Solution Structures of Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 and Their Interactions with the Platelet Integron αIIb Cytoplasmic Domain

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The calcium- and integrin-binding protein 1 (CIB1) is a ubiquitous Ca$^{2+}$-binding protein and a specific binding partner for the platelet integron αIIb cytoplasmic domain, which confers the key role of CIB1 in hemostasis. CIB1 is also known to be involved in apoptosis, embryogenesis, and the DNA damage response. In this study, the solution structures of both Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 were determined using solution-state NMR spectroscopy. The methyl groups of Ile, Leu, and Val were selectively protonated to compensate for the loss of protons due to deuteration. The solution structure of Ca$^{2+}$-CIB1 possesses smaller opened EF-hands in its C-domain compared with available crystal structures. Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 have similar structures, but the N-lobe of Mg$^{2+}$-CIB1 is slightly more opened than that of Ca$^{2+}$-CIB1. Additional NMR experiments, such as chemical shift perturbation and methyl group solvent accessibility as measured by a nitroxide surface probe, were carried out to further characterize the structures of Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 as well as their interactions with the integrin αIIb cytoplasmic domain. NMR measurements of backbone amide proton slow motion (microsecond to millisecond) dynamics confirmed that the C-terminal helix of Ca$^{2+}$-CIB1 is displaced upon αIIb binding. The EF-hand III of both Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 was identified to be directly involved in the interaction of CIB1 with αIIb. Together, these data illustrate that CIB1 behaves quite differently from related EF-hand regulatory calcium-binding proteins, such as calmodulin or neuronal calcium sensor proteins.

The calcium- and integrin-binding protein 1 (CIB1) is a member of the regulatory Ca$^{2+}$-binding helix-loop-helix or EF-hand protein family (1). CIB1 is a ubiquitous 191-residue (22 kDa) protein with several functions in cell signaling (2, 3). CIB1 has been reported to interact with a number of protein targets, including the platelet integron αIIb subunit (4), sphingosine kinase 1 (5), p21-activated kinase (6), apoptosis signal-regulating kinase (7), polo-like protein kinases (8), and a recently reported new target in the regulation of cardiac hypertrophy, calcineurin B (9). The interaction of CIB1 with the integrin αIIb subunit has been studied extensively (10–13). The integron αIIb subunit usually associates with the integrin β3 subunit, and the heterodimeric αIIbβ3 protein is involved in both so-called “inside-out” and “outside-in” signaling pathways (3). CIB1 is believed to be capable of specifically binding to the αIIb cytoplasmic domain and dissociating the αIIbβ3 dimer, which in turn triggers integrin activation (11, 12). The interactions between Ca$^{2+}$-CIB1 and a fragment of the integrin αIIb subunit (residues 983–1008, hereafter referred to as the αIIb peptide) have been suggested to mainly involve a hydrophobic pocket in the C-domain of Ca$^{2+}$-CIB1 with a dissociation constant in the submicromolar range (10).

The calcium-bound CIB1 (Ca$^{2+}$-CIB1) structure consists of four helix-loop-helix (EF-hand) Ca$^{2+}$-binding motifs (EF-I to EF-IV) (14, 15), in which only EF-III and EF-IV have the capacity to bind Ca$^{2+}$. In addition, CIB1 contains a myristoylated N-terminal extension in vivo (16), and a short C-terminal extension that was postulated to be involved in a displacement mechanism to increase its target-binding specificity (12). So far, two x-ray crystal structures of Ca$^{2+}$-CIB1 have been deposited in the Protein Data Bank, in which 1XO5 (15) is a monomer and 1Y1A (14) is a head-to-tail homodimer. These two crystal structures have a similar arrangement of the secondary structural elements, but large differences exist in their relative orientations of the N- and C-domains as well as the secondary structure of their N- and C-terminal extensions. Our initial residual dipolar coupling (RDC) NMR study (12) suggested that the monomeric crystal structure of CIB1 (1XO5) more closely resembles the overall conformation of the protein in solution, except for its N-terminal extension.

Because the magnesium ion is constantly present in the cytosol in millimolar concentrations, the magnesium-bound form of CIB1 (Mg$^{2+}$-CIB1) is considered to be the physiologically relevant form of CIB1 in the resting state of the cell (17). CIB1 has been found to bind Mg$^{2+}$ only at its EF-III site, but it still possesses an overall well folded tertiary structure (17). It is fur...
ther suggested that Mg$^{2+}$-CIB1 and Ca$^{2+}$-CIB1 have similar tertiary structures with subtle differences (18). In addition, as both Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 can bind to αβl with similar binding affinities, the role of CIB1 for being a specific Ca$^{2+}$ sensor has been brought into question (10). Therefore the high resolution structure and dynamics of Mg$^{2+}$-CIB1 are of interest to better understand the mechanism of activation of CIB1 and the regulation of the interactions of CIB1 with its binding targets.

