The Interaction between Calcium- and Integrin-binding Protein 1 and the αIIb Integrin Cytoplasmic Domain Involves a Novel C-terminal Displacement Mechanism*®

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Calcium- and integrin-binding protein 1 (CIB1) regulates platelet aggregation in hemostasis through a specific interaction with the αIIb cytoplasmic domain of platelet integrin αIIbβ3. In this work we report the structural characteristics of CIB1 in solution and the mechanistic details of its interaction with a synthetic peptide derived from the αIIb cytoplasmic domain. NMR spectroscopy experiments using perdeuterated CIB1 together with heteronuclear nuclear Overhauser effect experiments have revealed a well folded α-helical structure for both the ligand-free and αIIb-bound forms of the protein. Residual dipolar coupling experiments have shown that the N and C domains of CIB1 are positioned side by side, and chemical shift perturbation mapping has identified the αIIb-binding site as a hydrophobic channel spanning the entire C domain and part of the N domain. Data obtained with a truncated version of CIB1 suggest that the extreme C-terminal end of the protein weakly interacts with this channel in the absence of a biological target, but it is displaced by the αIIb cytoplasmic domain, suggesting a novel mechanism to increase binding specificity.

The platelet-specific heterodimeric transmembrane integrin receptor αIIbβ3 plays a central role in hemostasis and thrombosis (1). At the site of vascular injury, platelet agonists such as thrombin trigger “inside-out” signaling events that activate αIIbβ3, resulting in ligand binding by the integrin extracellular domains, integrin cross-linking, and ultimately, platelet aggregation. Ligand occupancy also generates “outside-in” signals that lead to granular secretion of ADP, cytoskeletal reorganization, and platelet spreading (2). The small EF-hand calcium-binding protein CIB1,4 (calcium- and integrin-binding protein 1, also known as CIB, calmyrin, KIP) binds specifically to the αIIb cytoplasmic domain (3), and the interaction has been implicated in both inside-out and outside-in signaling events (4–6). The binding of CIB1 to synthetic αIIb peptides can occur in vitro with a dissociation constant in the high nanomolar range (7, 8); however, the mechanistic details of the interaction are not well understood. Because inappropriate platelet activation is a major contributor to cardiovascular disease (9), understanding the interaction between CIB1 and αIIb could be an important step toward the development of novel anti-platelet therapeutics.

CIB1 shares significant sequence homology with calcineurin B (CnB), calcineurin homologous protein-1, and the neuronal calcium sensor (NCS) family of EF-hand proteins. Like these proteins, CIB1 is myristoylated on its N-terminal glycine residue and is membrane-associated in vivo (10, 11). However, myristoylation is not required for αIIb binding (7, 8, 10). Recent x-ray crystal structures (Protein Data Bank codes 1XO5 and 1Y1A) have shown that like its homologs, calcium-bound CIB1 (Ca2+-CIB1) folds into N- and C-terminal globular domains, each composed of two EF-hand motifs, with extended N- and C-terminal regions (12, 13). Ca2+ is bound to the canonical C domain EF-hands (EF-III and EF-IV) but not to the divergent N domain EF-hands (EF-I and EF-II), consistent with biochemical studies (14). Although the core dual helix-loop-helix EF-hand structure of Ca2+-CIB1 is very similar in both 1XO5 and 1Y1A, there are distinct differences in the protein’s oligomeric state, the orientation of the two domains, and the conformation of the N- and C-terminal extensions (supplemental Fig. 1). In 1XO5, Ca2+-CIB1 was crystallized as a monomer with similar domain orientation to CnB, calcineurin homologous protein-1, and the NCS proteins (13). CIB1 is also monomeric in solution as revealed by diffusion NMR spectroscopy (15), sedimentation equilibrium, and gel filtration studies (13, 16). However, in 1Y1A the protein was crystallized as a head-to-tail dimer, with a different domain orientation and structurally distinct N- and C-terminal extensions (12). The

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4 The abbreviations used are: CIB1, calcium- and integrin-binding protein 1; CnB, calcineurin B; NCS, neuronal calcium sensor; DTT, dithiothreitol; HSQC, heteronuclear single quantum coherence; TROSY, transverse relaxation optimized spectroscopy; CSP, chemical shift perturbation; ITC, isothermal titration calorimetry; RDC, residual dipolar couplings; CaM, calmodulin; sMLCKp, smooth muscle myosin light chain kinase.
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structure of the N-terminal extension is important since it would affect the placement of the myristoyl group and orientation of CIB1 with respect to the cytoplasmic membrane. The structure of the C-terminal extension is interesting because in each crystal structure it shields the C domain hydrophobic channel that had previously been proposed to be the αIIb-binding site based on mutagenesis and molecular modeling data (10, 17). In this study we have utilized solution NMR spectroscopy, optical spectroscopy and microcalorimetry of CIB1, and truncated CIB1 proteins to investigate the solution structure of Ca\(^{2+}\)-CIB1 and characterize its interaction with an acetylated synthetic peptide (αIIb-L) encompassing the entire αIIb cytoplasmic domain and part of the transmembrane domain (Ac-LVLAMWKTFKKRNRPPPLEEDEGQ-OH). Our data reveal the structural characteristics of peptide-free and peptide-bound Ca\(^{2+}\)-CIB1 in solution and reveals a novel binding mode for the αIIb cytoplasmic domain, in which the C-terminal extension of CIB1 plays an important role in controlling binding specificity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Wild type histidine\(^{10}\)-tagged-CIB1 (herein referred to as CIB1) was expressed in Escherichia coli strain ER2566 (New England Biolabs) from a pET19b vector (Novagen) as previously described (14). The total protein construct is 213 residues long (24 kDa) including N-terminal extension of CIB1, and truncated CIB1 proteins to investigate the solution structure of Ca\(^{2+}\)-CIB1 and characterize its interaction with an acetylated synthetic peptide (αIIb-L) encompassing the entire αIIb cytoplasmic domain and part of the transmembrane domain (Ac-LVLAMWKTFKKRNRPPPLEEDEGQ-OH). Our data reveal the structural characteristics of peptide-free and peptide-bound Ca\(^{2+}\)-CIB1 in solution and reveals a novel binding mode for the αIIb cytoplasmic domain, in which the C-terminal extension of CIB1 plays an important role in controlling binding specificity.

