Structural and Biochemical Characterization of CIB1 Delineates a New Family of EF-hand-containing Proteins*

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CIB1 (CIB) is an EF-hand-containing protein that binds multiple effector proteins, including the platelet αIIbβ3 integrin and several serine/threonine kinases and potentially modulates their function. The crystal structure for Ca2⁺-bound CIB1 has been determined at 2.0 Å resolution and reveals a compact α-helical protein containing four EF-hands, the last two of which bind calcium ions in the standard fashion seen in many other EF-hand proteins. CIB1 shares high structural similarity with calcineurin B and the neuronal calcium sensor (NCS) family of EF-hand-containing proteins. Most importantly, like calcineurin B and NCS proteins, which possess a large hydrophobic pocket necessary for ligand binding, CIB1 contains a hydrophobic pocket that has been implicated in ligand binding by previous mutational analysis. However, unlike several NCS proteins, Ca2⁺-bound CIB1 is largely monomeric or ligand-free. Differences in structure, oligomeric state, and phylogeny define a new family of CIB1-related proteins that extends from arthropods to humans.

One important mechanism by which cells respond to changes in Ca2⁺ levels involves the activation of EF-hand proteins, such as calmodulin. Upon Ca2⁺ binding, these proteins undergo structural changes that allow them to bind and activate target proteins. At least two specific structural changes can occur in EF-hand-containing proteins due to Ca2⁺ binding that facilitate signal transduction. The first type of change occurs when a hydrophobic binding pocket becomes accessible, thus facilitating ligand binding. The second type of change, termed a myristoyl switch, involves the extrusion of an N-terminal myristoyl group from a hydrophobic cavity in the protein, thereby leading to membrane localization, as described in recoverin (1) and related proteins. Recoverin and related proteins are termed neuronal calcium sensors (NCS) due to their neuronal expression pattern and high degree of homology (2). These proteins have a variety of functions; recoverin directly inhibits rhodopsin kinase (3), while a subgroup of NCS proteins that includes frequenin modulates synaptic activity, neurotransmitter release, and vesicle secretion (2). NCS proteins bind Ca2⁺ at levels just above basal Ca2⁺ concentrations and reach half maximal binding below 1 μM free Ca2⁺, which is a 10-fold higher affinity than that of calmodulin (4). Therefore, these proteins are fully active at intracellular Ca2⁺ concentrations that activate only a small fraction of calmodulin.

CIB1 (CIB, calmyrin, and KIP) is an EF-hand-containing protein that possesses many features of NCS proteins. CIB1 contains four EF-hand domains (denoted EF1–4), two of which bind Ca2⁺ (EF3 and EF4) with affinities of 1.9 and 0.54 μM, respectively (5). These values are comparable to the Ca2⁺ affinities seen in NCS proteins. In addition, CIB1 is N-terminally myristoylated (6), although it is unknown whether it functions as a Ca2⁺-myristoyl switch, despite sequence similarity in its myristoyl-binding pocket to the Ca2⁺-myristoyl switch protein recoverin (7, 8). The myristoyl group of CIB1 may target it to membranes, since CIB1 fractionates exclusively with membrane fractions in both nucleated (6) and non-nucleated (9) cells. Moreover, disruption of myristoylation by addition of an N-terminal myc tag causes CIB1 staining to shift from membranes to a diffuse cytosolic and nuclear pattern (6). However, myristoylation does not appear to be required for CIB1 binding to its ligands (9, 10).

Unlike NCS proteins, CIB1 is not exclusively neuronal, but is widely expressed (9). Moreover, CIB1 is less homologous in sequence to several NCS proteins, such as KChIP1 (46%), neuregulin (46%), and frequenin (43%) than they are to one another (2). In fact, CIB1 is more homologous to the EF-hand proteins calcineurin B (57% similarity) and calmodulin (54%). CIB1 was originally identified in a yeast two-hybrid screen as a binding partner for the cytoplasmic tail of the platelet integrin αIIb (11). Subsequently, additional CIB1-binding proteins were identified, including DNA-dependent protein kinase (12), the polo-like kinases Fnk and Snk (13), Rac3 (10), Pax3 (14), and presenilin 2 (6), and CIB1 has been shown to modify the function of some of these proteins (14, 15).

