoplasmic domains. Since CIB1 is mainly found as a myristoylated protein in the cytoplasm<sup>3,56</sup> and the binding interface on  $\alpha$ IIb primarily includes the fragment of 983–995,<sup>3</sup> it seems unlikely that  $\text{Ca}^{2+}$ -CIB1 will penetrate into the cell membrane or be engaged with a significantly large part of the  $\alpha$ IIb transmembrane domain. Therefore, a conformational change is likely to happen in  $\alpha$ IIb upon binding to  $\text{Ca}^{2+}$ -CIB1. We propose that the transmembrane helix of  $\alpha$ IIb will bend around W988 (the membrane-border residue for  $\alpha$ IIb-L) when binding to  $\text{Ca}^{2+}$ -CIB1 (Figure 7B,C). In this manner, the steric clash and the potential energy barrier caused by an intrusion of CIB1 into the membrane would be avoided.

Recent structural studies of the transmembrane domains<sup>57,58</sup> as well as the protein fragments including both the transmembrane and cytoplasmic domains of  $\alpha$ IIb $\beta$ <sup>3,7,8</sup> have shed some light on the molecular mechanism of the regulation of the activation of  $\alpha$ IIb $\beta$ 3. For the structure of the  $\alpha$ IIb $\beta$ 3 transmembrane domains, various approaches gave rise to almost converged structures (see detailed analysis in Yang et

al.<sup>7</sup> and Metcalf et al.<sup>57</sup>). However, the structure of the cytoplasmic domains of  $\alpha\text{IIb}\beta_3$  was reported to be diverse.<sup>7,8</sup> The importance of the previously suggested salt bridge between  $\alpha\text{IIb}$  R995 and  $\beta_3$  D723<sup>7,59</sup> that was considered for a long time to be critical for the association of  $\alpha\text{IIb}\beta_3$  has recently been brought into question,<sup>8,60</sup> consistent with the dynamic nature of the cytoplasmic domains of  $\alpha\text{IIb}\beta_3$ . The G<sub>991</sub>FFKR<sub>995</sub> motif on  $\alpha\text{IIb}$  is critical for the interactions between  $\alpha\text{IIb}$  and  $\beta_3$  as revealed in the  $\alpha\text{IIb}\beta_3$  complex structure determined using Cys-scanning-mutagenesis/disulfide-cross-linking/rosetta-modeling<sup>61</sup> and another  $\alpha\text{IIb}\beta_3$  complex NMR structure that has been determined using lipid bicelles as the solvent (PDB 2K9J).<sup>58</sup> On the basis of a comparison with these two structures, Ca<sup>2+</sup>-CIB1 seems to dissociate the  $\alpha\text{IIb}\beta_3$  heterodimer, which would potentially activate the  $\alpha\text{IIb}\beta_3$  complex<sup>14</sup> because the F992 and F993 side chains become buried in the hydrophobic pocket in the Ca<sup>2+</sup>-CIB1/ $\alpha\text{IIb-L}$  complex structure (Figure S5). It has also been reported that an F992A mutation in  $\alpha\text{IIb}$  may partially impair the interaction with  $\beta_3$  although both F992 and F993 in  $\alpha\text{IIb}$  point away from the  $\beta_3$  subunit in 2KNC.<sup>7</sup> Moreover, a potential conformational change of  $\alpha\text{IIb}$  upon binding to Ca<sup>2+</sup>-CIB1 (Figure 7) will also disturb the stability of the structure of the  $\alpha\text{IIb}\beta_3$  complex and cause the dissociation of these two subunits. On the basis of the classic studies indicating that the activation of integrin can be caused by the dissociation of the heterodimer,<sup>4</sup> Ca<sup>2+</sup>-CIB1 is highly likely able to disrupt the association of  $\alpha\text{IIb}$  and  $\beta_3$ .