Here, we present the solution structures of both Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1, as well as a biophysical and NMR dynamics characterization of the interaction of CIB1 with αβl. The construct used for CIB1 had a His tag and a linker, with a total molecular mass of ~24 kDa. Thus, the acquisition of high-quality NMR data required the use of transverse relaxation optimized spectroscopy-type experiments (19) and perdeuteration of the protein (20). The solution structures of Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 NMR were subsequently determined by using chemical shift information, two sets of backbone RDCs ($^{1}D_{CN}$ and $^{1}D_{NH}$) in combination with the NOEs from backbone amide protons (NH) as well as selectively labeled methyl groups (Ile/Leu/Val) in an otherwise perdeuterated protein sample. The Carr-Purcell-Meiboom-Gill (CPMG) NMR method for measuring slow motion (microsecond to millisecond scale) dynamics (21) was employed to study the CIB1 backbone dynamic behavior in both the free state and the αβl peptide-bound state. Additionally, the TEMPOL paramagnetic surface probe (22, 23) was used to assess the solvent accessibility of the methyl groups in the hydrophobic pocket in different states of the protein.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The CIB1 protein was prepared as described previously (17). The $^{13}$C,$^{2}$H,$^{15}$N-uniformly labeled sample was expressed and purified according to a published protocol (12), and this sample was used to acquire the backbone $^{1}D_{NH}$ and $^{1}D_{CN}$ RDCs. The $^{13}$C,$^{2}$H,$^{15}$N-uniformly and Ile-$\delta$-l$^{13}$CH$_{3}$,Leu,Val-$^{15}$CH$_{3}$,12CD$_{3}$-labeled sample was prepared by expression in M9 minimal media in D$_{2}$O supplemented with $^{15}$NH$_{4}$Cl, $^{13}$C-labeled glucose, and the methyl labeling precursors 2-keto-3-d$_{2}$-1,2,3,4-$^{13}$C-butyrate (Cambridge Isotope Laboratories) and 2-keto-3-methyl-3-d$_{1}$-1,2,3,4-$^{13}$C-butyrate (Cambridge Isotope Laboratories) (24); this sample was used for the acquisition of methyl assignment spectra. The $^{13}$C,$^{2}$H,$^{15}$N-uniformly and Ile-$\delta$-l$^{13}$CH$_{3}$,Leu,Val-$^{15}$CH$_{3}$,12CD$_{3}$-labeled CIB1 was prepared using M9 minimal media in D$_{2}$O supplemented with $^{15}$NH$_{4}$Cl, $^{13}$C-labeled glucose, and the methyl labeling precursors 2-keto-3-d$_{2}$-1,2,3,4-$^{13}$C-butyrate (Cambridge Isotope Laboratories) and 2-keto-3-methyl-3-d$_{1}$-1,2,3,4-$^{13}$C-butyrate (Cambridge Isotope Laboratories) (24); this sample was used to acquire the $^{15}$N-edited NOEY and $^{13}$C-edited NOEY spectra. Finally, the $^{2}$H,$^{15}$N-uniformly labeled sample was prepared with M9 minimal media in D$_{2}$O supplemented with $^{15}$NH$_{4}$Cl and d$_{6}$-glucose; this sample was used to record the CIB1 backbone CPMG-relaxation dispersion spectra. The synthetic 26-residue αβl peptide (Ac-LVLMWKVGFKNRP-PLEEDDEEGQ-OH) is the same as used in earlier studies (12), and it corresponds to amino acids 983–1008 of the platelet integrin αβl subunit, with Gln-1008 as the C-terminal residue. The synthetic peptide was purchased from GenScript Corp. and was over 95% pure as determined by mass spectrometry and high performance liquid chromatography. Protein and peptide concentrations were either determined by using the Bio-Rad protein assay kit or UV absorbance measurements based on the extinction coefficients $\varepsilon_{280} = 3105$ for CIB1 and $\varepsilon_{280} = 5500$ for the αβl peptide.

**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 cryo-probe. The CIB samples for the $^{13}$C-edited NOEY experiments contained 50 mM HEPES in 99.9% D$_{2}$O. For other NMR CIB1 samples with or without αβl, they all contain 0.6–0.7 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% D$_{2}$O, pH 7.5 ± 0.05. For Mg$^{2+}$-CIB1 samples, 0.5 mM EGTA and 6 mM MgCl$_{2}$ were added; for Ca$^{2+}$-CIB1, 4 mM CaCl$_{2}$ was added. The $^{13}$C,$^{2}$H,$^{15}$N-uniformly labeled samples were used to acquire two sets of backbone RDCs ($^{1}D_{CN}$ and $^{1}D_{NH}$). In this sample, 14 mg/ml pf1 phage (Asla-Biotech) was used to achieve partial precipitation of the protein molecules. $^{1}D_{NH}$ RDCs were measured using a two-dimensional IPAP-HSQC experiment (25) with complex points of 1024 × 1024. After linear prediction and zero filling, the digital resolution was 0.83 Hz/pt in the $^{15}$N dimension. $^{1}D_{CN}$ RDCs were measured using the three-dimensional IPAP-J-HNCO (CA) experiment (26), with 1024 × 128 × 40 complex points. The digital resolution was 2.06 Hz/pt in F2 ($^{13}$C) after linear prediction and zero filling. A scale factor of 4 was used in the measurement of the $^{1}D_{CN}$ RDC. The obtained $^{1}D_{NH}$ RDC of Mg$^{2+}$-CIB1 was analyzed against the solution structure of Ca$^{2+}$-CIB1 with the program PALES (27) to evaluate the correlation between the Mg$^{2+}$-CIB1 and Ca$^{2+}$-CIB1 solution structures. In the PALES analysis, a linear correlation indicates the degree to which the experimentally obtained RDCs correspond to an existing protein structure, with a high correlation factor R and a low quality factor Q indicating good agreement.

