Calcium-dependent properties of CIB binding to the integrin α Ib cytoplasmic domain and translocation to the platelet cytoskeleton

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The α Ib β 3 integrin receives signals in agonist-activated platelets, resulting in its conversion to an active conformation that binds fibrinogen, thereby mediating platelet aggregation. Fibrinogen binding to α Ib β 3 subsequently induces a cascade of intracellular signalling events. The molecular mechanisms of this bi-directional α Ib β 3-mediated signalling are unknown but may involve the binding of proteins to the integrin cytoplasmic domains. We reported previously the sequence of a novel 22-kDa, EF-hand-containing, protein termed CIB (calcium- and integrin-binding protein) that interacts specifically with the α Ib cytoplasmic domain in the yeast two-hybrid system. Further analysis of numerous tissues and cell lines indicated that CIB mRNA and protein are widely expressed. In addition, isothermal titration

calorimetry indicated that CIB binds to an α Ib cytoplasmic-domain peptide in a Ca^{2+} -dependent manner, with moderate affinity (K_d , 700 nM) and 1:1 stoichiometry. In aggregated platelets, endogenous CIB and α Ib β 3 translocate to the Triton X-100-insoluble cytoskeleton in a parallel manner, demonstrating that the cellular localization of CIB is regulated, potentially by α Ib β 3. Thus CIB may contribute to integrin-related functions by mechanisms involving Ca^{2+} -modulated binding to the α Ib cytoplasmic domain and changes in intracellular distribution.

Key words: EF-hand motif, fibrinogen, intracellular signalling, platelet activation, platelet aggregation.

INTRODUCTION

The integrin α Ib β 3, or glycoprotein IIb-IIIa, is the major integrin-adhesion receptor on platelets and mediates both platelet aggregation and clot retraction. In unstimulated platelets the majority of α Ib β 3 is present in a resting conformation or low-affinity state, and is unable to bind circulating plasma fibrinogen. However, upon platelet activation by agonists, α Ib β 3 receives intracellular signals (inside-out signalling) that allow its rapid conversion to a high-affinity state capable of binding soluble ligands [1]. Once fibrinogen binds to α Ib β 3, this integrin stimulates intracellular signalling (outside-in signalling), resulting in cytoskeletal rearrangement and recruitment of signalling molecules to the cytoskeleton, which are probably required for processes such as clot retraction [2]. The mechanism of integrin-mediated bi-directional signalling is unclear but molecular approaches have demonstrated that the short cytoplasmic tails of both the α and β subunits play an important role in both inside-out [3–5] and outside-in signalling [6]. The α Ib and β 3 cytoplasmic domains have been shown to interact with a number of proteins, such as talin [7], Shc and Grb2 [8]. Moreover, a novel 110-amino acid protein termed β 3-endonexin, which interacts with the β 3 cytoplasmic domain, was identified recently using the yeast two-hybrid system [9], and found to convert α Ib β 3 to an active state in Chinese hamster ovary cells [10].

We recently identified a novel 22-kDa calcium- and integrin-binding protein, termed CIB, that interacts specifically with the cytoplasmic domain of the integrin α Ib subunit [11]. CIB is

expressed in platelets and binds to heterodimeric α Ib β 3 *in vitro*. CIB contains at least two EF-hand domains and is homologous with other EF-hand containing proteins such as the regulatory molecules, calcineurin B (58 % homology) and calmodulin (CaM; 56 % homology) [12,13].

Here we show that CIB mRNA and protein are widely distributed in a variety of tissues. Moreover, CIB binds with moderate affinity and in a calcium-dependent manner to the α Ib cytoplasmic domain. In aggregated platelets, but not activated platelets without aggregation, endogenous CIB and α Ib translocate to the Triton X-100-insoluble cytoskeleton in a parallel manner. These results suggest that CIB might influence aspects of α Ib β 3 function in platelets, but may also have distinct roles in cells that do not express this integrin.

EXPERIMENTAL

Peptide synthesis

Two N-terminally acetylated peptides corresponding to the C-terminal 26 amino acids of α Ib [LVLAMWKVGFFKRNRP-PLEEDDEEG(E/Q)], were synthesized and HPLC-purified by The University of North Carolina at Chapel Hill/National Institute of Environmental Health Sciences Protein Chemistry Laboratory (Chapel Hill, NC, U.S.A.). Note that the peptides terminated with either a C-terminal glutamic acid (peptide E), as predicted by the cDNA sequence of α Ib [14], or a glutamine (peptide Q), as predicted by Calvete et al. [15] based on analysis

Abbreviations: CIB, calcium- and integrin-binding protein; CaM, calmodulin; ITC, isothermal titration calorimetry; mAb, monoclonal antibody.

