Calcium- and magnesium-dependent interactions between calcium- and integrin-binding protein and the integrin α Ib cytoplasmic domain

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Abstract

Calcium- and integrin-binding protein (CIB) is a small EF-hand calcium-binding protein that is involved in hemostasis through its interaction with the α Ib cytoplasmic domain of integrin α Ib β ₃. We have previously demonstrated that CIB lacks structural stability in the absence of divalent metal ions but that it acquires a well-folded conformation upon addition of Ca^{2+} or Mg^{2+} . Here, we have used fluorescence spectroscopy, NMR spectroscopy, and isothermal titration calorimetry to demonstrate that both Ca^{2+} -bound CIB (Ca^{2+} -CIB) and the Mg^{2+} -bound protein (Mg^{2+} -CIB) bind with high affinity and through a similar mechanism to α Ib cytoplasmic domain peptides, but that metal-free CIB (apo-CIB) binds in a different manner. The interactions are thermodynamically distinct for Ca^{2+} -CIB and Mg^{2+} -CIB, but involve hydrophobic interactions in each case. Since the Mg^{2+} concentration inside the cell is sufficient to saturate CIB at all times, our results imply that CIB would be capable of binding to the α Ib cytoplasmic domain independent of an intracellular Ca^{2+} stimulus in vivo. This raises the question of whether CIB can act as a Ca^{2+} sensor in α Ib β ₃ signaling or if other regulatory mechanisms such as fibrinogen-induced conformational changes in α Ib β ₃, post-translational modifications, or the binding of other accessory proteins mediate the interactions between CIB and α Ib β ₃. Differences in NMR spectra do suggest, however, that Ca^{2+} -binding to the Mg^{2+} -CIB- α Ib complex induces subtle structural changes that could further modulate the activity of α Ib β ₃.

Keywords: calcium- and integrin-binding protein; calcium; magnesium; integrin; peptide; isothermal titration calorimetry; nuclear magnetic resonance spectroscopy; fluorescence spectroscopy

Integrins are a large family of transmembrane glycoproteins that are important in mediating adhesion between cells as well as the attachment of cells to the extracellular matrix. The highly abundant platelet integrin α Ib β ₃

links the cytoplasmic and extracellular environments, acting as a bidirectional signaling molecule in the platelet aggregation process leading to blood clot formation (Calvete 2004). Platelet agonists trigger inside-out signaling, where ligand binding to the cytoplasmic domains causes conformational changes that increase the affinity of the extracellular domains for fibrinogen and the von Willebrand factor, initiating platelet aggregation. The subsequent ligand binding by the extracellular domains also triggers outside-in signaling events that increase the avidity of binding and enhance the aggregation process (Calvete 2004; Mould and Humphries 2004; Xiao et al. 2004). Despite their relatively small sizes, the cytoplasmic domains of α Ib and β ₃ interact with each other and many intracellular ligands and play an important role in

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Abbreviations: CIB, calcium- and integrin-binding protein; Ca^{2+} -CIB, calcium-bound CIB; Mg^{2+} -CIB, magnesium-bound CIB; apo-CIB, metal-free CIB; CaM, calmodulin; CnB, calcineurin B; can, calcineurin A; NCS, neuronal calcium sensor; TnC, troponin C; TnI, troponin I.

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mediating these signaling events (Vinogradova et al. 2002; Weljie et al. 2002).