A recently published three-dimensional multiple quantum (MQ) (H)CCMhm-TOCSY experiment was employed to assign the methyl groups of CIB1 (28). This methyl assignment approach requires preliminary knowledge of the chemical shifts of Ca and Cβ, which have been previously obtained from the backbone assignment work (12, 18, 29). Stereospecific assignments for Val and Leu methyl groups were obtained using the 10% $^{13}$C glucose labeling strategy (30) for Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 and Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 in complex with the αβl peptide. The methyl chemical shift perturbation (CSP) was calculated using Equation 1, which is revised based on a previously published study (31), in which the δH and δC indicate the proton and carbon chemical shift changes, respectively.

$$\text{CSP} = \sqrt{\delta H^2 + (\delta C/2)^2}$$  \hspace{1cm} (Eq. 1)

Titrations with 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxo (TEMPOL) (Sigma) were performed using 600 μM samples of $^{2}$H,$^{15}$N-uniformly and Ile-$\delta$-l$^{13}$CH$_{3}$,Leu,Val-$^{15}$CH$_{3}$,12CD$_{3}$-labeled CIB1 samples.
\(^{13}\text{CH}_3\), \(^{12}\text{CD}_3\)-labeled CIB1, being titrated with 6 eq of a freshly prepared concentrated stock solution of TEMPOL in 100 mM KCl, 99% \(\text{D}_2\text{O}\), pH 7.5 (not corrected for isotope effects).

The backbone amide \(^{15}\text{N}\) relaxation dispersion measurements were carried out using a CPMG relaxation dispersion experiment (21) implemented with an inter-scan delay of 2 s and a total constant CPMG length \((T_{CP})\) of 61.08 ms. Two sets of \(CT\)-CPMG experiments were acquired at CPMG field strengths \((\nu_{CPMG})\) of 50 and 500 Hz, respectively. For each CPMG field strength, two replicate data sets were collected each with 32 dummy scans and 64 scans per \(t\) point. Duplicates were co-added using NMRPipe prior to processing. Duplicates at each frequency were acquired and analyzed for standard error. Peaks affected by partial or total overlap were excluded from analysis. The NMR relaxation dispersion \((\Delta R_{2,\text{eff}})\) was quantified as (32) as shown in Equations 2–4,

\[
\Delta R_{2,\text{eff}} = R_{2,\text{eff}}(50\text{ Hz}) - R_{2,\text{eff}}(500\text{ Hz}) \quad (\text{Eq. 2})
\]

\[
R_{2,eff}(\nu_{CPMG}) = (-1/T_{CP})\ln(I_{CPMG}/I_0) \quad (\text{Eq. 3})
\]

\[
\Delta R_{2,eff} = (1/T_{CP})\ln(I_{500Hz}/I_{100Hz}) \quad (\text{Eq. 4})
\]

**Structure Calculations**—A two-stage simulated annealing approach (33) using the program XPLOR-NIH 2.18 (34) was implemented for the structure determination of \(\text{Ca}^{2+}-\text{CIB1}\) and \(\text{Mg}^{2+}-\text{CIB1}\). The experimental restraints include two sets of backbone RDCs \((^1D_{CN} \text{ and } ^3D_{NH})\) (26), the dihedral angle restraints predicted from NMR chemical shifts using TALOS (35), hydrogen bonding restraints from the Chemical Shift Index (36), NOEs (NH-NH, NH-methyl, and methyl-methyl), and metal ion binding coordinates (15). For the NH-NH and NH-methyl NOEs from the \(^{15}\text{N}\)-edited HSQC-NOESY experiment (mixing time 120 ms), two categories (1.8–5.0 and 1.8–6.0 \(\text{Å}\)) were created based on the peak intensities; for the methyl-methyl NOEs from \(^{13}\text{C}\)-HMOC-NOESY (mixing time 200 ms) acquired with a sample in \(\text{D}_2\text{O}\) solvent, two slightly larger categories (1.8–6.0 and 1.8–8.0 \(\text{Å}\)) were implemented. Because the N-terminal region of both \(\text{Ca}^{2+}-\text{CIB1}\) and \(\text{Mg}^{2+}-\text{CIB1}\), including the His tag, and the first six residues could not be observed by NMR, we excluded this region from structure calculation. The details of structure calculation were elaborated in the *supplemental material*. Procheck 3.5.4 (37) was used to check the calculated structures for stereochemical quality. The inter-helical angles of the EF-hands were measured using an in-house script. The structural differences between \(\text{Ca}^{2+}-\text{CIB1}\) and \(\text{Mg}^{2+}-\text{CIB1}\) were assessed with difference distance matrix plot (DDMP) (38).