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1 The abbreviations used are: NCS, neuronal calcium sensor; BTP, bis-Tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane); MES, 2-(N-morpholino)ethanesulfonic acid; AUC, analytical ultracentrifugation; r.m.s.d., root mean square deviation.
an α and β subunit that can exist in a resting conformation on unactivated cells and convert to an active conformation upon cell stimulation, resulting in a conformational change that allows the integrin to bind its adhesive protein ligands with higher affinity. The αIIbβ3 integrin is expressed only on platelets and their precursors, megakaryocytes, and is the most abundant integrin on these cells. CIB1 binding to αIIb appears to modulate the activation state of the integrin (16, 17), platelet spreading (18), and FAK activation (19). The CIB1 binding region of αIIb has been delineated, and corresponds to a membrane-proximal portion of the cytoplasmic tail, including several amino acids within the putative transmembrane domain (7). These binding residues of αIIb are likely to form an amphipathic α-helix (20, 21), which agrees with data showing that the hydrophobic crevices of EF-hand-containing proteins bind α-helical regions, such as the binding of calcineurin B to calcineurin A (22) and the binding of KChIP1 to the Kv4.2 channel (23).

The sequences of three CIB1 homologs (CIB2, -3, and -4) are also found in the human genome. CIB1 and some of its homologs have been identified in other vertebrates, and highly homologous sequences are present in the genome of more simple organisms such as *Drosophila melanogaster*. Sequence analysis indicates CIB1 and its homologs form a protein family distinct from those of the calcineurin B and the NCS families. To help determine the molecular mechanism for CIB1 binding to its various targets, we describe the x-ray crystal structure of Ca^{2+}-bound CIB1 to 2.0 Å.

**Materials and Methods**

**Protein Purification**—Human wild type CIB1 and CIB1 Δ1–8 (residues 9–191), each fused to an N-terminal hexahistidine tag, were expressed from pProEX HTX (Invitrogen) in *Escherichia coli* BL21(DES) cells. The final DNA sequence includes a hexahistidine Ni-affinity tag followed by a tobacco etch virus cleavage site at the N terminus; the tag was later removed from the protein as described below. CIB1 Δ1–8 was used for crystallization experiments. Wild-type CIB1 was used for gel filtration and analytical ultracentrifugation experiments.

Expression of CIB1 was initiated by inoculating 1 liter of LB broth with 100 ml of overnight culture and incubating at 37 °C. CIB1 expression was induced after 4 h by the addition of isopropyl 1-thio-D-galactopyranoside to a final concentration of 1 mM, and cells were incubated for an additional 3 h at 37 °C. Cells were centrifuged at 4000 rpm for 20 min and resuspended in Buffer A, composed of 20 mM Tris, 10 mM imidazole, 100 mM NaCl, 10% (v/v) glycerol, and 100 μM CaCl2 (pH 7.5) with 1 tablet of *Complete EDTA-free protease inhibitor cocktail (Roche)* (24, 25). Cells were lysed by one pass through an EmulsiFlex-C5 (Avestin) homogenizer, and lysed cells were centrifuged at 16,000 rpm for 2 h at 20 °C. Offset was determined to have been achieved by analyzing samples by SDS-PAGE. If additional cleavage was required, further incubation with additional tobacco etch virus was performed by overnight incubation with 4 ml of tobacco etch virus ( supplied by Worthington Biochemical) against 20 mM Bis-Tris propane, 300 mM NaCl, and 10% (v/v) glycerol in the mother liquor solution, followed by flash freezing in liquid nitrogen.

**Gel Filtration**—The Low Molecular Weight Gel Filtration Calibration Kit (Amersham Biosciences) was used to calibrate a Superdex S-75 gel filtration column (Amersham Biosciences) according to the manufacturer’s instructions. Briefly, 200 μl each of ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) at 5 mg/ml or albumin (67 kDa) and chymotrypsinogen A (25 kDa) were loaded onto a column pre-equilibrated with S-75 buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, and 2 mM calcium acetate) at 0.8 ml/min. The void volume (Vv) was determined by the elution volume of blue dextran (2000 kDa). Purified full-length CIB1 (250 μl of 4 mg/ml) was loaded onto the column that had been pre-equilibrated with 2 mM calcium acetate or 2 mM EGTA. The cytoplasmic αIIb peptide (Ac-LVLMKVGFFFFKRNPPLDEDEEQG-COOH) was added to CIB1 in a molar ratio of 2:1, with 200 ml of CIB1 (65 μM) and 100 μl of αIIb (146 μM) loaded.