## CONCLUSION

Taken all data together, a mechanism for activation of  $\alpha\text{IIb}\beta_3$  upon interacting with Ca<sup>2+</sup>-CIB1 can be proposed, that is similar to the action of the cytoskeletal protein talin,<sup>9</sup> where Ca<sup>2+</sup>-CIB1 dissociates the  $\alpha\text{IIb}\beta_3$  heterodimer and consequently activates integrin  $\alpha\text{IIb}\beta_3$  (Figure 8). Alternatively CIB1



**Figure 8.** A structural model for the mechanism of Ca<sup>2+</sup>-CIB1 regulation of integrin activation. (A) Inactivated  $\alpha\text{IIb}\beta_3$  complex, modified based on Yang et al.<sup>7</sup> (B) In the presence of Ca<sup>2+</sup>-CIB1, the  $\alpha\text{IIb}$  cytoplasmic subunit interacts with Ca<sup>2+</sup>-CIB1, with its N-terminal  $\alpha$ -helix buried in the hydrophobic pocket of the C-lobe of CIB1 and its C-terminal negatively charged tail being dynamic but likely being in contact with the N-lobe of CIB1. Subsequently,  $\alpha\text{IIb}$  and  $\beta_3$  are separated by Ca<sup>2+</sup>-CIB1 and this  $\alpha\text{IIb}\beta_3$  complex dissociates. The integrin  $\alpha\text{IIb}\beta_3$  dimer is activated by Ca<sup>2+</sup>-CIB1, which is similar to the activation by talin, a cytoskeleton protein, which binds to the  $\beta_3$  cytoplasmic domain.<sup>9</sup>

can maintain the activated state of  $\alpha\text{IIb}\beta_3$  by covering the binding site in  $\alpha\text{IIb}$  and preventing its association with  $\beta_3$ .

Since nearly identical HSQC NMR patterns were observed for Mg<sup>2+</sup>-CIB1 and for Ca<sup>2+</sup>-CIB1 upon interaction with  $\alpha\text{IIb-L}$  (Figure S4B), our data suggest that the same complex can form under various physiological conditions and is not calcium-dependent per se. This notion is supported by previous in vivo studies, in which  $\alpha\text{IIb}$  was shown to interact with CIB1 in both resting and stimulated platelets with no requirement for a rise in the intracellular calcium concentration.<sup>19</sup> In addition, it is possible that protein phosphatase 1 could compete with CIB1 for  $\alpha\text{IIb}$ , as these two proteins share the same binding motif (K<sub>989</sub>VGF<sub>992</sub>) on the  $\alpha\text{IIb}$  cytoplasmic domain.<sup>62</sup>

In this work, we have used a reverse cross-saturation NMR approach to detect the interface on Ca<sup>2+</sup>-CIB1 that recognizes the bound  $\alpha\text{IIb-L}$  peptide. In these experiments, we selectively irradiated the smaller partner in the protein–protein complex, and the results from our work (Figure 4 and Figure 5) demonstrate that it is possible to identify the hydrophobic residues that are involved in the interactions between CIB1 and  $\alpha\text{IIb}$ . This represents a powerful way of using the NMR cross-saturation experiment, where normally the larger partner has been irradiated to achieve more effective spin-diffusion.<sup>26,47</sup> Many protein complexes currently under investigation can be represented by studying a large protein and a synthetic peptide to represent the intact protein partner or a protein domain. Provided that the proteins of interest can be bacterially expressed, deuterated, and selectively protonated on methyl groups, the reverse cross-saturation experiment with methyl detection should become a useful NMR approach for better understanding protein–protein interactions in general.

## ASSOCIATED CONTENT

### Supporting Information

Table S1 provides the statistics of the NMR-based structure calculation of Ca<sup>2+</sup>-CIB1 in complex with  $\alpha\text{IIb-L}$ . The interhelical angle (Table S2) and Q factor (Table S3) analyses for the structures of Ca<sup>2+</sup>-CIB1 free and in complex with  $\alpha\text{IIb-L}$  were also included. Figure S1 shows the NH-RDC (pf1) fitting for the structures of Ca<sup>2+</sup>-CIB1 free and in complex with  $\alpha\text{IIb-L}$ . Figure S2 shows the 1-D <sup>1</sup>H NMR spectra illustrating the reverse cross-saturation approach employed in this work; Figure S3 shows the crystal structure of Ca<sup>2+</sup>-CIB1 (PDB 1XO5); Figure S4 shows the superposition of 2-D NMR HSQC spectra demonstrating the roles of the acidic tail of  $\alpha\text{IIb-L}$ , as well as Ca<sup>2+</sup> and Mg<sup>2+</sup> in the regulation of the CIB1/ $\alpha\text{IIb}$  interactions; Figure S5 shows the mapping of critical residues in  $\alpha\text{IIb-L}$  onto the complex structure obtained in this work. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