Calcium- and integrin-binding protein (CIB) is a 22-kDa calcium (Ca^{2+})-binding protein of the helix-loop-helix EF-hand superfamily. It was originally identified as a specific binding partner for the αIIb cytoplasmic domain of platelet integrin (Naik et al. 1997), but has since been suggested to bind to several other proteins (Wu and Lieber 1997; Kauselmann et al. 1999; Stabler et al. 1999; Ito et al. 2000; Fang et al. 2001; Haataja et al. 2002; Henderson et al. 2002; Hollenbach et al. 2002; Whitehouse et al. 2002). Both in vivo and in vitro binding studies using intact $\alpha\text{IIb}\beta_3$ as well as αIIb cytoplasmic domain peptides have shown that CIB specifically binds to the cytoplasmic domain of αIIb and that its affinity is enhanced in the presence of Ca^{2+} (Shock et al. 1999; Barry et al. 2002; Tsuboi 2002). CIB is also myristoylated on its N terminus (Stabler et al. 1999), although it binds to αIIb independent of myristoylation (Shock et al. 1999; Tsuboi 2002). The sequence homology of CIB to calmodulin (CaM), calcineurin B (CnB), and members of the neuronal calcium sensor (NCS) family suggests that CIB might function as a Ca^{2+} sensor in $\alpha\text{IIb}\beta_3$ -mediated inside-out signaling (Hwang and Vogel 2000). Indeed CIB binds two Ca^{2+} ions with its C-domain EF-hands EF-III and EF-IV with dissociation constants (K_d) near 1.9 μM and 0.5 μM , respectively (Yamniuk et al. 2004). However, there have also been reports of apo-CIB binding to αIIb (Shock et al. 1999; Vallar et al. 1999; Tsuboi 2002). Moreover, unlike many other Ca^{2+} -regulatory proteins, a single Mg^{2+} binds to EF-III of CIB ($K_d = 120 \mu\text{M}$) and causes conformational changes similar to those induced by Ca^{2+} (Yamniuk et al. 2004). The binding of either Ca^{2+} or Mg^{2+} to CIB significantly increases the structural stability of the protein in comparison to apo-CIB, which is a molten globule (Yamniuk et al. 2004). Here we have studied the binding of αIIb cytoplasmic domain peptides to all three forms of CIB, metal-free CIB (apo-CIB), Ca^{2+} -bound CIB (Ca^{2+} -CIB), and Mg^{2+} -bound CIB (Mg^{2+} -CIB). Our results demonstrate that both Ca^{2+} -CIB and Mg^{2+} -CIB bind strongly to the αIIb cytoplasmic domain in a similar manner, whereas apo-CIB binds differently and with lower affinity. Because the free intracellular Mg^{2+} concentration in platelets is maintained between 0.5 and 1.3 mM (Matsuno et al. 1993), our results suggest that CIB would be capable of binding αIIb independent of a Ca^{2+} stimulus in vivo. Therefore, other regulatory mechanisms are likely involved in vivo, in mediating the interactions of CIB with αIIb .

Results

Fluorescence spectroscopy

We first studied the interactions between CIB and the cytoplasmic domain of αIIb by fluorescence spectroscopy, taking advantage of the single Trp residue in the αIIb

cytoplasmic domain (W988). Studying peptide binding directly in this fashion is possible here because CIB does not contain any Trp residues in its sequence. Titrations were performed to establish the stoichiometry and affinity of binding, while steady state fluorescence emission spectra and quenching studies were used to determine the environment of W988 in each complex. Studies were performed using a "long" peptide ($\alpha\text{IIb-L}$) encompassing the entire cytoplasmic domain or a "short" peptide ($\alpha\text{IIb-S}$) encompassing only the membrane proximal region. To test the role of ionic interactions all experiments were performed in the presence and absence of 100 mM KCl. NMR studies indicate that the conformation of CIB is unchanged between 0 and 100 mM KCl and therefore any salt-dependent binding effects are unlikely to be caused by structural modifications (data not shown).

The steady state fluorescence emission spectrum of free $\alpha\text{IIb-L}$ is characteristic of a solvent-exposed Trp having an emission wavelength maximum near 355 nm (Fig. 1A). Addition of Ca^{2+} -CIB increased the steady state emission intensity up to a stoichiometry of 1:1 with a K_d near 0.2 μM , consistent with a previous study (Table 1; Barry et al. 2002). Interestingly, we found that peptide binding to Mg^{2+} -CIB was also stoichiometric with a comparable affinity, suggesting that a similar interaction can occur in the presence of Mg^{2+} . In each complex the emission wavelength maximum shifted only 2–4 nm to shorter wavelengths, indicating that although the Trp is held rigidly, it is not buried within a hydrophobic binding pocket. Fluorescence quenching studies also indicated that W988 is on the surface of each complex since the fluorescence emission was quenched $\sim 70\%$ as efficiently when bound to either Ca^{2+} -CIB or Mg^{2+} -CIB in comparison to the free peptide (Table 1). Experiments performed in the absence of salt gave very similar results to those in the presence of 100 mM KCl, suggesting that ionic interactions play a minor role in the binding of Ca^{2+} -CIB or Mg^{2+} -CIB to $\alpha\text{IIb-L}$. All studies with $\alpha\text{IIb-S}$ also gave similar results to those with $\alpha\text{IIb-L}$, although the emission wavelength maxima were shifted an additional ~ 2 –4 nm toward shorter wavelengths, and binding to Mg^{2+} -CIB was about fivefold weaker than with the full-length peptide (Table 1). The similar interactions with each peptide and lack of salt dependency suggest that both Ca^{2+} -CIB and Mg^{2+} -CIB bind to the membrane proximal region of αIIb predominantly through hydrophobic interactions.