**Analytical Ultracentrifugation**—Purified full-length CIB1 was equilibrated to AUC buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and either 2 mM (CH₃COO)₂Ca or 2 mM EGTA) using a Vivaisin 10 MWCO concentrator (Vivascience, Hannover, Germany). The final concentrations of CIB1 were ~230, ~180, and ~80 μg/ml, with A₂₆₀ values of ~0.6, 0.4, and 0.2, respectively. Experiments with the cytoplasmic–αIIb-LVLMKVGFFFFKRNPPLDEDEEQG-COOH, a 1:1 molar ratio of αIIb to CIB1 was used. Equivalent concentrations of αIIb peptide were used in the sample and reference cells. Prior to centrifugation, impurities of the αIIb peptide were eliminated by performing a buffer exchange in a stirred cell (Amicon) with a 1.000 NMWL Ultrafiltration Membrane (Millipore) of regenerated cellulose. Analytical ultracentrifugation was performed using a Beckman Optima XL-I Ultracentrifuge, a Ti-60 rotor and a 6-sector cell (1.2-cm path length). For CIB1 protein alone, samples were centrifuged at 16,000 rpm for 26 h at 20 °C. Offset was determined by meniscus depletion by centrifugation at 45,000 rpm for 6 h. For CIB1 plus αIIb samples, centrifugation was performed at 16,000 rpm for 32 h at 20 °C, and offset was determined by centrifugation at 40,000 rpm for 8 h. Equilibrium was determined to have been reached when sedimentation velocity was 1.51 e₄₀ and the sedimentation velocity of CIB1 plus αIIb was equipped with a slit that allowed the divergence to be tuned for the sample. This slit setting was optimized by maximizing I₀(1) on equivalent screening images. The crystal was mounted in an arbitrary orientation, and data were collected at cryogenic temperatures in a single continuous scan. Both incident and diffracted beam helium beam paths were used to minimize air absorption and scattering. Data collection statistics are given in Table I.

**Structure Determination and Refinement**—Calculation of the Matthews number of CIB1 (as determined by X-ray diffraction) was 1.87 (26) and represented CIB1 in a single molecule per unit cell. The program SHELX (27) was used to locate the positions of anomalous scatterers from the chromium data. We used the “single-wavelength anomalous diffraction” script (to 3.0 Å) provided on the SOLVE website to search for the eight highest peaks, reasoning that these might correspond to individual calcium ions (with a higher ΔF’ at 2.990 Å compared with sulfur) bound to each of the EP-hands in the asymmetric unit. SOLVE easily found eight sites with occupancies...
ranging from 0.147 to 0.522. The overall Z score from SOLVE was 30.9, and the figure of merit was 0.39. Only two C-terminal EF-hands per CIB1 monomer are occupied by individual calcium ions. There are, however, two more calcium ions per CIB1 monomer that bind at surface positions and are most likely due to the high Ca\(^{2+}\) concentration (300 mM) in the crystallization medium. That gives a total of eight calcium ions in the asymmetric unit. The top eight sites found by SOLVE were not all calcium ions; three were sulfur atoms.

The program RESOLVE (27) was used to find non-crystallographic symmetry and carry out “statistical” solvent flattening. Three pairs of the eight sites were related by NCS, and the overlap of NCS-related density was 0.76 in RESOLVE. The solvent-flattened electron density map from RESOLVE was used to trace the chain of one of the two monomers of CIB1 in the asymmetric unit. The process was guided by the structure of calcineurin B (PDB code ITCO), which is 57% homologous to CIB1. The sequence in the helices surrounding the EF-hands 13 and 14 in molecule A) or neighboring a disordered loop (residue 146 respectively; however, these residues were either at the N terminus (residues (0.3%) were found in generously allowed and forbidden regions, respectively. From the Ramachandran plot, 2 (0.6%) and 1 residues (2.5%) were matched easily to density; however, the density in the EF-hand loops was not as continuous and clear. An anomalous difference Fourier map using the phases from the eight SOLVE sites produced a new set of sites, which turned out to be sulfur and lower occupancy calcium atoms. The chain tracing was verified using the sulfur sites from cysteine and methionine residues. Once a preliminary model of the first molecule in the asymmetric unit was built, molecular replacement using the CCP4 program AMoRe (28) was used to position the second molecule. Independently determined positions of calcium and sulfur atoms determined by SOLVE verified this solution.

The density for the second molecule is significantly weaker, especially in the second half of the molecule that contains the two EF-hand calcium ions. The temperature factors for this molecule in initial rounds of conjugate gradient or simulated annealing refinement using CNS (29) were quite high (average temperature factors for all atoms in molecules 1 and 2 were typically ~40 and ~70 Å\(^{2}\), respectively). \(R_{	ext{free}}\) values were just below 30% for these refinements, using either the 2.0 Å data set collected at the synchrotron, or the chromium data set to 2.8 Å. If the packing is inspected, the second molecule (“B”) has fewer intermolecular contacts in the crystal, apparently giving it a more flexible position and thereby more average disorder as indicated by high temperature factors. A TLS refinement using the CCP4 program Refmac5 (43) to 2.0 Å using each subunit and its associated calcium ions as a TLS “group,” and using tight geometrical restraints (matrix diagonal weighting term = 0.05), gave a model with \(R_{	ext{work}} = 21.2\%\) and \(R_{	ext{free}} = 25.7\%\). The r.m.s. bond and angle deviations were 0.01 Å and 1.1°, respectively. From the Ramachandran plot, 2 (0.6%) and 1 residues (0.3%) were found in generously allowed and forbidden regions, respectively; however, these residues were either at the N terminus (residues 13 and 14 in molecule A) or neighboring a disordered loop (residue 146 in molecule B) where the density is poor.