**Generation of Mutant CIB1 Proteins**—The CIB1ΔH10 construct was generated by introducing a stop codon (TGA) in place of the Ser-180 codon (AGC) using the QuickChange site-directed mutagenesis kit (Stratagene) and the forward and reverse primers 5’-CAGCATGATGATACGGCCGCAGA-3’ (FWD) and 5’-GCTCGAAAATCTGGTCGCGGTGATCATGAC-3’ (REV). The coding region for residues 96–191 of CIB1 was amplified from the CIB1-pET19b template using PCR and the primers 5’-AGATT TCTGGATCATATGACGGCTATGGTTTGCAGC-3’ (FWD) and 5’-TTAGCAGCGGATCCCTACAGCAC-3’ (REV). The purified PCR product and empty pET19b expression vector were each digested with Ndel and BamHI (New England Biolabs) and then ligated using T4 DNA ligase (Invitrogen) to generate the CIB1-C-pET19b construct. The integrity of both constructs was confirmed by DNA sequencing, and each protein was purified to homogeneity identically to wild type CIB1. Concentrations were determined using the predicted molar extinction coefficient for CIB1 (ε\(_{276}\) = 2900), and CIB1-C (ε\(_{276}\) = 1450).

**Peptides**—All peptides were synthesized commercially and determined to be more than 95% pure by matrix-assisted laser desorption/ionization mass spectroscopy and high pressure liquid chromatography. Peptide αIIb-L (Ac-LVLAMWKTFKKRNRPPPLEEDDEEQ-OH) corresponds to amino acids 983–1008 of the platelet integrin αIIb subunit, with Gln-1008 as the C-terminal residue (20). Peptide H10p (Ac-SPDFASSFKIVL-OH) corresponds to residues 180–191 of CIB1. Peptide smooth muscle myosin light chain kinase smMLCKp (Ac-ARKWQKGTHAVRAIGRLSS-NH₂) is the calmodulin (CaM)-binding site of chicken smooth muscle myosin light chain kinase, encompassing amino acids 36–55. The concentration of each peptide was determined using their predicted molar extinction coefficients: αIIb-L, ε\(_{280}\) = 5690; H10p, ε\(_{258}\) = 390; smMLCKp, ε\(_{280}\) = 5690.

**NMR Spectroscopy**—All NMR spectra were acquired on Bruker AVANCE 500 MHz or 700 MHz NMR spectrometers equipped with either a triple resonance inverse cryoprobe with single axis z gradient or triple-axis gradient triple broad band inverse detection (TBI) probe. Samples used for resonance assignments contained 650 μM \(^{2}H,^{13}C,^{15}N\)-labeled CIB1 in 20 mM HEPES, 100 mM KCl, 10 mM d\(_{10}\)-dithiothreitol (d\(_{10}\)-DTT), 3 mM CaCl\(_{2}\), 10% D\(_{2}\)O, ~0.5 mM NaN\(_{3}\), pH 7.5 ± 0.1, with and without 720 μM (1.1 molar equivalent) αIIb-L. Sequence-specific assignments of the \(^{1}H,^{13}C,^{13}B,^{15}N\) resonances for Ca\(^{2+}\)-CIB1 and Ca\(^{2+}\)-CIB1 in complex with αIIb-L were obtained manually using \(^{1}H,^{13}N\) heteronuclear single quantum coherence (HNSQC) spectra and a combination of transverse relaxation optimized spectroscopy (TROSY)-based triple-resonance experiments including three-dimensional HNCA,CB, HN(CO)CA,CB, and HNCO experiments (21–23). All spectra were acquired at 310 K. Proton chemical shifts were referenced to the internal standard, 2,2-dimethyl-2-silapentane-5-sulfonate, and both \(^{13}C\) and \(^{15}N\) were referenced indirectly (24). Spectral analysis was performed using NMRPipe/NMRDraw (25) and NMRView (26) software, and chemical shift values were corrected for shifts induced by TROSY and perdeuteration (27).