**Data Deposition**—Backbone and methyl chemical shift assignments for \(\text{Ca}^{2+}-\text{CIB1}\) and \(\text{Mg}^{2+}-\text{CIB1}\) have been deposited in the BioMagResBank with the accession numbers 17329 and 17328, respectively. The coordinates of the calculated structure ensembles and corresponding experimental restraints used in the structure calculation have been deposited in the Protein Data Bank with the accession codes 2L4H for \(\text{Ca}^{2+}-\text{CIB1}\) and 2L4I for \(\text{Mg}^{2+}-\text{CIB1}\), respectively.

**Supplemental Material**—This section contains the details of the structure calculation procedure of using a two-stage low temperature simulated annealing. It also contains the complete assignments of the methyl resonances for \(\text{Ca}^{2+}-\text{CIB1}\), \(\text{Mg}^{2+}-\text{CIB1}\), and \(\text{Ca}^{2+}-\text{CIB1}\) bound to the \(\text{a}1\text{lb}\) peptide and \(\text{Mg}^{2+}-\text{CIB1}\) bound to the \(\text{a}1\text{lb}\) peptide. In addition, the Hsqc spectra of TEMPOL titration in \(\text{Ca}^{2+}-\text{CIB1}/\text{a}1\text{lb}\) and \(\text{Mg}^{2+}-\text{CIB1}/\text{a}1\text{lb}\) are also included.

**RESULTS**

**Secondary Structure of \(\text{Ca}^{2+}-\text{CIB1}\) and \(\text{Mg}^{2+}-\text{CIB1}\)**—Two crystal structures of \(\text{Ca}^{2+}-\text{CIB1}\) have been reported, in which one is a monomer (15) and the other one is a head-to-tail homodimer (14) with a glutathione (GSH) molecule noncovalently bound to the N-domain of chain B. These two crystal structures have significant differences in the relative orientation of the N- and C-domains (12, 39). Even though the amide NH-RDC analysis results suggested that the monomer crystal structure of \(\text{CIB1}\) (Protein Data Bank 1X05) more closely resembles the conformation of the protein in solution (12), the secondary structure of 1X05 differs from the solution state conformation in the N- and C-terminal extensions (Fig. 1). To some extent, the structural differences for these extensions can be explained by the presence of weak \(\text{Ca}^{2+}\)-binding sites and the extremely high calcium concentration used to induce crystallization (39). In contrast, 1Y1A has good agreement with the NMR study in terms of secondary structure, including the N- and C-terminal extensions (Fig. 1). In the solution state, \(\text{Mg}^{2+}-\text{CIB1}\) has an overall similar secondary structure arrangement as \(\text{Ca}^{2+}-\text{CIB1}\) (Fig. 1), but the \(\text{Mg}^{2+}\) can only occupy EF-III of \(\text{CIB1}\) (17), leaving site EF-IV in intermediate conformational exchange between the \(\text{Mg}^{2+}\)-bound and -unbound states. This is similar to other calcium-binding proteins, e.g. calmodulin (40) and soybean calmodulin isoform 4 (41). Unfortunately, this means that most resonances in the C-terminal portion (residues 158–191) are missing for \(\text{Mg}^{2+}-\text{CIB1}\) (18). In addition, backbone heteronuclear \(^{1}H,^{15}N\) NOE dynamics for both \(\text{Ca}^{2+}-\text{CIB1}\) (12) and \(\text{Mg}^{2+}-\text{CIB1}\) (18) suggest the notion that the N-terminal end (residues 8–20) of \(\text{CIB1}\) should adopt relatively rigid secondary structure rather than being an unstructured flexible tail.

**Methyl Group Assignments and Their Utilization in the Structure Determination**—To compensate for the loss of proton signals caused by deuteration of the protein, the methyl groups of the hydrophobic residues Ile-\(\delta\)-\(\text{L}^{13}\text{CH}_3\)-Leu,Val-\(\text{L}^{13}\text{CH}_3\)-\(\text{L}^{12}\text{CD}_3\) were selectively labeled with a published protocol (24). The methyl groups of Ile, Leu, and Val in \(\text{Ca}^{2+}-\text{CIB1}\) and \(\text{Mg}^{2+}-\text{CIB1}\) could be assigned using a three-dimensional multiple quantum (MQ) (H)CCmHm-TOCSY (28) experiment. The stereospecific assignment of the methyl groups of the \(\text{Ca}^{2+}-\text{CIB1}\) (supplemental Fig. 1A), \(\text{Mg}^{2+}-\text{CIB1}\) (supplemental Fig. 1B), \(\text{Ca}^{2+}-\text{CIB1}\) in complex with the \(\text{a}1\text{lb}\) peptide (supplemental Fig. 2A), and \(\text{Mg}^{2+}-\text{CIB1}\) in complex with the \(\text{a}1\text{lb}\) peptide (supplemental Fig. 2B), obtained by using the 10\% \(\text{L}^{13}\text{C}\)-glucose approach (30), are displayed in the supplemental material. For \(\text{Mg}^{2+}-\text{CIB1}\), the backbone assignment for the C-terminal region (residues 158–191) is largely missing, but the methyls on this fragment could still be assigned by referring to the assignment of \(\text{Ca}^{2+}-\text{CIB1}\) (supplemental Fig. 1A) as well as the knowledge about the numbers of Ile/Leu/Val residues in \(\text{CIB1}\). The methyl-labeled \(\text{CIB1}\) samples have been used for the
acquisition of $^{15}$N-, and $^{13}$C-edited NOESY spectra, and the NOEs obtained in this manner were used in the structure determination of Ca$^{2+}$/H11001-CIB1 and Mg$^{2+}$/H11001-CIB1.