### RESULTS AND DISCUSSION

**Overall Structure of CIB1 and Geometry of Ca\(^{2+}\) Binding—**

Full-length CIB1 consistently yielded twinned, poorly formed crystals. Because proteins homologous to CIB1, such as recoverin, have a disordered N terminus (31), and secondary structure suggests that this region is also disordered in CIB1, CIB1 \(\Gamma_{1} = 8\) was used for crystallization and structure determination.
The structure of CIB1 was solved by single-wavelength anomalous diffraction phasing using chromium radiation (λ = 2.29 Å, Rigaku/MSC Inc., The Woodlands, TX). These data were collected to 2.7 Å, but phases were eventually extended to 2.0 Å using native data sets collected both in-house and at the SER-CAT beamline at Advanced Photon Source (Argonne, IL). There are two molecules of CIB1 in the asymmetric unit. The electron density (Fig. 1A) has been fit with models of CIB1 encompassing residues 12–191 except for a disordered loop between residues 137 and 142 in molecule A and 136–145 in molecule B.

The overall fold of CIB1 is shown in Fig. 1B and is very similar to other Ca2+-binding proteins containing four EF-hands, such as calmodulin and calcineurin B. However, we observed that only EF-hands 3 and 4 of CIB1 bind Ca2+ (Fig. 1, B and C), as previously demonstrated by NMR (5). Calcium ions bind canonical EF-hands with high affinity due to acidic residues at positions 1, 3, 5, and 12, where residues at positions 1, 3, and 5 contribute individual ligands to the coordination sphere and the residue at position 12 contributes two ligands (32). In most EF-hands, a backbone carbonyl at position 7 as well as a water molecule that is hydrogen-bonded to a sidechain oxygen from position 7 complete the coordination sphere.

Calcium ions are not bound in EF1 and EF2. It is not surprising that Ca2+ is absent from EF1, because it has an additional eight amino acids within the loop that normally binds Ca2+. Following this extended loop region in EF1, the helix that typically corresponds to the second helix in an EF-hand is kinked at Pro-62, a residue conserved in calmodulin and calcineurin B, thereby creating two smaller helices (H3a and H3b) (Fig. 1C). EF2 is less radically modified from a canonical EF-hand domain. For example, many of the residues (positions 1, 3, and 5) within the loop that are typically acidic and chelate calcium ions have been replaced with serine or alanine. The EF2 loop also contains 13 residues rather than 12, with the extra residue, Pro-82, inserted after position 3.

A high concentration of Ca2+ (300 mM calcium acetate) was necessary for crystallization, presumably explaining why each monomer of CIB1 binds an additional two Ca2+ ions in positions not typically associated with EF-hand domains (see Table I). There are six water molecules in the octahedral coordination sphere of each of these Ca2+ ions, indicating that both binding events are of low affinity and most likely not biologically relevant, which is consistent with previous binding data (5).

The helices of EF-hands reorient in relation to one another when Ca2+ is bound (33). The interhelical angle between the E and F helices is used as a measure of whether these helices display a Ca2+-bound (open) conformation or a Ca2+-free (closed) conformation. In a closed conformation, E and F helices are close to one another and the interhelical angle (180° − θ) is large. In an open conformation, when Ca2+ is present, the E

### Table I

| Data collection | Source | Chromium | APS |
|-----------------|--------|----------|-----|
| λ (Å)           | 2.29   | 1.00     |     |
| Space group     | P2₁    | P2₁      |     |
| Unit cell (Å)   | 56.61, 50.98, 77.13 | 56.66, 51.01, 77.21 |
| Resolution (Å)  | 40–2.0 | 40–2.0   |     |
| Reflections     | 633,650 | 589,209  |     |
| Unique reflections | 12,013 | 29,442   |     |
| Rmerge (%)      | 4.6 (12.6) | 5.8 (23.4) |
| Completeness (%)| 94.1 (88.1) | 95.4 (81.0) |
| Redundancy      | 10.4 (8.4) | 7.3 (6.2)  |     |
| (dof)           | 54.0 (14.5) | 32 (5.8)  |     |
| Data processing | HKL2000 | HKL2000  |     |
| Anomalous scatterers per A.U. | 8 Ca, 3 S |     |
| Calculated anomalous (∆F/F) (%) | 3.7 |     |

| Refinement and model statistics |       |       |
|---------------------------------|-------|-------|
| Resolution (Å)                  | 40–2.0| 40–2.0|
| Number of protein atoms         | 2781  | 2 molecules/A.U. |
| Number of waters                | 397   |       |
| Rmerge (%)                      | 0     |       |
| Rcryst (%)                      | 21.2% (24.4) | 25.7% (24.4) |
| Rfree (%)                       | 25.7% (30.2) | 21.2% (24.4) |
| r.m.s.d. bonds (Å)              | 0.01  |       |
| r.m.s.d. angles (°)             | 1.10  |       |
| Ramachandran plot               |       |       |
| % in most favored regions       | 92.5  |       |
| % in additional allowed regions | 6.6   |       |
| % in disallowed regions         | 0.3   |       |