The secondary structure of Ca\(^{2+}\)-CIB1 alone and in complex with αIIb-L was determined using the weighted average secondary shift method, where the chemical shift deviation from random coil for the backbone \(^{13}C\), \(^{15}N\) and N nuclei are normalized to the random coil range for each nucleus, and then averaged (Equation 1).

\[
W_{ASS} = \frac{\frac{3}{2}[(\delta C_{\alpha} - \delta C_{\alpha}) + 1.7(\delta C - \delta C_{\alpha}) + 0.35(\delta N - \delta N_{\alpha})]}{(\delta HN)^2 + (\delta N/5)^2 + (\delta C_{\alpha}/2)^2 + (\delta C/2)^2}
\]

(Eq. 1)

Chemical shift changes between Ca\(^{2+}\)-CIB1 and the Ca\(^{2+}\)-CIB1-αIIb-L complex were analyzed using the chemical shift perturbation (CSP) method described by Wingfield and co-workers (28), but for illustrative purposes the contributions of the HN and N nuclei are displayed separately from the contribution of the Ca and CB nuclei (Equation 2).

\[
CSP = \sqrt{(\Delta HN)^2 + (\Delta N/5)^2 + (\Delta C_{\alpha}/2)^2 + (\Delta C/2)^2}
\]

(Eq. 2)
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![Diagram of secondary structure and backbone flexibility of $\mathrm{Ca}^{2+}$-CIB1](image)

$[\mathrm{H}]^{15}\mathrm{N}$ NOE experiments were acquired at 310 K and 700 MHz with and without $[\mathrm{H}]$ saturation using a recycle delay of 4.5 s (29). Samples consisted of 600 $\mu\text{M}$ $^{15}\text{N}$-labeled CIB1 in 20 mM HEPES, 100 mM KCl, 10 mM $d_{10}$-DTT, 3 mM CaCl$_2$, 10% D$_2$O, ~0.5 mM NaN$_3$, pH 7.5 ± 0.1, with and without 660 $\mu\text{M}$ $\alpha$IIb-L. $[\mathrm{H}]^{15}\mathrm{N}$ NOE data were processed and analyzed using NMRPipe/NMRDraw and NMRView software, with a noise floor of 7 and 10% for $\mathrm{Ca}^{2+}$-CIB1 and $\mathrm{Ca}^{2+}$-CIB1- $\alpha$IIb-L, respectively.

$[\mathrm{H}]^{15}\mathrm{N}$ backbone dipolar couplings were measured at 310 K and 700 MHz using a sensitivity-enhanced IPAP-type $[\mathrm{H}]^{15}\text{N}$ HSQC experiment (30). Isotropic samples contained 650 $\mu\text{M}$ $^{15}\text{N}$-labeled CIB1 in 20 mM HEPES, 100 mM KCl, 10 mM $d_{10}$-DTT, 3 mM CaCl$_2$, 10% D$_2$O, ~0.5 mM NaN$_3$, pH 7.5 ± 0.1, and aligned samples contained 500 $\mu\text{M}$ $^{15}\text{N}$-labeled CIB1 in the same buffer plus 12 mg/ml Pf1 phage (Asla Biotech). Data processing and analysis were performed using NMRPipe/NMRDraw, NMRView, and PALES software (31). In the PALES analysis the correlation between residual dipolar couplings (RDC) data and a protein structure is given by the correlation coefficient ($R$) and the quality factor ($Q$), which each range from 0 to 1, with a better correlation represented by higher $R$ values and lower $Q$ values.

**Isothermal Titration Calorimetry**—All isothermal titration calorimetry (ITC) experiments were performed on a MicroCal VP-ITC microcalorimeter. Protein preparation included an overnight incubation in 10 mM DTT to reduce any disulfide bonds and subsequent DTT removal by gel filtration as previously described (14). Titrations consisted of sequential injections of 300–500 $\mu\text{M}$ CIB1, CIB1A-H10, or CIB1-C in 20 mM HEPES, 100 mM KCl, 2 mM CaCl$_2$, pH 7.5, into a sample cell containing 10–20 $\mu\text{M}$ $\alpha$IIb-L or H10p in the same buffer at temperatures ranging from 20 to 37 °C. The non-linear heat of dilution exhibited by CIB1A-H10 required the subtraction of a complete reference experiment for each peptide titration at all temperatures. Titrations of 400 $\mu\text{M}$ smMLCKp into 20 mM CIB1 or CIB1A-H10 were also performed under similar conditions. All data were fit to a one-site binding model using MicroCal Origin software to obtain values for the stoichiometry ($N$), association constant ($K_a$), and enthalpy change ($\Delta H$), whereas values for the entropy change ($T\Delta S$) were calculated using standard thermodynamic equations.

**Fluorescence Spectroscopy**—Steady state 8-anilino-1-naphalenesulfonate (ANS) fluorescence spectra were recorded at 37 °C on a Varian Cary Eclipse spectrofluorimeter using samples of 40 $\mu\text{M}$ ANS in 20 mM HEPES, 100 mM KCl, 2 mM CaCl$_2$, 1 mM DTT, pH 7.5 ± 0.1, with and without 10 $\mu\text{M}$ CIB1 or CIB1A-H10. Samples were excited at 370 nm using 5 nm excitation slits, and steady state fluorescence emission spectra were recorded from 400 to 600 nm at a scan rate of 600 nm/min using 10-nm emission slits.