vogel@ucalgary.ca

### Present Address

<sup>†</sup>Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, Canada, M5G 1X5

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This research has been supported by the Canadian Institutes of Health Research (CIHR). H.J.V. holds a Scientist award from the Alberta Heritage Foundation for Medical Research, now



called Alberta Innovates Health Solutions (AHFMR/AIHS). H.H. was the recipient of an AHFMR Studentship award. We thank the late Dr. Deane McIntyre for maintaining the NMR instrumentation and Dr. Hiroaki Ishida for helpful discussions. Dr. Ryan McKay at NANUC (National High Field Nuclear Magnetic Resonance Centre) is gratefully acknowledged for his assistance in attempting to record intermolecular NOEs between  $\text{Ca}^{2+}$ -CIB1 and  $\alpha\text{IIb-L}$ .

## REFERENCES

- (1) Luo, B. H.; Carman, C. V.; Springer, T. A. *Annu. Rev. Immunol.* **2007**, *25*, 619–647.
- (2) Hynes, R. O. *Cell* **2002**, *110*, 673–687.
- (3) Leisner, T. M.; Yuan, W. P.; DeNofrio, J. C.; Liu, J.; Parise, L. V. *Curr. Opin. Hematol.* **2007**, *14*, 255–261.
- (4) Kim, M.; Carman, C. V.; Springer, T. A. *Science* **2003**, *301*, 1720–1725.
- (5) Ulmer, T. S.; Yaspan, B.; Ginsberg, M. H.; Campbell, I. D. *Biochemistry* **2001**, *40*, 7498–7508.
- (6) Weljie, A. M.; Hwang, P. M.; Vogel, H. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5878–5883.
- (7) Yang, J.; Ma, Y. Q.; Page, R. C.; Misra, S.; Plow, E. F.; Qin, J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 17729–17734.
- (8) Metcalf, D. G.; Moore, D. T.; Wu, Y.; Kielec, J. M.; Molnar, K.; Valentine, K. G.; Wand, A. J.; Bennett, J. S.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 22481–22486.
- (9) Wegener, K. L.; Partridge, A. W.; Han, J.; Pickford, A. R.; Liddington, R. C.; Ginsberg, M. H.; Campbell, I. D. *Cell* **2007**, *128*, 171–182.
- (10) Naik, U. P.; Patel, P. M.; Parise, L. V. *J. Biol. Chem.* **1997**, *272*, 4651–4654.
- (11) Gifford, J. L.; Walsh, M. P.; Vogel, H. J. *Biochem. J.* **2007**, *405*, 199–221.
- (12) Yamniuk, A. P.; Vogel, H. J. *Protein Sci.* **2005**, *14*, 1429–1437.
- (13) Barry, W. T.; Boudignon-Proudhon, C.; Shock, D. D.; McFadden, A.; Weiss, J. M.; Sondek, J.; Parise, L. V. *J. Biol. Chem.* **2002**, *277*, 28877–28883.
- (14) Tsuboi, S. *J. Biol. Chem.* **2002**, *277*, 1919–1923.
- (15) Burgoyne, R. D. *Biochim. Biophys. Acta* **2004**, *1742*, 59–68.
- (16) Yamniuk, A. P.; Nguyen, L. T.; Hoang, T. T.; Vogel, H. J. *Biochemistry* **2004**, *43*, 2558–2568.