For Fig. 1C, the side chains of Asp-161 (position 1), Asp-163, Asp-165, and Glu-172 (position 12) coordinate Ca2+, with Glu-172 being a bidentate ligand. Thr-167 (position 7) ligates Ca2+ through its carbonyl oxygen and also coordinates a water molecule that ligates Ca2+. Similarly, in EF3 (Fig. 1C), the Ca2+ is coordinated by the side chains of Asp-116 (position 1), Asp-118, Asp-120, and Asp-127 as well as the carbonyl of Thr-122 (position 7) and two water molecules. One ligating water molecule is bridged through an additional water molecule interacting with the side chain of Thr-122, and the other ligating water molecule is seen in only one molecule of the asymmetric unit. In EF4, this water molecule is replaced by the bidentate ligation of Glu-172 (position 12). Relative to EF4, the ligation sphere of the Ca2+ in EF3 is less well ordered, which is consistent with the fact that EF3 binds Ca2+ with a lower affinity than EF4 (5).
similar, they indicate that EF2, which does not contain Ca2$^+$, and 111.5°, respectively. Because these angles are so
similar interhelical angles, with values of 116.4°,
To determine whether the EF-hands of CIB1 displayed an open
calmodulin has interhelical angles between 85° and 100° (23).
ac.uk/msd-srv/ssm/). As expected, a number of mammalian
was shown in Fig. 2 (24). In addition to these proteins, an
alignment of these proteins based on the output from MSD-fold
molecule in the asymmetric unit) were compared with other
mammalian Ca2$^+$-binding proteins. From this analysis, we conclude
for calcineurin B and KChIP1
A
B
CIB1 & calcineurin B
CIB1 & KChIP1
FIG. 2. Superposition of CIB1 with its closest structural ho-
and F helices are typically farther apart and the interhelical
angles are smaller. For example, Ca2$^+$-free calmodulin has inter-
helical angles between 130° and 140°, whereas Ca2$^+$-bound
calmodulin has interhelical angles between 85° and 100° (23).
To determine whether the EF-hands of CIB1 displayed an open
or closed conformation, we calculated the interhelical angles
using the program interhix (33). EF2, EF3, and EF4 of CIB1
display similar interhelical angles, with values of 116.4°,
113.2°, and 111.5°, respectively. Because these angles are so
similar, they indicate that EF2, which does not contain Ca2$^+$,
adopts an open conformation like those of Ca2$^+$-bound EF3 and
EF4. This open conformation is maintained by hydrogen bonds
across the EF2 loop involving residues that typically bind Ca2$^+$
(positions 1, 3, 7, and 13). EF1, which is highly degenerate, has
an interhelical angle of 125.5°, which is indicative of a more
closed conformation.
CIB1 and Its Structural Relationship to Other EF-hand-
containing Proteins—To compare the structure of CIB1 to other
EF-hand-containing proteins, the coordinates of CIB1 (the first
molecule in the asymmetric unit) were compared with other
proteins in the Protein Data Bank using MSD-fold (www.ebi.
ac.uk/msd-srv/ssm/). As expected, a number of mammalian
EF-hand-containing proteins showed significant homology to
CIB1, the two most significant being calcinurin B (PDB code
1TCO, Z-score of 5.2, r.m.s.d. of 2.5 Å over 136 residues, 27%
sequence identity) and KChIP1 (Z-score of 3.9, r.m.s.d. of 2.6 Å
over 136 residues, 24% sequence identity). The structural
alignment of these proteins based on the output from MSD-fold
is shown in Fig. 2 (A and B). In addition to these proteins, an
Arabidopsis protein (AtCBL2, PDB code 1UHN) (34) was found
to have strong structural homology to CIB1 (Z-score of 5.1,
r.m.s.d. of 2.4 Å over 137 residues, 24% sequence identity). This
protein is part of a distinct calcineurin B-like family of Arabi-
dopsis proteins that interact with a novel family of plant serine/threonine protein kinases (35).
Although CIB1 closely aligns to other EF-hand-containing
proteins in EF2, EF3, and EF4, significant differences appear
within EF1 and the N-terminal region. As stated previously,
CIB1 contains a long insertion within the EF1 loop that is not
found in any of the structures of EF-hand-containing proteins
solved to date, including calcinurin B and KChIP1. The geomet-
ries of helices H3a and H3b (Fig. 1C), including the bend of
the helices due to the proline residue described previously, are
recapitulated in calcineurin B (Fig. 2A), but not in KChIP1