Solution Structures of Ca$^{2+}$/H11001-CIB1 and Mg$^{2+}$/H11001-CIB1—To understand the molecular details of the regulatory mechanism for the interactions of CIB1 with its various targets, we determined the solution structures of Ca$^{2+}$/H11001-CIB1 and Mg$^{2+}$/H11001-CIB1. In our calculation, a two-stage low temperature simulated annealing approach (33) was employed to refine the crystal structure of Ca$^{2+}$/H11001-CIB1 because of high similarity in secondary structure between 1XO5 and the results predicted by the NMR chemical shift index. Moreover, 1XO5 is a monomeric protein, and our previous NMR data are consistent with the protein being in a monomeric form in solution (12, 42). The same protocol has been successfully employed in the structure determination of several other proteins (33, 41, 43, 44). The $^{13}$C- and $^{15}$N-edited NOESY provided distance restraints for our structure calculation in addition to the dihedral angle, hydrogen bonds, and the RDC restraints for the protein backbone. The calculation results are summarized in Table 1. The refined solution structure of Ca$^{2+}$/H11001-CIB1 is overall similar to the 1XO5 crystal structure with a backbone r.m.s.d. of 2.71 Å for the well defined regions (residues 24–136 and 146–191). In the newly determined Ca$^{2+}$/H11001-CIB1 solution structure, the secondary structures of the N- and C-terminal extensions (H0 and H10) have been corrected to be consistent with the solution state studies (12) and the other crystal structure 1Y1A.
Interestingly, we found that the solution structure of Ca\(^{2+}\)-CIB1 has a more closed conformation than 1X05. For EF-hand calcium-binding proteins, the interhelical angles have been widely employed to characterize the opening of EF-hands (41, 45–47). We used an in-house script to analyze the interhelical angles of each EF-hand in Ca\(^{2+}\)-CIB1 structures (1X05, 1Y1A, and the solution structure) (Table 2) with smaller angles indicating larger openings. Site EF-I was excluded from this analysis because it is atypical as it contains several short α-helices (Fig. 1). In the Ca\(^{2+}\)-CIB1 solution structure, site EF-II in the N-domain has a similar opening of 119.9 ° ± 2.2 ° compared with 1X05 (116.4 °); however, the EF-III (122.8 ° ± 1.7 °) and EF-IV (122.2 ° ± 0.7 °) sites in the C-domain have smaller openings compared with 1X05 (EF-III 113.3 ° and EF-IV 109.0 °) (Table 2). Therefore, sites EF-III and EF-IV in the solution structure of Ca\(^{2+}\)-CIB1 are about 10° less open compared with 1X05 (Fig. 3C), which is similar to the case of calmodulin (CaM), where the solution structure is also found to be more closed than the crystal structure (46).

It has been suggested that the solution structure of Mg\(^{2+}\)-CIB1 is quite similar to that of Ca\(^{2+}\)-CIB1 on the basis of NMR-HSQC and fluorescence spectroscopy data combined with mutagenesis results (18); however, it has also been suggested that the relatively small structural differences between Mg\(^{2+}\)-CIB1 and Ca\(^{2+}\)-CIB1 may contribute to their different thermodynamic behavior in their interactions with the calb peptide (10). Therefore, the structure determination for Mg\(^{2+}\)-CIB1 was also conducted. The \(^{1}D_{NH}\) RDC values for Mg\(^{2+}\)-CIB1 show that there is a good correlation between the Mg\(^{2+}\)-CIB1 and Ca\(^{2+}\)-CIB1 solution structures (Fig. 2A) with a relatively high correlation factor of \(R = 0.93\) and a quality factor of \(Q = 36\%\). In fact, the differences between these two structures are distributed throughout the whole protein sequence with the outliers Leu-13, Tyr-16, Glu-26, Phe-55, Asp-93, Ala-111, and Cys-134 (Fig. 2B). Because the backbone resonances of residues 158–191 are largely missing, we chose to only present the solution structure for residues 8–157 for Mg\(^{2+}\)-CIB1. The solution structure of Mg\(^{2+}\)-CIB1 is overall similar to the Ca\(^{2+}\)-CIB1 solution structure with an r.m.s.d. of 3.28 Å for the well defined area (residues 24–136 and 146–157) (Fig. 3E). The interhelical angles were also measured to evaluate the opening of the EF-hands in Mg\(^{2+}\)-CIB1 (Table 2). Site EF-III in the C-domain of Mg\(^{2+}\)-CIB1 has a similar degree of opening compared with Ca\(^{2+}\)-CIB1. However, the interhelical angle (113.2 ° ± 1.1 °) of site EF-II in the N-domain of Mg\(^{2+}\)-CIB1 is slightly more open than Ca\(^{2+}\)-CIB1 (119.9 ° ± 2.2 °) (Fig. 3F and Table 2); this result was unexpected compared with several other calcium-binding proteins, e.g. CaBp1 (45) and CaM (33).