- (17) Huang, H.; Ishida, H.; Yamniuk, A. P.; Vogel, H. J. *J. Biol. Chem.* **2011**, *286*, 17181–17192.
- (18) Yamniuk, A. P.; Ishida, H.; Vogel, H. J. *J. Biol. Chem.* **2006**, *281*, 26455–26464.
- (19) Tsuboi, S.; Nonoyama, S.; Ochs, H. D. *EMBO Rep.* **2006**, *7*, 506–511.
- (20) Yuan, W. P.; Leisner, T. M.; McFadden, A. W.; Wang, Z. Y.; Larson, M. K.; Clark, S.; Boudignon-Proudhon, C.; Lam, S. C. T.; Parise, L. V. *J. Cell. Biol.* **2006**, *172*, 169–175.
- (21) Tugarinov, V.; Kanelis, V.; Kay, L. E. *Nat. Protoc.* **2006**, *1*, 749–754.
- (22) Hansen, M. R.; Mueller, L.; Pardi, A. *Nat. Struct. Biol.* **1998**, *5*, 1065–1074.
- (23) Ruckert, M.; Otting, G. *J. Am. Chem. Soc.* **2000**, *122*, 7793–7797.
- (24) Yang, D. W.; Venters, R. A.; Mueller, G. A.; Choy, W. Y.; Kay, L. E. *J. Biomol. NMR* **1999**, *14*, 333–343.
- (25) Cordier, F.; Dingley, A. J.; Grzesiek, S. *J. Biomol. NMR* **1999**, *13*, 175–180.
- (26) Takahashi, H.; Miyazawa, M.; Ina, Y.; Fukunishi, Y.; Mizukoshi, Y.; Nakamura, H.; Shimada, I. *J. Biomol. NMR* **2006**, *34*, 167–177.
- (27) Chou, J. J.; Li, S. P.; Bax, A. *J. Biomol. NMR* **2000**, *18*, 217–227.
- (28) Cornilescu, G.; Delaglio, F.; Bax, A. *J. Biomol. NMR* **1999**, *13*, 289–302.
- (29) Venters, R. A.; Farmer, B. T.; Fierke, C. A.; Spicer, L. J. *Mol. Biol.* **1996**, *264*, 1101–1116.
- (30) Huang, H.; Ishida, H.; Vogel, H. J. *Protein Sci.* **2010**, *19*, 475–485.
- (31) Chou, J. J.; Li, S. P.; Klee, C. B.; Bax, A. *Nat. Struct. Biol.* **2001**, *8*, 990–997.
- (32) Park, C. J.; Lee, J. H.; Choi, B. S. *Nucleic Acids Res.* **2005**, *33*, 4172–4181.
- (33) Pennestri, M.; Melino, S.; Contessa, G. M.; Casavola, E. C.; Paci, M.; Ragnini-Wilson, A.; Cicero, D. O. *J. Biol. Chem.* **2007**, *282*, 667–679.
- (34) Verdone, G.; Corazza, A.; Colebrooke, S. A.; Cicero, D.; Eliseo, T.; Boyd, J.; Doliana, R.; Fogolari, F.; Viglino, P.; Colombatti, A.; Campbell, I. D.; Esposito, G. *J. Biomol. NMR* **2009**, *43*, 79–96.
- (35) Gifford, J. L.; Ishida, H.; Vogel, H. J. *J. Biomol. NMR* **2011**, *50*, 71–81.
- (36) Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. *Bioinformatics* **2006**, *22*, 195–201.
- (37) Dominguez, C.; Boelens, R.; Bonvin, A. M. J. *J. Am. Chem. Soc.* **2003**, *125*, 1731–1737.
- (38) De Vries, S. J.; van Dijk, M.; Bonvin, A. M. J. *J. Nat. Protoc.* **2010**, *5*, 883–897.
- (39) Hwang, P. M.; Vogel, H. J. *J. Mol. Recognit.* **2000**, *13*, 83–92.
- (40) Laskowski, R. A.; Macarthur, M. W.; Moss, D. S.; Thornton, J. M. *J. Appl. Crystallogr.* **1993**, *26*, 283–291.