**RESULTS**

**NMR Characterization of $\mathrm{Ca}^{2+}$-CIB1**—Using TROSY-based triple resonance NMR spectroscopy experiments, we were able to...
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**TABLE 1**

Correlation between the residual dipolar couplings determined by solution NMR spectroscopy and those calculated from the x-ray crystal structures of Ca\(^{2+}\)-CIB1, 1XOS, and 1Y1A

| Region analyzed | 1XOS  | 1Y1A   | 1XOS  | 1Y1A   |
|-----------------|-------|--------|-------|--------|
|                 | R     | Q      | R     | Q      |
| Full protein (1–191) | 0.90  | 0.43   | 0.60  | 0.80   |
| ΔH0 (24–191)     | 0.96  | 0.27   | 0.61  | 0.80   |
| ΔH10 (1–179)     | 0.91  | 0.42   | 0.60  | 0.81   |
| N domain (1–99)  | 0.87  | 0.49   | 0.89  | 0.45   |
| N domain ΔH0 (24–99) | 0.97  | 0.23   | 0.90  | 0.43   |
| C domain (105–191) | 0.95  | 0.29   | 0.96  | 0.29   |
| C domain ΔH10 (105–179) | 0.97  | 0.24   | 0.97  | 0.25   |

Therefore, we used the chemical shift data to predict the secondary structure of Ca\(^{2+}\)-CIB1 in solution. As shown in Fig. 1, A and B, the NMR data confirmed that Ca\(^{2+}\)-CIB1 is highly \(\alpha\)-helical and composed of four EF-hand motifs. Both of the canonical Ca\(^{2+}\) binding EF-hand motifs in the C domain (EF-III and EF-IV) show the expected helix-loop-helix structure with a short \(\beta\)-strand in the center of each Ca\(^{2+}\) binding loop. EF-II within the N domain also displays the typical helix-loop-helix structure, whereas the more divergent EF-I consists of a long \(\alpha\)-helix (H1) and then a mixture of short helical segments (H2, H3a, and H3b) and connecting loops. Importantly, an N-terminal \(\alpha\)-helix (H0) from K10-D18 is clearly observed in the NMR data, similar to crystal structure 1Y1A. The C-terminal extension adopts a short \(\alpha\)-helical segment from Asp-182—Ser-185 followed by an extended conformation from Ser-186—Leu-191, which is also similar to the secondary structure observed in 1Y1A. However, the signals for the last three residues (Phe-187—Leu-191) displayed greater than average line-broadening, suggesting that these residues might be partially solvent-exposed or in chemical exchange between multiple conformations (supplemental Fig. 2A).

The backbone flexibility of Ca\(^{2+}\)-CIB1 was further examined using \(^{1}\)H-\(^{15}\)N NOE experiments. This analysis revealed that almost the entire protein backbone has flexibility consistent with typical well folded globular domains, having average \(^{1}\)H-\(^{15}\)N NOE values near ~0.8 (Fig. 1C). This includes the residues within the short central linker between the N and C domains, suggesting that the two domains might adopt a fixed orientation in solution. The most flexible region of the protein is the loop connecting EF-III and EF-IV, which also has very high temperature factors in the crystallographic data (12, 13). The \(^{1}\)H-\(^{15}\)N NOE values also decrease toward the C terminus, suggesting increased mobility, but unfortunately the signals for the last two residues (U189—L191) could not be analyzed in the \(^{1}\)H-\(^{15}\)N NOE data due to chemical exchange broadening.

**Domain Orientation of Ca\(^{2+}\)-CIB1 Revealed by Residual Dipolar Couplings**—To determine the N and C domain orientation of Ca\(^{2+}\)-CIB1 in solution, we measured one-bond backbone \(^{1}\)H,\(^{15}\)N RDC and compared the results to the RDC predicted from crystal structures 1XOS and 1Y1A (Fig. 2, Table 1). Analysis of the entire protein backbone revealed a much better correlation between the NMR data and the RDC predicted from crystal structure 1XOS in comparison to 1Y1A. However, a good correlation was observed when the N and C domains of
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**A**

![Secondary structure and backbone flexibility of Ca\textsuperscript{2+}-CIB1 bound to αIIb-L.](image)