Similar studies using apo-CIB produced very different results than those obtained in the presence of Ca^{2+} or Mg^{2+} . Most striking were a 22–24 nm blue shift for the fluorescence emission of both $\alpha\text{IIb-L}$ and $\alpha\text{IIb-S}$ when bound to apo-CIB and an almost complete lack of quenching by CsCl (Table 1). These results suggest that W988 is nearly completely buried within a hydrophobic pocket when bound to apo-CIB. In the presence of

Figure 1. Interactions between CIB and α Ib peptides studied by fluorescence spectroscopy. (A) Steady state fluorescence emission spectra of free α Ib-L (—), and α Ib-L in the presence of 1.2 molar equivalents of Ca²⁺-CIB (---), Mg²⁺-CIB (···), or apo-CIB (—•—). Titrations of Ca²⁺-CIB (B), Mg²⁺-CIB (C), or apo-CIB (D) into α Ib-L in the absence (●) or presence (■) of 100 mM KCl or similar titrations into α Ib-S in the absence (▼) or presence (▲) of 100 mM KCl. The K_d was calculated from the best fit to the titration data (—), except for the titrations of apo-CIB into α Ib-S, which were not fit due to nonspecific interactions and are instead connected by dotted lines (···▼···) and (···▲···).

100 mM KCl the changes in the fluorescence intensity also saturated near a stoichiometry of 1:1 and suggested that binding was of similar affinity to the interactions in the presence of Ca²⁺ or Mg²⁺. However, binding was approximately fivefold weaker in the absence of salt and an order of magnitude weaker in each case when the K_d was calculated from the change in emission wavelength maxima (Table 1). This suggests that peptide binding to apo-CIB is weaker than in the presence of Ca²⁺ or Mg²⁺. Titrations of α Ib-S with apo-CIB exhibited nonspecific binding of several peptides to a single apo-CIB molecule both in the presence and absence of salt, and therefore the K_d was not calculated (Fig. 1D). The reason

for this nonspecific binding is likely because apo-CIB is a negatively charged, flexible molten globule with exposed nonpolar surfaces (Yamniuk et al. 2004) and α Ib-S is small and contains both nonpolar and basic regions. Because of this nonspecific binding we restricted our remaining studies to the interactions with α Ib-L, which encompasses the entire cytoplasmic domain of α Ib.

Isothermal titration calorimetry

Thermodynamic analysis of α Ib-L binding to CIB was performed by isothermal titration calorimetry (ITC).

Table 1. Fluorescence parameters for α Ib-peptides binding to CIB

	$\Delta\lambda_{\max}$ (nm) ^a	ΔI (%) ^a	K_d (μ M)	K_{sv} (M ⁻¹)
α Ib-L	—	—	—	0.96 (0.74)
α Ib-L + Ca ²⁺ -CIB	-2 (-4)	78 (96)	0.20 (0.44)	0.71 (0.72)
α Ib-L + Mg ²⁺ -CIB	-3 (-3)	99 (109)	0.23 (0.18)	0.75 (0.71)
α Ib-L + apo-CIB	-23 (-22)	56 (74)	0.20 (1.12) 1.54 (14.2) ^b	0.08 (0.06)
α Ib-S	—	—	—	1.00 (0.84)
α Ib-S + Ca ²⁺ -CIB	-6 (-4)	101 (103)	0.30 (0.66)	0.74 (0.82)
α Ib-S + Mg ²⁺ -CIB	-6 (-5)	130 (131)	1.53 (1.10)	0.74 (0.82)
α Ib-S + apo-CIB	-24 (-24)	161 (191)	—	0.04 (0.11)

Numbers in parentheses indicate experiments done in the absence of salt.

^a Changes in the emission wavelength maxima ($\Delta\lambda_{\max}$) and intensity (ΔI) are with respect to the free peptides.

^b Values calculated from $\Delta\lambda_{\max}$ rather than ΔI .

Figure 2. Representative isothermal titration calorimetry experiments for α IIB-L binding to Ca^{2+} -CIB (A), Mg^{2+} -CIB (B), and apo-CIB (C) in 20 mM HEPES, 100 mM KCl (pH 7.3) at 37°C. The *top* panels display the baseline corrected calorimetric titration data while the *bottom* panels display the derived binding isotherms for each experiment.