Because the structural differences between Ca\(^{2+}\)-CIB1 and Mg\(^{2+}\)-CIB1 are relatively small, a DDMP (Fig. 4) was used to illustrate the subtle differences. Overall, the differences are distributed all over the examined fragments (residues 8–157) with the most significant differences in helix 3b and 5 (Fig. 4). Because we have observed that the EF-II (containing H4 and H5) of Mg\(^{2+}\)-CIB1 is more open than that of Ca\(^{2+}\)-CIB1 (Fig. 3), the H3b is another new site showing difference between these two structures. Because site EF-I (containing H2, H3a and H3b) has several short α-helices (Fig. 1), it was not examined for an inter-helical angle.

**TABLE 2**

Interhelical angles of the EF-hands in two crystal structures of Ca\(^{2+}\)-CIB1 (1X05 and 1Y1A) and the Ca\(^{2+}\)-CIB1 and Mg\(^{2+}\)-CIB1 solution structures

| Helix pair       | 1X05 (x-ray) | 1Y1A (x-ray) | Ca\(^{2+}\)-CIB1 (NMR) | Mg\(^{2+}\)-CIB1 (NMR) |
|------------------|-------------|-------------|------------------------|------------------------|
| H4/H5 (EF-II)   | 116.4       | 128.0       | 119.9 ± 2.2            | 113.2 ± 1.1            |
| H6/H7 (EF-III)  | 113.3       | 116.6       | 122.8 ± 1.7            | 121.2 ± 1.2            |
| H8/H9 (EF-IV)   | 109.0       | 112.2       | 122.2 ± 0.7            | ND*                    |

* ND means not determined.

**FIGURE 2. Correlation between the Ca\(^{2+}\)-CIB1 and Mg\(^{2+}\)-CIB1 solution structures revealed by RDC analysis.** The backbone NH-RDC of Mg\(^{2+}\)-CIB1 shows a good correlation with the solution structure of Ca\(^{2+}\)-CIB1 (A), in which the outliers are labeled. The absolute values of the difference between the observed RDC and calculated RDC are plotted as a function of the primary sequence (B), and a horizontal line highlights the outliers.
Solution Structures of Ca$^{2+}$- and Mg$^{2+}$-CIB1

Methyl Groups Probe the Interaction between CIB1 and αIIb—The methyl groups of the αIIb peptide-bound forms of Ca$^{2+}$- and Mg$^{2+}$-CIB1 have been assigned as well (supplemental Fig. 2). The binding of the αIIb peptide induces significant CSP, which are shown in Fig. 6, A and D. For Ca$^{2+}$-CIB1 (Fig. 6, A–C), the methyl-containing residues Ile-114, Leu-123, Leu-131, Leu-135, Ile-168, and Ile-177 have the largest CSP values, and they are likely in the interface between CIB1 and αIIb.

Unexpectedly, Mg$^{2+}$-CIB1 appears to have more methyl groups exposed to the solvent than Ca$^{2+}$-CIB1 as follows: five Ile (Ile-73, Ile-114, Ile-153, Ile-177, and Ile-189), seven Leu (Leu-19, Leu-64, Leu-95, Leu-131, Leu-135, Leu-144, and Leu-191), and three Val (Val-97, Val-132, and Val-176) (Fig. 5B). When 5 mM Ca$^{2+}$ was subsequently added into the Mg$^{2+}$-CIB1/TEMPOL sample to replace the Mg$^{2+}$ and occupy the metal binding loops of EF-III and EF-IV of CIB1 (17), an identical HSQC spectrum was obtained compared with the Ca$^{2+}$-CIB1/TEMPOL (result not shown). We conclude that the difference of the methyl group exposure is essentially caused by the intrinsic structural and dynamic differences between Mg$^{2+}$- and Ca$^{2+}$-CIB1.

The experiments were also done with saturating amounts of the αIIb peptide added into the TEMPOL saturated Ca$^{2+}$- (supplemental Fig. 3A) and Mg$^{2+}$-CIB1 (supplemental Fig. 3B) samples. Similar to the results obtained with peptide-bound CaM (22), addition of the αIIb peptide results in a regular binding mode of CIB1 in the presence of TEMPOL. In both cases (supplemental Fig. 3, A and B), Ca$^{2+}$-CIB1/αIIb/TEMPOL and Mg$^{2+}$-CIB1/αIIb/TEMPOL show HSQC spectra identical to the protein complex samples without TEMPOL, except for the weakened signal of the α1 and α2 methyl groups on Leu-95 and the γ1 methyl group on Val-132. Given that almost all methyl groups (except for Leu-95 and Val-132) have recovered their intensity compared with samples with no TEMPOL added, it seems likely that binding of the αIIb peptide sterically prevents the access of TEMPOL to the hydrophobic pockets in both Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1. The solvent exposure of residues Leu-95 and Val-132 in the complexes may be caused by the remaining structural plasticity of the complex. Moreover, these data suggest that the complex structures of Ca$^{2+}$-CIB1/αIIb and Mg$^{2+}$-CIB1/αIIb will be quite similar.
IIb binding, suggesting that this area in the N-domain of Ca\(^{2+}\)-CIB1 could also be involved in the interaction with IIb.