**B**

![NMR spectra of similar quality to the peptide-free protein and, consequently, also required perdeuteration and TROSY to obtain resonance assignments (supplemental Fig. 2B). Most of the residues that did not give rise to HSQC signals in peptide-free Ca\textsuperscript{2+}-CIB1 were also not observed in the presence of the αIIb-L peptide, indicating that peptide binding does not alter the exchange in these regions of the protein (supplemental Fig. 2C). The chemical shift analysis also revealed that the secondary structure of Ca\textsuperscript{2+}-CIB1 remains intact in complex with αIIb-L (Fig. 3A), which is typical for EF-hand proteins in complex with their targets (34). Moreover, the \(^{1}H\)-\(^{15}N\) NOE data showed similar backbone flexibility (or lack thereof) for α-helices H0-H9, their connecting loops, and all four short β-strands (Fig. 3B). The one distinct difference that is observed upon peptide binding is that the NMR signals for much of the C-terminal extension (Asp-182, Phe-183, Ser-185—Lys-188) become broadened beyond detection, suggesting that this region undergoes conformational exchange on the intermediate NMR timescale. Moreover, the previously exchange-broadened signals for Ile-189, Val-190, and Leu-191 at the extreme C terminus dramatically increase in

either 1XO5 or 1Y1A were analyzed separately. This suggests that the poor overall correlation with 1Y1A is due to a difference in domain orientation rather than domain structure and that the domains of Ca\textsuperscript{2+}-CIB1 are oriented side by side in solution, similar to the monomeric crystal form, 1XO5.

Omitting the N-terminal extension from the analysis significantly improves the correlation between the NMR data and 1XO5 but not 1Y1A (Table 1). This confirms that the N-terminal extension adopts an α-helical structure (H0) and orientation similar to 1Y1A rather than the extended conformation observed in 1XO5, which is also consistent with the secondary structure analysis. Omitting the C-terminal extension from the analysis of the C domain improves the correlation with each crystal structure only marginally. However, only three residues were available for RDC analysis in this region (Ser-180, Ser-186, Val-190) due to overlap and low signal intensity, making it difficult to exclude the conformation observed in either of the crystal structures. Considering the aforementioned chemical exchange in this region, it is also possible that the C-terminal extension simply exhibits some conformational flexibility.

**NMR Characterization of the Ca\textsuperscript{2+}-CIB1-αIIb-L Complex—**

The complex of Ca\textsuperscript{2+}-CIB1 with the αIIb-L peptide produced NMR spectra of similar quality to the peptide-free protein and, intensity and display very small or negative \(^{1}H\)-\(^{15}N\) NOE values (0.3-0.1) characteristic of a high degree of conformational flexibility (Fig. 3). Therefore, these data indicate that αIIb-L binding significantly increases the flexibility of the C-terminal extension of Ca\textsuperscript{2+}-CIB1.

**Identification of the αIIb-binding Site on Ca\textsuperscript{2+}-CIB1—**

To further characterize the regions of Ca\textsuperscript{2+}-CIB1 that are affected by αIIb-L binding, CSP analysis was performed. This analysis demonstrated that αIIb-L binding has the largest effect on nonpolar residues of Ca\textsuperscript{2+}-CIB1, consistent with the predominantly hydrophobic interaction predicted from previous experiments (7, 8, 10). The largest CSP values were generally to residues from the C domain, especially residues on the hydrophobic face of H6, H7, and H8, as well as most of H9 and the C-terminal extension (Fig. 4). However, significant changes were also observed in regions of the N domain, suggesting that both domains interact with the peptide. In the N domain the largest CSP values were within the loop between H0 and H1, Arg-33, and H3a/H3b as well as the C-terminal end of H5.

Mapping the CSP data onto the structure of Ca\textsuperscript{2+}-CIB1 localizes the αIIb-binding site to the face of each domain that is opposite to the Ca\textsuperscript{2+} binding loops (functional and non-functional) (Fig. 4, B and C). H5 and residues from H3a/H3b form a
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**FIGURE 4. Identification of the αIb-L-binding site on Ca$^{2+}$-CIB1.**
A. CSP induced upon αIb-L binding to Ca$^{2+}$-CIB1 plotted as a function of amino acid residue. The CSP for HN and N are shown together as open bars, whereas the CSP for Ca and CB are shown together as solid bars. The secondary structure of Ca$^{2+}$-CIB1 is shown in the top part of the panel with α-helices and β-strands represented by boxes and arrows, respectively, and residues that were unavailable for analysis are represented by solid circles in the lower part of the panel. B, residues with CSP > 0.1 are mapped in red onto the backbone structure of Ca$^{2+}$-CIB1 (1X05). Ca$^{2+}$ ions are represented by black spheres, and α-helices H0–H10 are labeled. C, space-fill representation of B including side chains. For clarity, the C-terminal extension (Ser-180—Leu-191) of Ca$^{2+}$-CIB1 is shown in green line representations in both B and C, and Arg-33 is indicated in panels A, B, and C. D, Ribbon representation of the x-ray crystal structure of Ca$^{2+}$-CnB bound to residues 347–372 of CnA (Protein Data Bank code 1AUI).

small hydrophobic pocket on the surface of the N domain that is solvent-exposed in the absence of peptide. The linker between H0 and H1 interacts with the C-terminal region of H5 in both 1X05 and 1Y1A and is likely perturbed by the movement of this helix in the complex. The side chain of Arg-33 projects toward this pocket (Fig. 4, B and C) and could be involved in a salt-bridge with the acidic C terminus of αIIb, as was initially proposed based on homology modeling studies (17). The large upfield Ca and C$/beta$ shifts for Arg-33 are also consistent with salt bridge formation (Fig. 4A). However, we note that this electrostatic interaction has little effect on the binding affinity since neither the R32A/R33A double mutation nor removal of the acidic αIIb-L. C terminus has a significant effect on the interaction (8, 10).