Under all conditions the binding was exothermic, and the data were best fit to a single site binding model (Fig. 2). The calorimetrically derived K_d values for α IIB-L binding to Ca^{2+} -CIB or Mg^{2+} -CIB were similar to each other and in the high nanomolar range, but slightly larger than the values calculated by fluorescence spectroscopy (Table 2). All thermodynamic parameters were similar in the absence of salt, confirming again that electrostatic interactions are not prevalent in either complex. The interactions were also thermodynamically distinct, with α IIB-L binding to Ca^{2+} -CIB proceeding with small but favorable changes in both enthalpy and entropy, whereas binding to Mg^{2+} -CIB was considerably more enthalpically favorable and entropically unfavorable (Table 2). Both ΔH and

$T\Delta S$ displayed strong linear but opposite temperature dependence known as enthalpy–entropy compensation (Fig. 3; Cornish-Bowden 2002). Therefore, the changes in ΔH and $T\Delta S$ offset to give similar binding free energy changes over the entire temperature range. The slope of the temperature dependence of ΔH was used to calculate the change in heat capacity (ΔC_p) for binding to each form of the protein. This in turn can be correlated to the change in solvent accessible nonpolar surface area accompanying peptide binding (Loladze et al. 2001). Importantly, the ΔC_p for α IIB-L binding to Ca^{2+} -CIB (-1.1 kJ/mol K) and Mg^{2+} -CIB (-1.3 kJ/mol K) were each negative and of similar magnitude, indicating that the amount of nonpolar surface buried upon formation

Table 2. Thermodynamics of α IIB-L binding to CIB as determined by isothermal titration calorimetry in 20 mM HEPES, 100 mM KCl (pH 7.3)

Conditions ^a	Temp. (°C)	<i>N</i>	K_d (μM)	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
Ca^{2+} -CIB	20	1.0 ± 0.0	0.42 ± 0.06	-10.7 ± 0.1	25.1	-35.8
Ca^{2+} -CIB	25	1.0 ± 0.0	0.64 ± 0.10	-15.6 ± 0.3	19.8	-35.4
Ca^{2+} -CIB	30	1.0 ± 0.0	0.66 ± 0.03	-22.0 ± 0.1	13.9	-35.9
Ca^{2+} -CIB ^b	30	1.0 ± 0.0	0.65 ± 0.13	-21.8 ± 0.4	14.1	-35.9
Ca^{2+} -CIB	37	0.9 ± 0.0	1.41 ± 0.12	-28.5 ± 0.5	6.2	-34.8
Mg^{2+} -CIB	20	1.2 ± 0.0	0.27 ± 0.05	-27.5 ± 0.4	9.4	-36.9
Mg^{2+} -CIB	25	1.1 ± 0.0	0.47 ± 0.07	-33.5 ± 0.4	2.6	-36.1
Mg^{2+} -CIB	30	1.1 ± 0.0	0.47 ± 0.05	-39.0 ± 0.4	-2.3	-36.7
Mg^{2+} -CIB ^b	30	0.9 ± 0.0	0.24 ± 0.03	-41.0 ± 0.4	-2.5	-38.5
Mg^{2+} -CIB	37	1.1 ± 0.0	1.02 ± 0.10	-50.2 ± 0.8	-14.6	-35.6
$\text{Ca}^{2+}(\text{Mg}^{2+})$ -CIB	20	1.0 ± 0.0	0.53 ± 0.07	-11.8 ± 0.2	23.4	-35.2
$\text{Ca}^{2+}(\text{Mg}^{2+})$ -CIB	30	0.9 ± 0.0	0.36 ± 0.05	-22.0 ± 0.3	15.4	-37.4
$\text{Ca}^{2+}(\text{Mg}^{2+})$ -CIB	37	0.8 ± 0.0	0.79 ± 0.20	-31.9 ± 1.2	4.3	-36.2
Apo-CIB	37	0.4 ± 0.1	3.89 ± 1.60	-58.3 ± 14.7	26.1	-32.2

^aExperiments were performed in 2 mM CaCl_2 (Ca^{2+} -CIB), 4 mM MgCl_2 + 1 mM EGTA (Mg^{2+} -CIB), or 2 mM CaCl_2 + 4 mM MgCl_2 ($\text{Ca}^{2+}(\text{Mg}^{2+})$ -CIB).

^bExperiments were performed in 20 mM HEPES (pH 7.3) without KCl.