Mg\(^{2+}\)-CIB1 shows a similar pattern as Ca\(^{2+}\)-CIB1 when interacting with the IIb peptide (Fig. 6, D–F), with the Ile-114, Leu-123, Leu-128, Leu-131, Leu-135, Ile-168, and Ile-177 having the largest CSP values. All the above residues in the C-domain of CIB1, and again some residues around Leu-64 in the N-domain are also slightly affected by the IIb binding.

To further map the IIb interactions with CIB1, the methyl groups were classified based on the CSP and mapped onto the

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**FIGURE 4. Difference distance matrix plot to compare the structural differences between Ca\(^{2+}\)-CIB1 and Mg\(^{2+}\)-CIB1 (residues 8–157).** The secondary structure was labeled with filled boxes indicating helices and arrows indicating \(\beta\)-strands. The program DDMP gives real values of differences concerning C\(\alpha\) atoms in two structures. For example, the distance between the C\(\alpha\) atoms of \(i\) and \(j\) residues in the Ca\(^{2+}\)-CIB1 structure is 10 Å, and the corresponding distance in structure Mg\(^{2+}\)-CIB1 is 15 Å, and hence \([\text{Ca}^{2+}\cdot\text{CIB1}])_{ij} - [\text{Mg}^{2+}\cdot\text{CIB1}])_{ij} = 10 - 15 = -5\ Å. The red and blue color shading is indicated above the figure.
solution structures of Ca\(^{2+}\)- and Mg\(^{2+}\)-CIB1 (Fig. 6B). In Fig. 6B, the C-terminal extension (residues 179–191) of Ca\(^{2+}\)-CIB1 solution structure is colored golden to demonstrate the C-terminal displacement mechanism. Overall, it seems that the major driving force for the interactions between Ca\(^{2+}\) and Mg\(^{2+}\)-CIB1 is the hydrophobic interaction that occurs in the C-domain, whereas the area in the N-domain around Leu-64 is also involved in this interaction. Specifically, the 7th helix (H7) is clearly extensively involved in the interaction of CIB1 with the αIIB peptide in both the Ca\(^{2+}\)-bound (Fig. 6C) and Mg\(^{2+}\)-bound (Fig. 6F) states, with the majority of the methyl group CSP located on H7.

**Microsecond Dynamics Reveals the Plasticity of CIB1 and the CIB1-αIIB Complex**—The heteronuclear \(^{1}H\)-\(^{15}N\) NOE dynamic approach has been employed previously to study the fast motion dynamics (nanosecond to picosecond time scale) of Ca\(^{2+}\)-CIB1, Mg\(^{2+}\)-CIB1 in complex with the αIIB peptide (12), and Ca\(^{2+}\)-CIB1 (29) to analyze the backbone flexibility of the CIB1 protein under different conditions. It has been widely accepted that slow motion dynamics (microsecond to millisecond time scale) is directly relevant to the biological function of many proteins (20, 49, 50). In particular, solution dynamics CPMG relaxation dispersion measurements have been used to characterize protein conformational exchange (21), ligand-binding site chemical exchange (51), and allostery mapping (32). In our studies, the CPMG slow motion dynamics experiments for the backbone residues of Ca\(^{2+}\)-CIB1, Mg\(^{2+}\)-CIB1, as well as Ca\(^{2+}\)-CIB1 and Mg\(^{2+}\)-CIB1 in complex with the αIIB peptide were carried out. The change in effective relaxation rates \(\Delta R_2,\text{eff} = R_2,\text{eff} (500 \text{ Hz}) - R_2,\text{eff} (50 \text{ Hz})\) can be used to characterize the intrinsic slow motion (microsecond to millisecond time scale) of the protein as well as its contributions to
exchange upon interaction with αIIb. The N- and C-domains of Ca\textsuperscript{2+}-CIB1 have been observed to have different types of slow motion dynamics (Fig. 7B). The ΔR\textsubscript{2,eff} values for the N-domain of Ca\textsuperscript{2+}-CIB1 are between 0 and 7 s\textsuperscript{-1} with an evenly distributed fluctuation throughout the entire N-domain (Fig. 7B), indicating the plasticity of the N-domain of Ca\textsuperscript{2+}-CIB1. In contrast, the ΔR\textsubscript{2,eff} values for the C-domain are relatively more convergent, with the exceptions of the loop area between EF-III and EF-IV (residues 138–143) and the C-terminal extension (residues 184–191). Once Ca\textsuperscript{2+}-CIB1 binds the αIIb peptide, Ca\textsuperscript{2+}-CIB1 displays an overall increased conformational and/or chemical exchange behavior, with most residues falling in the ΔR\textsubscript{2,eff} value range of 0–15 s\textsuperscript{-1}. Helices 6, 7, 9, and 10 in the C-domain of the CIB1-αIIb complex demonstrate signifi-
cantly increased exchange rates upon binding the αIIb peptide, suggesting their involvement in the interaction with the αIIb peptide. In the Ca\(^{2+}\)-CIB1-αIIb complex, the loop area in the C-domain of Ca\(^{2+}\)-CIB1 retained slow motion flexibility, and the C-terminal end has acquired increased slow motion with residues 174–191 having a significantly higher $\Delta R_{2, eff}$ (Fig. 7C).
than the nearby C-domain (residues 160–173). These observations provide additional support for the proposed C-terminal displacement mechanism (12). The slow motional on/off switching of the C-terminal extension not only allows for the interactions between Ca$^{2+}$-CIB1 and the αIIb peptide, it also prevents nonspecific interactions between other nonpolar substances and the hydrophobic pocket of the C-domain of Ca$^{2+}$-CIB1 (12).