The most perturbed regions within the C domain are a hydrophobic cleft formed by EF-III and EF-IV and the C-terminal extension that shields this cleft in the absence of αIIb-L. Together with the N domain, this cleft forms a continuous hydrophobic channel that is similar to the CnA-binding site of CnB (Fig. 4D). Several of the residues within the C domain portion of this channel have also been shown to be essential for αIIb binding by mutagenesis (10). In fact, previous studies have suggested that the C domain of Ca$^{2+}$-CIB1 may be sufficient for αIIb binding, likely interacting with the hydrophobic N-terminal region of the αIIb cytoplasmic/transmembrane domain (5, 10, 17). To test this hypothesis, we generated a C domain construct (CIB1-C) consisting of residues 96–191 of CIB1 and an N-terminal His$_{10}$ tag. Like full-length Ca$^{2+}$-CIB1 (14), circular dichroism spectra showed that CIB1-C is folded and α-helical in the presence of Ca$^{2+}$ (data not shown). However, ITC experiments revealed no significant interaction between Ca$^{2+}$-CIB1-C and αIIb-L (Fig. 5A). Subsequent NMR spectra showed that despite maintaining a folded α-helical structure, the tertiary structure of the isolated C domain is not identical to the C domain structure in the full-length Ca$^{2+}$-CIB1 protein (Fig. 5B). In addition to confirming the importance of the N domain in αIIb-binding, this suggests that an interdomain interaction is necessary to maintain the C domain in the correct native conformation.

**Defining the Function of the C-terminal Extension**—The change in flexibility and large CSP values for the C-terminal extension of Ca$^{2+}$-CIB1 suggests that it must undergo a large conformational change upon binding of the αIIb-L peptide. To determine whether this region is directly involved in αIIb-L binding or is simply displaced from the binding site by the peptide, we generated a deletion mutant of CIB1 having the entire C-terminal extension (Ser-180—Leu-191) removed (CIB11AH10). Steady state fluorescence spectroscopy experiments using the hydrophobic probe 8-anilino-1-naphalenesulfonate show that Ca$^{2+}$-CIB11AH10 has a significantly larger solvent-exposed hydrophobic surface than full-length Ca$^{2+}$-CIB1 (Fig. 6A). This is consistent with the hypothesis that removal of the C-terminal extension exposes the hydrophobic channel of the C domain. ITC experiments
clearly demonstrate that Ca\(^{2+}\)-CIB1ΔH10 retains the ability to bind to the αIIb-L peptide with similar affinity to the wild type protein (K_o ≈ 10^6) (Fig. 6B, Table 2) (8). Therefore, the C terminus of Ca\(^{2+}\)-CIB1 must not be directly involved in binding to the peptide but instead must be displaced from the binding site. The more negative change in heat capacity associated with αIIb-L binding to Ca\(^{2+}\)-CIB1ΔH10 (−1.6 kJ/mol K) in comparison to full-length Ca\(^{2+}\)-CIB1 (−1.1 kJ/mol K) (8) indicates that a larger hydrophobic surface becomes buried in the complex with the truncated protein, which is also consistent with the C terminus displacement model. Additional ITC experiments revealed no interaction between Ca\(^{2+}\)-CIB1ΔH10 and an acetylated synthetic peptide (H10p) encompassing the truncated portion of the C-terminal extension (Ac-180SPDFASSFKIVL191) of CIB1 (Fig. 6B). This indicates that the C-terminal extension itself has a low intrinsic affinity for the C domain. Together with our NMR data, these results suggest that the C-terminal extension interacts weakly with the C domain and is easily displaced by αIIb-L.

In our ITC experiments we noted a distinct heat of dissociation for Ca\(^{2+}\)-CIB1ΔH10 (Fig. 6B, top panel) that was not observed with wild type Ca\(^{2+}\)-CIB1. This suggested that removal of the C terminus causes weak self-association of Ca\(^{2+}\)-CIB1ΔH10 at high concentrations in the ITC syringe but dissociation upon dilution into the ITC cell, which is not surprising considering the increase in hydrophobic surface area for this truncated protein (Fig. 6A). However, this behav-

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**FIGURE 5. Structural and αIIb-L binding properties of CIB1-C.**

A, top panel, base-line corrected raw ITC titrations of Ca\(^{2+}\)-CIB1-C or Ca\(^{2+}\)-CIB1 into αIIb-L at 37 °C. Lower panel, derived binding isotherms for the Ca\(^{2+}\)-CIB1-C (○) or Ca\(^{2+}\)-CIB1 (■) titrations after subtraction of the heat of dilution control experiments. The data for Ca\(^{2+}\)-CIB1 were taken Yamniuk and Vogel (8). B, overlay of the 1\(^H\),1\(^5\)N HSQC NMR spectra for Ca\(^{2+}\)-\(^1\(^5\)N\)-labeled CIB1 (black) and Ca\(^{2+}\)-\(^1\(^5\)N\)-CIB1-C (red) suggest that the isolated C domain is not completely folded correctly (spectra recorded at 37 °C and 500 MHz). Some C domain peaks for Ca\(^{2+}\)-CIB1 are labeled.