(Fig. 2B); this proline residue causes a kink in these helices
that is also conserved in calmodulin. However, due to the large
insertion in the EF1 loop, helices H3a and H3b in CIB1 do not
superimpose on the equivalent helices in calcineurin B, despite
the similar proline-induced helical break.
The N-terminal regions of calcineurin B and NCS proteins
point away from the center of the molecule, but the equivalent
portion of CIB1 points to the center of the protein and forms
a number of interactions with helix H5 and H6 (Fig. 2). As crystal
packing interactions also occur in this region and the temper-
ature factors for the N terminus are generally higher than in
the helices of the EF-hands, it is not clear if this conformation
of the N terminus is biologically significant.
Finally, the C terminus of CIB1 forms an α-helix that is
similar in position to the C-terminal helix of KChIP1 (Fig. 2).
Because the C terminus plays an important role in target
recognition both in calcineurin B and in KChIP1, this region
will be discussed in greater detail in a later section (“Potential
Ligand Binding Site of CIB1”).
Sequence Alignment—Several proteins in the sequence data
base from arthropods to humans share significant sequence ho-
mony to CIB1. Three proteins in humans with high homology to
CIB1 are CIB2 (also known as KIP2), CIB3 (also known as KIP3),
and CIB4, which share 59%, 62%, and 64% similarity, respec-
tively. There is currently no published data on CIB3, and only the
expression profile and chromosomal location have been deter-
mined for CIB2 (36). CIB4 (NCBI accession number XP_059399)
is named by the present authors based on a sequence from an automated computational analysis of the human genome.
Based on the sequence alignment (Fig. 3A), all CIB homologs
have a large insertion within EF1, strongly suggesting that
this EF-hand cannot bind Ca2$^+$ in any of the CIB homologs.
Similarly, all CIB homologs contain a proline equivalent to
Pro-62 of CIB1 that causes a kink between helices H3a and
H3b (Fig. 3A), which is important for the overall structure of
the hydrophobic binding pocket (see subsequent section). How-
ever, one notable difference among CIB homologs is that the
EF2 loop (between helices H4 and H5) of CIB2 and CIB3
contains more acidic residues than CIB1, indicating that CIB2
and CIB3 may each bind an additional Ca2$^+$ ion within this
region. Differences in the number of bound Ca2$^+$ ions among
homologous proteins are not surprising, in that differences
in Ca2$^+$ stoichiometry are observed among members of the NCS
family. For example, KChIP1 binds Ca2$^+$ ions in EF-hands 3
and 4 (23), recoverin binds Ca2$^+$ ions in EF-hands 2 and 3 (1),
and frequenin binds Ca2$^+$ ions in EF-hands 2, 3 and 4 (37).
The dendrogram in Fig. 3B depicts the sequence relatedness
of CIB1 and its homologs, as well as a number of other mam-
nalian Ca2$^+$-binding proteins. From this analysis, we conclude
that CIB-like proteins, found in species from arthropods to
humans, form a family distinct from both the calcineurin B-like
and NCS proteins. Members of the CIB family share closest
**FIG. 3. Relationship of CIB1 with some of its nearest homologs.** A, sequence alignment of CIB1 with its three human homologs (CIB2, CIB3, and CIB4), calcineurin B and two NCS proteins, KChIP1 and frequentin. Alignment of CIB1 with CIB2, CIB3, and CIB4 was performed using ClustalX (30) and then merged with the structural alignment from MSD-fold (www.ebi.ac.uk/msd-srv/ssm/) of CIB1 with calcineurin B (PDB code 1TCO), KChIP1 (PDB code 1S6C), and human frequentin (PDB code 1G8I). The positions of helices (red tubes), loop regions (gray lines), and disordered regions (gray dotted lines) in the CIB1 structure are shown above the alignment, and every tenth residue in CIB1 is marked by a dot above the alignment. Residues in CIB1 involved in Ca²⁺ binding are colored red, and residues that correspond to the CIB1 hydrophobic binding...
homology to calcineurin B and its relatives, but sequence homology is still quite low between CIB family members and calcineurin B (30% sequence identity). Among the four human CIB proteins, CIB2 and CIB3 are more ancestral, because CIB proteins from *Drosophila melanogaster*, *Anopheles gambiae*, and *Danio rerio* more strongly resemble these proteins.