Similarly, the Mg$^{2+}$-CIB1-αIIb complex is more dynamic than Mg$^{2+}$-CIB1 alone (Fig. 7, E and F). Although the C-terminal end (residues 158–191) of Mg$^{2+}$-CIB1 could only be partially assigned (18), the corresponding part of Mg$^{2+}$-CIB1, once complexed with the αIIb peptide, can be almost fully assigned. Apparently, the presence of the αIIb peptide increases the binding affinity of the EF-IV loop for Mg$^{2+}$ resulting in the stabilization of the structure in the EF-IV region (29). Comparison of the ΔR$_{2}$off of Mg$^{2+}$-CIB1 and the αIIb-bound form of the protein reveals that H7 and H9 could be directly involved in the interaction with the αIIb peptide, which is consistent with the CSP results (Fig. 6F). In addition, for both Ca$^{2+}$- and Mg$^{2+}$-CIB1, H0 and partially H1 in the N-terminal domain have enhanced exchange upon binding αIIb, which might imply the involvement of the N-terminal myristoyl group in these interactions, and this has been observed in the case of sphingosine kinase 1 (5).

**DISCUSSION**

In this study, the solution structures of Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 were determined by using several solution NMR restraints, including two sets of backbone RDCs and NOEs (NH-NH, NH-methyl, and methyl-methyl). The Ca$^{2+}$-CIB1 structure that was obtained shows a relatively small deviation from the 1XO5 crystal structure, with an r.m.s.d. of 2.71 Å. However, the opening of sites EF-III and EF-IV in Ca$^{2+}$-CIB1 solution structure are smaller than in 1XO5 by 6–10° (Table 2), resulting in the activation of integrin.

For the interaction of CIB1 and the αIIb peptide, even though both the backbone (12) and methyl CSP suggest that the hydrophobic interactions between the N-terminal end of the αIIb peptide and the C-domain of the CIB provide the major driving force, the N-domain of CIB1 is still likely involved in this interaction, e.g. with Arg-33 (12), forming electrostatic salt bridges. Ca$^{2+}$-CIB1 has been predicted to take an orientation with the α-helical N-terminal end of the αIIb peptide buried into the hydrophobic pocket of Ca$^{2+}$-CIB1 and the negatively charged and extended C-terminal end of the αIIb peptide interacting with the N-domain of Ca$^{2+}$-CIB1 (53). We suggest that the side chain of Lys-65 also forms electrostatic interactions with αIIb based on the relatively large chemical shift changes observed around Leu-64 (Fig. 6). The regulation of integrin activity is intriguing, and a role for CIB1 in the regulation of αIIbβ3 activation has been suggested by several groups (3, 11). Salt bridges (e.g. αIIb (Arg-995)/β3 (Asp-723) and αIIb (Arg-995)/β3 (Glu-726)) and aromatic ring stacking (e.g. αIIb (Phe-992 to Phe-993)/β3 (Trp-715)) between αIIb and β3 of integrin are important in maintaining the heterodimeric structure of the αIIbβ3 complex (54, 55). We suggest that CIB1 could interrupt these interactions between αIIb and β3 and further dissociate the αIIb and β3, resulting in the activation of integrin.

It has been discussed that there are three typical mechanisms for the regulation of neuronal calcium sensor proteins (56), which is a protein family homologous to CIB1 as follows: (i) the calcium-independent type, e.g. frequenin; (ii) the calcium-myristoyl switch, e.g. recoverin; and (iii) a relatively small structural but substantial change in dynamics, e.g. GCAPI. Like the neuronal calcium sensor proteins, CIB1 possesses two lobes that interact with each other, unlike CaM, where the two lobes
rotate almost independently (57). However, based on what we observed in these studies, it seems that CIB1 has a distinct regulatory mechanism, which resembles the aforementioned type (iii) mechanism with relatively small structural differences between Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 and slightly different degree opening of site EF-II. The CPMG experiments also indicate the flexible loop between EF-III and EF-IV (residues 138–145) of Mg$^{2+}$-CIB1, and Ca$^{2+}$-CIB1 might be involved in the regulation of the activities of CIB1 by working as a spring-like apparatus to adjust the size of the hydrophobic pocket in the C-domain upon binding various targets. Taken together, the comparison of the solution structures of Ca$^{2+}$-CIB1 and Mg$^{2+}$-CIB1 leads to the conclusion that Mg$^{2+}$-CIB1 in the cytoplasm has a more open and more dynamic conformation compared with Ca$^{2+}$-CIB1, which is likely to contribute to its physiological functions.

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