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**FIGURE 6. Surface properties and peptide binding to Ca\(^{2+}\)-CIB1ΔH10.**

A, steady state fluorescence emission spectra of 8-anilino-1-naphalenesulfonate alone (solid line) in the presence of full-length Ca\(^{2+}\)-CIB1 (dashed line) or in the presence of Ca\(^{2+}\)-CIB1ΔH10 (dotted line) show an increase in hydrophobic surface for the C-terminal-truncated protein. a.u., arbitrary units. B, top panel, base-line-corrected raw ITC data for the titration of Ca\(^{2+}\)-CIB1ΔH10 into buffer (heat of dilution), peptide H10p (H10p), or peptide αIIb-L (αIIb-L) performed at 37 °C. Bottom panel, derived binding isotherms for the H10p (■) and αIIb-L (△) titrations after subtraction of the H10p control experiment. C, top panel, base-line-corrected raw ITC data and (bottom panel) derived binding isotherm for titrations of smMLCKp into Ca\(^{2+}\)-CIB1 (■) or Ca\(^{2+}\)-CIB1ΔH10 (△) performed at 37 °C. The binding to Ca\(^{2+}\)-CIB1 was too weak to fit the data to any binding model, whereas the titration data with Ca\(^{2+}\)-CIB1ΔH10 could be fit to a one-site model with a K_o of ≈4 × 10^6 M\(^{-1}\). Note that there was essentially no heat of dilution for smMLCKp itself (not shown).

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**TABLE 2**

Thermodynamics of \( \text{Ca}^{2+} \)-CIB1ΔH10 binding to \( \alpha_{IIb} \)-L or smMLCKp, as determined by ITC

The values and errors for the 37 °C experiments with \( \alpha_{IIb} \)-L are the average and S.D. of three independent titrations. All other errors are calculated from the curve fitting. Note that there is no error associated with \( \Delta S \) because it is a calculated value.

| Peptide      | Temperature | \( N \) | \( K_a \) | \( \Delta H \) | \( \Delta S \) |
|--------------|-------------|--------|----------|-------------|-------------|
| \( \alpha_{IIb} \)-L | 37°C | 1.2 ± 0.0 | \( 1.4 \pm 0.1 \times 10^6 \) | -35.0 ± 3.6 | 1.5 |
|               | 33°C | 1.2 ± 0.0 | \( 1.1 \pm 0.2 \times 10^6 \) | -31.9 ± 1.1 | 3.5 |
|               | 30°C | 1.2 ± 0.0 | \( 1.2 \pm 0.3 \times 10^6 \) | -23.9 ± 0.8 | 11.4 |
|               | 25°C | 1.6 ± 0.0 | \( 8.2 \pm 2.1 \times 10^6 \) | -16.6 ± 0.7 | 17.2 |
|               | 20°C | 1.5 ± 0.1 | \( 2.2 \pm 1.1 \times 10^6 \) | -9.1 ± 0.5 | 26.5 |
| smMLCKp      | 37°C | 1.4 ± 0.2 | \( 4.2 \pm 1.8 \times 10^6 \) | -17.1 ± 5.0 | 10.4 |

* The increased stoichiometry of \( \alpha_{IIb} \)-L binding to \( \text{Ca}^{2+} \)-CIB1ΔH10 at lower temperatures is likely due to increased self-association of \( \text{Ca}^{2+} \)-CIB1ΔH10.

**FIGURE 7.** Model for the interaction between CIB1 and the \( \alpha_{IIb} \) subunit of platelet integrin \( \alpha_{IIb}\beta_3 \). A, in resting platelets the CIB1 binding region of \( \alpha_{IIb} \) is sequestered by \( \beta_3 \) and is partially buried within the cytoplasmic membrane. B, agonist-induced disruption of interactions between the cytoplasmic and transmembrane domains of \( \alpha_{IIb} \) and \( \beta_3 \) exposes the CIB1-binding site of \( \alpha_{IIb} \). The binding of CIB1 to \( \alpha_{IIb} \) involves the displacement of the C-terminal extension (green).

The NMR spectroscopy studies of \( \text{Ca}^{2+} \)-CIB1 have shown that the core helix-loop-helix EF-hand structure of the two lobes is similar in solution to the structures observed in each of the recent crystal forms of the protein. However, the N domain and C domain are oriented side by side, similar to the monomeric crystal form 1X05, consistent with the monomeric state of the protein in solution. The relatively high \( [\text{H}] \)-\( [\text{N}] \) NOE values for the central linker are consistent with a folded structure rather than a flexible loop, and these data together with the interdomain interactions suggested from studies with CIB1-C imply that the N and C domains of \( \text{Ca}^{2+} \)-CIB1 tumble together in solution with a fixed orientation. This contrasts with CaM, which has a flexible central linker (\( [\text{H}] \)-\( [\text{N}] \) NOE values of 0.2–0.4) and N and C domains, which tumble independently in solution (36). An interdomain interaction would result in more rapid T2 relaxation for the domains of \( \text{Ca}^{2+} \)-CIB1 in comparison to the independently tumbling domains of CaM and could explain why perdeuteration was necessary to obtain NMR spectra of suitable quality for resonance assignment. Interdomain interactions are also important to the structure and function of CnB (37) and many NCS proteins, and in fact a single mutation in the domain interface of some NCS proteins can severely alter their activity (38). The close association of the N and C domains is one feature that distinguishes this class of EF-hand proteins from CaM, troponin C, and related proteins, which have independent domains that retain similar structures and ligand binding properties in isolation (39, 40).