Figure 3. Temperature dependence of the enthalpy (solid symbols) or entropy (open symbols) of α Ib-L binding to CIB in the presence of 2 mM Ca²⁺ (circles), 4 mM Mg²⁺ (squares), or 2 mM Ca²⁺ and 4 mM Mg²⁺ (triangles). The linear regression is shown as solid, dotted, and dashed lines, respectively.

of each complex is comparable. In the presence of saturating concentrations of both Ca²⁺ and Mg²⁺, the thermodynamics of binding were similar to Ca²⁺ alone, consistent with the higher affinity of CIB for Ca²⁺ over Mg²⁺ (Yamniuk et al. 2004).

The binding of α Ib-L to apo-CIB was also exothermic, as seen in Figure 2C. However, the stoichiometry was less than 1, and the binding was weaker than in the presence of Ca²⁺ or Mg²⁺ (Table 2). The calorimetric heat change also displayed a slow return to the baseline that required increased time between injections, which is a characteristic of nonspecific interactions. Due to the weaker binding and more noisy enthalpy data, the estimated thermodynamic parameters for apo-CIB binding to α Ib-L are less reliable than those obtained for Ca²⁺-CIB or Mg²⁺-CIB.

NMR spectroscopy

To investigate the extent of peptide-induced conformational changes in CIB, we recorded heteronuclear ¹H, ¹⁵N-HSQC NMR spectra of uniformly ¹⁵N-labeled Ca²⁺-CIB, Mg²⁺-CIB, and apo-CIB, in the presence and absence of unlabeled α Ib-L. These spectra are essentially the fingerprint for the protein where each peak represents a backbone or side chain amide group. As has been discussed in more detail previously (Yamniuk et al. 2004), the spectra of Ca²⁺-CIB and Mg²⁺-CIB are characteristic of folded proteins, where the downfield Gly121 and Gly166 peaks represent Ca²⁺ or Mg²⁺ coordination at EF-III or EF-IV, respectively (Fig. 4A,B). Less than the expected number of peaks are observed in each spectrum, presumably due to chemical exchange broadening from weak self-association or flexible internal regions. There are also more distinct backbone amide peaks detected for Ca²⁺-CIB (~160) than Mg²⁺-CIB (~140) in the absence of peptide, consistent with greater structural stability in the presence of Ca²⁺ than Mg²⁺. The binding of α Ib-L induces chemical shift

changes for most of the peaks in both the Ca²⁺-CIB and Mg²⁺-CIB spectra, indicative of large peptide-induced conformational changes in each form of the protein. Interestingly ~160 distinct backbone amide peaks are now observed in the spectra of each complex, indicating that peptide binding stabilizes the structure of some previously exchange-broadened regions of Mg²⁺-CIB. This includes a peak for G166 of Mg²⁺-CIB that was not observed in the absence of α Ib-L, suggesting that the structure of EF-IV of Mg²⁺-CIB is stabilized in the complex, possibly by Mg²⁺ binding directly to this site. Also noteworthy is that many of the peaks in each complex have similar chemical shifts, indicating that regions of the protein adopt similar structures when bound to α Ib-L in the presence of Ca²⁺ or Mg²⁺ (Fig. 4D). However these spectra are not identical, indicating that Ca²⁺ binding to the Mg²⁺-CIB- α Ib-L complex does induce some small conformational changes.

The spectrum of apo-CIB exhibits severe chemical exchange broadening and chemical shift degeneracy characteristic of a molten globule (Fig. 4C). In the presence of α Ib-L an increase in chemical exchange was observed consistent with a weak interaction, and also showing that peptide binding does not stabilize the structure of apo-CIB. Attempts to improve the apo-CIB

Figure 4. ¹H, ¹⁵N HSQC NMR spectra of uniformly ¹⁵N-labeled Ca²⁺-CIB (A), Mg²⁺-CIB (B), and apo-CIB (C) in the presence and absence of unlabeled α Ib-L at 37°C. (D) Overlaid spectra of the Ca²⁺-CIB- α Ib-L and Mg²⁺-CIB- α Ib-L complexes. G121 and G166 represent Gly6 of the Ca²⁺-binding loop from EF-III and EF-IV, respectively, as determined previously (Yamniuk et al. 2004). Note that the G166 peaks for Ca²⁺-CIB in the presence and absence of α Ib-L overlap in A, whereas G166 of Mg²⁺-CIB is only observed in the presence of α Ib-L in B.

NMR spectra by varying protein concentration, temperature, pH, salt, or adding various detergents have been unsuccessful, indicating that the structural instability of apo-CIB is intrinsic to the protein and not dependent on experimental conditions.