**Oligomeric State of CIB1**—Many NCS homologs of CIB1 form dimers or tetramers under various conditions. For instance, KChIP3 tends to form tetramers at protein concentrations higher than 20 mM and stable dimers at low protein concentrations (38). Similarly, neurocalcin forms a dimer in the crystal (39) and in solution when Ca\(^{2+}\) is present (40) but forms a monomer when Ca\(^{2+}\) is absent (40). Conversely, GCAP-2 forms dimers in the absence of Ca\(^{2+}\) and monomers in the presence of Ca\(^{2+}\) (40). Also, unmyristoylated recoverin forms a dimer within the asymmetric unit of the crystal structure (31), whereas KChIP1 also forms dimers, but only in the presence of ligand (23). Residues involved in KChIP1 dimerization are poorly conserved in CIB1 (Fig. 3A).

To determine whether CIB1 forms oligomers in the presence or absence of Ca\(^{2+}\) and ligand, we first performed gel filtration (Fig. 4A). In the presence of Ca\(^{2+}\), CIB1 eluted at a volume corresponding to 30.6 kDa, which is larger than its molecular mass of 21.7 kDa, but smaller than the expected size of a dimer, which would be 43.4 kDa (Fig. 4A). The anomalous elution volume of CIB1 may be accounted for by the fact that gel filtration columns are typically calibrated using spherical proteins as standards, while EF-hand-containing proteins have a more oblong “dumbbell-shape.” Similarly, GCAP-2, which has a molecular mass of 23.8 kDa, exhibits an anomalous elution volume, with the monomer eluting at 30.6 kDa and the dimer eluting at 57 kDa (40). Apo-CIB1, or CIB1 in the absence of Ca\(^{2+}\), elutes at 32.5 kDa, consistent with NMR data of apo-CIB1, which displays a conformation that is more extended than that of Ca\(^{2+}\)-CIB1 (5). An extended conformation is also seen in Ca\(^{2+}\)-free recoverin, as opposed to its more compact conformation when Ca\(^{2+}\) is present (1). Ca\(^{2+}\)-CIB1 in the presence of the entire IIb cytoplasmic peptide eluted at 35 kDa, which is representative of the molecular mass of monomeric CIB1 plus the molecular mass of the peptide (3.1 kDa).

To confirm the gel filtration results, we performed sedimentation equilibrium of purified recombinant full-length CIB1 in the presence of either 2 mM (CH\(_3\)COO)\(_2\)Ca or 2 mM EGTA by analytical ultracentrifugation (AUC). These experiments were also performed in the presence of the IIb peptide, with high, medium, and low concentrations of CIB1 as described under “Experimental Procedures.” Molecular weight determinations are given for the intermediate concentration (150–190 mM), and the other concentrations give similar results. The AUC data fit to a molecular mass for Ca\(^{2+}\)-CIB1 of 26.9 kDa compared with a calculated value of 21.7 kDa, with an offset of 0.05 (Fig. 4B). This indicates an equilibrium favoring monomers with the pocket (see Fig. 5) and that are involved in dimerization in rat KChIP1 (PDB code 1SC6) are highlighted with brown and green circles, respectively. B, evolutionary relationship of CIB1 with other Ca\(^{2+}\)-binding proteins. The dendrogram (derived as described under “Experimental Procedures”) is colored to highlight the distinct families of CIB-like proteins (purple), calcineurin B-like proteins (blue), and NCS proteins (green). The proteins are human unless otherwise stated, and their NCBI accession numbers are included.

**FIG. 4.** CIB1 primarily forms monomers in the presence or absence of Ca\(^{2+}\) and the αIIb cytoplasmic domain. A, elution profiles of apo-CIB1, Ca\(^{2+}\)-CIB1, and Ca\(^{2+}\)-CIB1 plus αIIb cytoplasmic tail peptide were determined using a Superdex S-75 column (Amersham Biosciences). The molecular mass standards used were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The elution volume ($V_e$) of blue dextran was used to determined the void volume ($V_o$). B, sedimentation equilibrium data of Ca\(^{2+}\)-CIB1, apo-CIB1, and Ca\(^{2+}\)-CIB1 plus αIIb by analytical ultracentrifugation. Molecular weights fit using a one-state model are posted on each graph.
Crystal Structure of CIB1

Residues within the hydrophobic binding pocket of CIB1 are denoted in the sequence alignment on Fig. 3A.

In the case of calcineurin B, calcineurin A binds in a hydrophobic channel formed from residues in helices H1, H2, H3, and H7 (equivalent to H1, H3, H4, and H8 in CIB1) and the C-terminal region (see Fig. 5) (22). In KChIP1, the Kv4.2 peptide binds as a continuation of the C-terminal helix H10 in a hydrophobic channel formed by EF1 and EF2 (23). Helix H10 of KChIP1 is in a similar position to the C-terminal helix of CIB1. Similarly placed hydrophobic channels have been found in other NCS proteins (37, 39, 41), although structures of these proteins with their ligands have not yet been reported.