- (41) Clore, G. M.; Starich, M. R.; Bewley, C. A.; Cai, M. L.; Kuszewski, J. *J. Am. Chem. Soc.* **1999**, *121*, 6513–6514.
- (42) Prestegard, J. H.; Bougault, C. M.; Kishore, A. I. *Chem. Rev.* **2004**, *104*, 3519–3540.
- (43) Meiler, J.; Prompers, J. J.; Peti, W.; Griesinger, C.; Bruschweiler, R. *J. Am. Chem. Soc.* **2001**, *123*, 6098–6107.
- (44) Fischer, M. W.; Losonczy, J. A.; Weaver, J. L.; Prestegard, J. H. *Biochemistry* **1999**, *38*, 9013–9022.
- (45) Jain, N. U.; Wyckoff, T. J.; Raetz, C. R.; Prestegard, J. H. *J. Mol. Biol.* **2004**, *343*, 1379–1389.
- (46) Cornilescu, G.; Marquardt, J. L.; Ottinger, M.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 6836–6837.
- (47) Takahashi, H.; Nakanishi, T.; Kami, K.; Arata, Y.; Shimada, I. *Nat. Struct. Biol.* **2000**, *7*, 220–223.
- (48) Shimada, I. *Methods Enzymol.* **2005**, *394*, 483–506.
- (49) Kalk, A.; Berendsen, H. J. C. *J. Magn. Reson.* **1976**, *24*, 343–366.
- (50) Takeda, M.; Terasawa, H.; Sakakura, M.; Yamaguchi, Y.; Kajiwara, M.; Kawashima, H.; Miyasaka, M.; Shimada, I. *J. Biol. Chem.* **2003**, *278*, 43550–43555.
- (51) Shimada, I.; Ueda, T.; Matsumoto, M.; Sakakura, M.; Osawa, M.; Takeuchi, K.; Nishida, N.; Takahashi, H. *Prog. Nucl. Magn. Reson. Spectrosc.* **2009**, *54*, 123–140.
- (52) Ikura, M.; Clore, G. M.; Gronenborn, A. M.; Zhu, G.; Klee, C. B.; Bax, A. *Science* **1992**, *256*, 632–638.
- (53) Post, C. B. *Curr. Opin. Struct. Biol.* **2003**, *13*, 581–588.
- (54) Koloka, V.; Christofidou, E. D.; Vaxevelanis, S.; Dimitriou, A. A.; Tsikaris, V.; Tselepis, A. D.; Panou-Pomonis, E.; Sakarellos-Daitsiotis, M.; Tsoukatos, D. C. *Platelets* **2008**, *19*, 502–511.
- (55) Yamniuk, A. P.; Gifford, J. L.; Linse, S.; Vogel, H. J. *Biochemistry* **2008**, *47*, 1696–1707.
- (56) Yamniuk, A. P.; Vogel, H. J. *Calcium Binding Proteins* **2006**, *1*, 150–155.
- (57) Metcalf, D. G.; Kulp, D. W.; Bennett, J. S.; DeGrado, W. F. *J. Mol. Biol.* **2009**, *392*, 1087–1101.
- (58) Lau, T. L.; Kim, C.; Ginsberg, M. H.; Ulmer, T. S. *EMBO J.* **2009**, *28*, 1351–1361.
- (59) Vinogradova, O.; Velyvis, A.; Velyviene, A.; Hu, B.; Haas, T. A.; Plow, E. F.; Qin, J. *Cell* **2002**, *110*, 587–597.
- (60) Wang, W.; Luo, B. H. *J. Cell. Biochem.* **2010**, *109*, 447–452.
- (61) Zhu, J.; Luo, B. H.; Barth, P.; Schonbrun, J.; Baker, D.; Springer, T. A. *Mol. Cell* **2009**, *34*, 234–249.
- (62) Vijayan, K. V.; Liu, Y.; Li, T. T.; Bray, P. F. *J. Biol. Chem.* **2004**, *279*, 33039–33042.
- (63) Koradi, R.; Billeter, M.; Wuthrich, K. *J. Mol. Graphics* **1996**, *14*, 51–55.