Discussion

Previous studies have shown that CIB is capable of binding to the α IIB cytoplasmic domain in the absence of divalent metal ions, and that its affinity increases in the presence of Ca^{2+} (Shock et al. 1999; Vallar et al. 1999; Barry et al. 2002; Tsuboi 2002). Our structural studies have demonstrated that apo-CIB is a molten globule, but adopts a folded structure in the presence of Ca^{2+} or Mg^{2+} (Yamniuk et al. 2004). Here we demonstrate that both Ca^{2+} -CIB and Mg^{2+} -CIB bind to α IIB peptides with high affinity and by a similar binding mode, but that binding to apo-CIB is of lower affinity and involves a different mechanism. The apo-CIB binding mechanism might involve some nonspecific interactions between the exposed hydrophobic surfaces of apo-CIB and the nonpolar residues of α IIB, including W988, as evidenced by the slow ITC baseline recovery and binding of several α IIB-S peptides observed by fluorescence spectroscopy. However, the interaction with apo-CIB is unlikely to be of biological importance since CIB would never exist in vivo devoid of divalent metal ions due to the high intracellular Mg^{2+} concentration. Instead, Mg^{2+} -CIB would be the major intracellular form of the protein, with Ca^{2+} potentially displacing Mg^{2+} upon intracellular Ca^{2+} influx in a stimulated cell.

Both fluorescence spectroscopy and ITC demonstrate that the similar mode of α IIB-binding to Ca^{2+} -CIB and Mg^{2+} -CIB involves predominantly hydrophobic interactions with little contribution from electrostatic interactions. Studies using mutant α IIB peptides have indicated that L983, W988, F992, and F993 of α IIB are particularly important in binding, and that this region of α IIB likely interacts with the C domain of CIB (Barry et al. 2002). We also found that W988 of α IIB-L is on the surface of the complex with either Ca^{2+} -CIB or Mg^{2+} -CIB, similar to W352 of CnA bound to CnB, but unlike many CaM-binding peptides where the Trp is completely shielded from solvent by the protein (Crivici and Ikura 1995; Ke and Huai 2003; Yamniuk and Vogel 2004). This supports the previous proposal that the CIB- α IIB interaction is similar to the CnB-CnA interaction (Barry et al. 2002). Peptide binding to Ca^{2+} -CIB or Mg^{2+} -CIB is also accompanied by comparable changes in heat capacity, indicating that the nonpolar binding interface is similar in the complexes with Ca^{2+} -CIB or Mg^{2+} -CIB. However, the ΔCp of α IIB-L binding to CIB was considerably less than that associated with peptides binding to other EF-hand

Ca^{2+} -regulatory proteins such as CaM (Wintrobe and Privalov 1997; Brokx et al. 2001) and S100P (Gribenko et al. 2002), suggesting that the hydrophobic binding surface of CIB is somewhat smaller than these proteins. In fact the ΔCp associated with the CIB- α IIB-L interaction (~ -1.2 kJ/mol K) is quite similar to that associated with peptides binding to a single domain of CaM (~ -1.6 kJ/mol K) (Brokx et al. 2001), suggesting that α IIB-L binds to only one domain of CIB, possibly the C domain as suggested by Parise and coworkers (Barry et al. 2002).

The high affinity interaction between CIB and α IIB in the presence of Mg^{2+} indicates that CIB should be capable of binding to the integrin in vivo independent of a Ca^{2+} stimulus. This feature distinguishes CIB from many related Ca^{2+} -regulatory proteins that show strict Ca^{2+} -dependent target protein binding (Ikura 1996; Bhattacharya et al. 2004). However, the majority of evidence suggests that CIB binds only to activated α IIB β_3 , indicating that it does not remain constitutively bound in vivo (Vallar et al. 1999; Naik and Naik 2003). Therefore, other mechanisms must regulate the interaction between CIB and α IIB in vivo. For example, the membrane proximal CIB-binding region of α IIB also interacts with the β_3 cytoplasmic domain, and it is this interaction that is believed to maintain α IIB β_3 in an inactive conformation (Hughes et al. 1995, 1996; Vinogradova et al. 2002; Weljie et al. 2002). This association between the α IIB and β_3 cytoplasmic domains could prevent the binding of CIB in vivo. Alternatively, CIB binding could be regulated by other accessory proteins that associate with either the α IIB or β_3 domains. The protein phosphatase PP1 was recently shown to interact with the $^{989}\text{KVG}^{992}$ region of α IIB, but to dissociate upon α IIB β_3 binding to fibrinogen (Vijayan et al. 2004). Likewise the ion channel-forming protein ICln has been shown to influence the activity of α IIB β_3 through a high affinity interaction with the $^{989}\text{KVGFFKR}^{995}$ region of α IIB (Larkin et al. 2004). Since these binding regions also overlap with the CIB-binding domain, dissociation of PP1 or ICln might be a prerequisite to CIB binding. Other proteins such as the β_3 binding protein Talin might also be required to disrupt the α IIB- β_3 interaction (Vinogradova et al. 2002; Garcia-Alvarez et al. 2003; Tadokoro et al. 2003), which could free α IIB for a subsequent binding to CIB. Moreover, CIB contains several consensus phosphorylation sequences (Naik et al. 1997) that might require dephosphorylation prior to α IIB-binding, while phosphorylation of Tyr and Thr residues of the β_3 integrin tail has also been demonstrated (Jenkins et al. 1998; Lerea et al. 1999; Kirk et al. 2000) and this in turn could also fine-tune its interactions with the α IIB domain.