CIB1 contains a large hydrophobic channel just following the C terminus of the molecule, as in KChIP1, but the binding pocket is considerably narrower than that of KChIP1. This is in part due to the bend at Pro-61 and the associated position of helix H3b, which pushes further into the hydrophobic binding pocket relative to the similar helix in KChIP1. Binding studies have shown that CIB1 binds to a minimal 15-amino acid region of integrin αIIb (7), which corresponds to four α-helical turns. This channel (~18 Å across) would be too small to bind four turns of an α-helix (~25 Å). However, this hydrophobic channel continues, albeit in a narrower form, on either side of the H10 helix, so residues within this C-terminal helix could also be important in peptide binding. Indeed, it has been demonstrated that residues within this C-terminal helix (17) are critical for CIB1 binding to the αIIb cytoplasmic tail. In addition, prior to this structure, a homology model of CIB1 was generated (7) based on the structure of calcineurin B bound to calcineurin A (PDB code 1TCO). Based on this model, four residues (Leu-115, Leu-131, Ile-153, and Phe-173) were mutated and found to affect αIIb integrin binding without grossly affecting the CIB1 structure (as measured by circular dichroism). In our structure, these residues are buried under helix H10 and are thus not surface-accessible. However, loss of integrin binding via these mutations may be explained by their causing local movements of helix H10, which then would affect the structure of the hydrophobic binding groove. Another possibility is that upon integrin binding, helix H10 undergoes a conformational change such that a larger ligand binding groove is formed. In the structure of the Arabidopsis EF-hand-containing protein AtCLB2 (34), the C terminus completely covers the hydrophobic binding groove, and consequently it has been suggested that, upon ligand binding, the C terminus is released from this groove. For CIB1, these studies indicate the importance of the hydrophobic binding groove and the H10 helix. Additional structural and mutational studies will aid in further defining the role of this region in CIB1 binding to effectors.

In conclusion, the crystal structure of CIB1 reveals significant structural similarity to other EF-hand-containing proteins and confirms that only EF-hands 3 and 4 bind Ca²⁺ through canonical interactions characteristic of EF-hand domains. The major differences between CIB1 relative to the NCS and calcineurin B families are in the overall structure of EF1 and the N-terminal portions. The position of the C-terminal helix of CIB1 is similar to that of KChIP1, and like KChIP1, CIB1 contains a hydrophobic binding pocket near this helix. This hydrophobic binding pocket is likely to bind αIIb and possibly other targets; as shown previously, residues within this area are critical for binding αIIb (7). A family of proteins related to CIB1 has been discovered that is distinct from its closest homologs, calcineurin B and the NCS protein family. Future structural studies of CIB1 and CIB-family members with high affinity binding peptides from target proteins such as integrin

Potential Ligand Binding Site of CIB1—EF-hand-containing proteins contain hydrophobic pockets that bind the amphipathic α-helices of their ligands. These binding pockets exist on the opposite side of the molecule from the Ca²⁺-binding sites of EF-hands 3 and 4. In Fig. 5, the molecular surfaces of CIB1, KChIP1, and calcineurin B are colored by hydrophobic potential, and the positions of the portions of calcineurin A and Kv4.2 bound in calcineurin B and KChIP1, respectively, are traced.

ability to form dimers for a small percentage of molecules. The molecular mass from AUC for apo-CIB1 was 23.6 kDa, with an offset of ~0.021, indicating a population almost entirely monomeric. To determine whether Ca²⁺-CIB1 forms dimers in the presence of ligand, we performed experiments with equivalent concentrations of αIIb peptide in the sample and reference cells. The molecular mass determined from AUC was 25.5 kDa (offset of ~0.255), compared with a calculated molecular mass for a 1:1 CIB1-peptide complex of 24.8 kDa, indicating the prevalence of a 1:1 complex of CIB1 and αIIb. The high offset values obtained for experiments with αIIb are indicative of the difficulty in precisely matching the αIIb concentration in the reference and sample cells. Taken together, these data indicate that CIB1, unlike NCS proteins, remains monomeric in the presence and absence of Ca²⁺ and ligand.

FIG. 5. CIB1 contains a strongly hydrophobic channel that is preserved in other EF-hand-containing proteins. Coordinates for CIB1 (PDB code 1X05), calcineurin B (PDB code 1TCO), and KChIP1 (PDB code 1S6C) were read by the program SYBYL version 6.9.1 (www.tripos.com), hydrogens were added, and MOLCAD molecular surfaces were generated and colored to represent the spectrum of hydrophobic potential (red, highly hydrophobic; blue, highly hydrophilic). The orientation of CIB1 in this molecular surface representation is very similar to that in Fig. 1B. The region of calcineurin A in contact with calcineurin B, as well as the Kv4.2 peptide, are shown as helical worms in the binding sites of calcineurin B and KChIP1, respectively, and their boundaries are enumerated. Positions of the C termini of CIB1, calcineurin B, and KChIP1 on these surfaces are labeled, as are selected secondary structural elements.
cryoEM, in combination with further biochemical and cellular assays, will extend our understanding of the role of this family of proteins in cellular signaling.

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