Another characteristic that distinguishes CIB1 from CaM is the mechanism of target protein binding. Like CaM and the NCS proteins, the side-by-side domain orientation of the \( \text{Ca}^{2+} \)-CIB1 domains generates a single binding surface. This differs from the N and C lobes of CaM, which adopt numerous distinct orientations to accommodate different target sequences (41). CaM also lacks N- and C-terminal extensions, and its hydrophobic target protein binding surfaces are exposed to the solvent in the absence of a binding partner, whereas the binding site of CIB1 is partially shielded by the C-terminal extension. The interaction between the C-terminal extension and the C domain of CIB1 is conceivable since downstream of Asp-182 it has hydrophobic/basic character and a similar amino acid content to the CIB1 binding region of \( \alpha_{IIb} \). Our interpretation is that this shielding mechanism is necessary to prevent self-association and reduce nonspecific binding of CIB1 to the hydrophobic sequences of non-biological targets. However, our data suggest that the C terminus is only weakly coordinated in this hydrophobic channel and can be easily displaced by \( \alpha_{IIb} \),
thereby providing a simple mechanism to increase target specificity. Sequestering the target binding site of CIB1 could be important since it is mostly composed of hydrophobic Leu, Ile, Val, and Phe residues, in contrast to the binding patches of CaM, which are very rich in more polar Met residues (42–44). The hydrophobic face of the EF-hands in many CIB1 homologs also have a similar amino acid content to CIB1 and are either partially or completely covered by their C-terminal extensions (45). However, the primary target binding site of recoverin has been mapped to the N domain (46), and truncation of the C-terminal extension from the NCS protein KChIP1 completely abolishes binding to its biological target (47). Therefore, the C-terminal extensions of different members within this protein family appear to have evolved other unique functions as well.

Our NMR data have shown that the N-terminal extension of Ca\(^{2+}\)-CIB1 adopts a structure similar to that observed in 1Y1A, including an \(\alpha\)-helix (H0) from Lys-10—Asp-18. Therefore, the extended structure of this region in 1X05 must be an artifact of crystal formation, as it was initially hypothesized by Parise and co-workers (13). The position of this H0 helix implies a role in extruding the myristoyl group into solution away from the protein for interaction with the cytoplasmic membrane. The glycerine-rich extreme N terminus of Ca\(^{2+}\)-CIB1 probably acts as a flexible tether between the myristoyl group and H0, allowing the membrane-anchored protein to adjust its orientation in solution to optimally interact with the \(\alpha\)IIb cytoplasmic/transmembrane domain. The N-terminal region of many CIB1 homologs also have similarly placed \(\alpha\)-helices and flexible N termini that perform a similar function (47, 48). Like CnB (49) and some NCS proteins (50), the association of CIB1 with biological membranes is Ca\(^{2+}\)-independent, indicating that the protein does not utilize a Ca\(^{2+}\)-myristoyl switch mechanism (51). This suggests the potential for constitutive membrane tethering of CIB1, which would increase the probability of binding to the membrane-anchored \(\alpha\)IIb cytoplasmic/transmembrane sequence. The binding of CIB1 to \(\alpha\)IIb is also Ca\(^{2+}\)-independent, since the binding is of similar affinity in vitro in the presence of Ca\(^{2+}\) or physiological Mg\(^{2+}\) concentrations (8). Therefore, regulation of the CIB1-\(\alpha\)IIb interaction is likely achieved in vivo through a dynamic interplay between conformational changes in the \(\alpha\)IIb and \(\beta_3\) subunits and the competitive binding of other regulatory proteins to the cytoplasmic domains. For example, Johansson and co-workers (52) have suggested that the primary CIB1 binding region of \(\alpha\)IIb may alternate between cytoplasmic and membrane-buried environments depending on the activation state of \(\alpha\)IIb\(\beta_3\) (Fig. 7). The binding of CIB1 might also require disrupting interactions between the \(\alpha\)IIb and \(\beta_3\) cytoplasmic or transmembrane domains themselves (53, 54). Once the binding site becomes accessible, regulation could be fine-tuned through competition between CIB1 and other proteins that have overlapping binding sites (55) or through phosphorylation of the integrin domains or CIB1 itself. We note with interest that the C-terminal extension of CIB1, which becomes exposed upon binding to \(\alpha\)IIb, contains a consensus protein kinase C (PKC) phosphorylation site (3), and PKC has been linked to \(\alpha\)IIb\(\beta_3\) signaling (56). The interplay between these different regulatory mechanisms would allow for strict control of the activation state of \(\alpha\)IIb\(\beta_3\) and the hemostatic processes that it regulates.

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