Although CIB is capable of binding to α IIB in the absence of Ca^{2+} , subtle Ca^{2+} -induced conformational changes

in CIB could be important for regulating the activity of α Ib β_3 . Indeed although the NMR spectra of Ca²⁺-CIB or Mg²⁺-CIB bound to α Ib-L were similar, they were not identical. This type of mechanism takes place in the troponin complex in vertebrate skeletal muscle where the Mg²⁺-bound C domain of troponin C (TnC) constitutively tethers the protein to troponin I (TnI), awaiting subtle Ca²⁺-induced conformational changes that activate the complex (Zot and Potter 1982; Finley et al. 2000). Notably, Mg²⁺ binds to EF-III but not EF-IV of TnC in the absence of TnI, but to both EF-III and EF-IV in the presence of TnI (Finley et al. 2000). Moreover the Δ Cp associated with Ca²⁺-TnC or Mg²⁺-TnC binding to TnI are nearly identical (Calvert et al. 2000). These characteristics were also observed with CIB. Although N-terminal myristoylation of CIB is not required for binding to α Ib, a Ca²⁺-induced myristoyl switch mechanism similar to the NCS proteins (Ames and Ikura 2002; O'Callaghan and Burgoyne 2003) could also target CIB toward the cytoplasmic membrane, which would increase the probability of an interaction with α Ib. Therefore, although the Mg²⁺-dependent binding of CIB to α Ib can occur in the absence of Ca²⁺, Ca²⁺-binding to CIB could potentially alter its interactions with α Ib β_3 and contribute to the regulation of integrin-mediated signaling events.

Materials and methods

Protein expression, purification, and peptide synthesis

Unlabeled and uniformly ¹⁵N-labeled CIB were expressed and purified as previously described (Yamniuk et al. 2004). Both peptides used in this study were synthesized commercially and were determined to be more than 95% pure by high pressure liquid chromatography and matrix-assisted laser desorption/ionization mass spectroscopy. Peptide α Ib-L (Ac-LVLAMWKVGFFKRNRPPEEDDEEGQ-OH) corresponds to amino acids 983–1008 of the α Ib subunit of platelet integrin α Ib β_3 , and comprises the entire cytoplasmic domain. This peptide contains the E1008Q post-translational modification that was suggested by mass spectrometry (Calvete et al. 1990). Peptide α Ib-S (Ac-LVLAMWKVGFFKRN-NH₂) is a shortened version of α Ib-L containing only the membrane proximal region of α Ib.

Fluorescence spectroscopy

All fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorimeter. In each experiment the Trp residue of α Ib-L or α Ib-S was selectively excited at 295 nm using an excitation slit width of 5 nm, and emission spectra were recorded from 300 to 450 nm using an emission slit width of 10 nm. All samples consisted of 8–10 μ M peptide in 20 mM HEPES, 1 mM DTT (pH 7.3) with or without 100 mM KCl. Studies with Ca²⁺-CIB were performed in 2 mM CaCl₂, while Mg²⁺-CIB samples contained 2 mM MgCl₂ and 0.5 mM EGTA, and apo-CIB samples contained 2 mM EDTA and

2 mM EGTA. This strategy for producing Ca²⁺-, Mg²⁺-, and apo-CIB has been described previously (Yamniuk et al. 2004). Titration experiments involved sequential addition of microliter volumes of 100–200 μ M CIB in the respective buffer into 1-ml samples of α Ib-L or α Ib-S. The changes in fluorescence intensity at the emission wavelength maxima of each complex were used to calculate the K_d for the interaction. In the case of α Ib-L binding to apo-CIB, a second estimate for the K_d was obtained using the wavelength change of this emission maximum ($\Delta\lambda_{\max}$). For fluorescence quenching experiments, 1-ml samples of 10 μ M peptide saturated with 12 μ M CIB were titrated with aliquots from a 5 M solution of CsCl to a final concentration of 1.4 M, and the fluorescence emission intensity was recorded at 351 nm for Ca²⁺-CIB and Mg²⁺-CIB, 333 nm for apo-CIB, and 355 nm for the unbound peptides. A total of six titration points were used in each case to generate Stern-Volmer plots that follow Equation 1:

$$I_0/I = K_{sv}[Q] \quad (1)$$

where Q is the quencher CsCl, I_0 is the fluorescence intensity at 0 M CsCl, I is the intensity at each titration point, and K_{sv} is the quenching constant. All Stern-Volmer plots were linear and there were no changes in the emission wavelength maxima during the CsCl titrations, indicating that no significant structural changes occurred in the presence of large amounts of CsCl.

Isothermal titration calorimetry

All ITC experiments were performed on a MicroCal VP-ITC microcalorimeter. CIB was dissolved in 20 mM HEPES, 100 mM KCl (pH 7.3) and 2–10 mM DTT, and incubated at room temperature for several hours to reduce all sulfhydryl groups. Since the presence of DTT causes undesirable noise in ITC baselines, it was removed prior to ITC analysis by passing the samples through an Econo-Pac 10DG column (BioRad) equilibrated with 20 mM HEPES, 100 mM KCl (pH 7.3). Then concentrated metal ion or chelator solutions were added to give final concentrations of 2 mM CaCl₂ for Ca²⁺-CIB, 4 mM MgCl₂ and 1 mM EGTA for Mg²⁺-CIB, 2 mM CaCl₂ and 4 mM MgCl₂ for Ca²⁺(Mg²⁺)-CIB, or 2 mM EDTA and 2 mM EGTA for apo-CIB. The α Ib-L peptide was simply dissolved in the respective ITC buffer. Titrations consisted of 4–11- μ L injections of 280–500 μ M CIB into a 1.43-mL sample cell containing 15–25 μ M α Ib-L at various temperatures. The heat of dilution was estimated from the heat of injection after saturation and subtracted before performing the curve fitting (Pierce et al. 1999). The stoichiometry (N), association constant (K_a), and enthalpy change (ΔH) were each obtained directly from the data, while the entropy change (ΔS) and Gibbs free energy change (ΔG) were calculated using Equations 2 and 3:

$$\Delta G = -RT \ln K_a \quad (2)$$

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

By performing the titrations at multiple temperatures, the change in heat capacity associated with binding was also calculated using Equation 4:

$$\Delta C_p = d\Delta H/dT \quad (4)$$

All data were fit to a one-site model using MicroCal Origin software.

NMR spectroscopy

All ^1H , ^{15}N HSQC NMR spectra were recorded at 37°C on a Bruker Avance 500 NMR spectrometer equipped with a triple resonance inverse cryoprobe with a single axis Z-gradient. Samples of $570\ \mu\text{M}$ CIB in 20 mM HEPES, 100 mM KCl, 10 mM $\text{d}_{10}\text{-DTT}$, 10% D_2O (pH 7.5 ± 0.1) with an additional 3 mM CaCl_2 for Ca^{2+} -CIB, 5 mM MgCl_2 + 1 mM EGTA for Mg^{2+} -CIB, or 2.5 mM EDTA + 2.5 mM EGTA for apo-CIB, were titrated with 1.1–1.2 mM $\alpha\text{IIb-L}$ in the respective buffer. Each spectrum was recorded with sweep widths of 8012.82 Hz and 1600.0 Hz and carrier frequencies of 500.13235 MHz and 50.68365 MHz for the ^1H and ^{15}N dimensions, respectively. Quadrature detection in the F1 dimension was obtained using the echo/anti-echo time-proportional phase increment method. A total of 2048×256 real data points were recorded in ^1H and ^{15}N , respectively, with 16 scans for peptide-free CIB and 48 scans for peptide-bound CIB in order to obtain a similar signal-to-noise ratio with the peptide-diluted samples. Each spectrum was zero filled once in each dimension and referenced in ^1H to 0 ppm using the DSS signal in the one-dimensional ^1H spectrum of each sample and in ^{15}N using the conversion factor of 0.101329118 as suggested by Sykes and coworkers (Wishart et al. 1995). Data analysis was performed using NMRpipe and NMRDraw software (Delaglio et al. 1995).

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