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of platelet α IIB by MS. A truncated α IIB peptide with sequence Ac-AMWKVGFFKRNRPPLEE (where Ac is acetyl) was synthesized and HPLC-purified by the University of North Carolina at Chapel Hill Department of Microbiology Protein Sequencing and Peptide Synthesis Core Facility.

Antibodies

The affinity purified mouse monoclonal antibody (mAb) UN2 was raised against recombinant purified CIB and characterized as described previously [11]. Affinity purified anti- α IIB polyclonal antibody SEW8 was a generous gift from Dr. Peter Newman, Blood Center of Southeastern Wisconsin, Milwaukee, WI, U.S.A. All other reagents were of analytical grade.

Northern-blot analysis

Human multiple-tissue Northern blots (Clontech, Palo Alto, CA, U.S.A.) were probed with 32 P-labelled CIB cDNA (5 ng/ml) following the manufacturer's protocol. Blots were subjected to autoradiography overnight. The blots were stripped to remove the initial probe and reprobed with β -actin cDNA to determine the relative amounts of the cDNA loaded in each lane, which were similar.

Recombinant CIB

PCR was used to amplify the coding sequence for CIB and to introduce 5' *Bgl*II and 3' *Eco*RI restriction sites. The purified PCR product was digested with *Bgl*II and *Eco*RI, ligated into pGEX-2T (Pharmacia, Piscataway, NJ, U.S.A.) between *Bam*HI and *Eco*RI sites, sequenced and subsequently expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase. *E. coli* were grown at 37 °C and induced with 0.1 mM IPTG for 2.25 h. Cell pellets from 6 litres of *E. coli* culture were resuspended in buffer A (1 × PBS, pH 7.5, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM PMSF and 5 mM dithiothreitol) and lysed three times in a French press (1.1 × 10⁸ Pa). Cell lysate was clarified by centrifugation, loaded on to a 25-ml glutathione-Sepharose 4B column (Pharmacia), washed with 20 mM Tris/HCl (pH 7.5)/137 mM NaCl and eluted with 25 ml of 10 mM reduced glutathione in elution buffer (50 mM Tris/HCl, pH 8.0). The eluate was incubated with bovine α -thrombin (Pharmacia; 10 units/mg of fusion protein) at ambient temperature for 2–4 h, resulting in > 99% cleavage of fusion protein. CIB was further purified by gel-exclusion chromatography using a HiPrep 16/60 S-200 column equilibrated in 20 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 mM EDTA/1 mM EGTA/10% glycerol, followed by passage over glutathione-Sepharose 4B to remove residual glutathione S-transferase. A final purification step was performed on an 8-ml Resource Q column (Pharmacia) equilibrated with 20 mM Tris/HCl (pH 7.5)/10 mM NaCl/10% (v/v) glycerol. The protein was eluted with a linear gradient of 0–500 mM NaCl, concentrated by ultrafiltration (Amicon) with a YM-10 membrane, and stored at –80 °C. CIB constituted > 99% of total protein as determined on Commassie Brilliant Blue-stained polyacrylamide gels. Recombinant CIB encodes the full-length protein as well as an N-terminal dipeptide cloning artifact (glycine-serine), previously part of the thrombin-recognition sequence.

Isothermal titration calorimetry (ITC)

ITC was performed using an Omega Microcal calorimeter (Microcal, Northhampton, MA, U.S.A.). CIB, lyophilized bovine brain CaM (Calbiochem, San Diego, CA, U.S.A.) and α IIB

peptides were dialysed extensively against ITC buffer (20 mM Hepes, pH 7.5, 137 mM NaCl and 5 mM CaCl₂) prior to all titrations. Concentrations of CIB (ϵ_{280} , 2560 M⁻¹·cm⁻¹), CaM (ϵ_{280} , 2560 M⁻¹·cm⁻¹) and α IIB peptides (ϵ_{280} , 5690 M⁻¹·cm⁻¹) were determined as described previously [16]. CIB and α IIB cytoplasmic-domain peptides were diluted to 50 µM and 2.1 mM respectively with ITC buffer and used for titrations maintained at 25 °C and consisting of 20 5-µl injections of peptide into CIB at 300-s intervals. Similar titrations of ITC buffer into CIB, α IIB peptides E and Q into ITC buffer and ITC buffer into ITC buffer were performed to determine heats of dilution and mixing. Titrations of peptide Q (1.25 mM) into CaM (50 µM) as a negative control were performed as above for CIB. The enthalpy of binding was determined as described in [17]. Binding isotherms and curve fitting were obtained using Origin for ITC analysis software (Microcal). For experiments without calcium, calcium was removed from CIB by one of two procedures. First, the α IIB peptide and CIB were dialysed for a minimum of 24 h against a modified ITC buffer (20 mM Hepes, pH 7.5, 137 mM NaCl, 5 mM EDTA and 5 mM EGTA) supplemented with 1 g/100 ml Chelex 100 resin (BioRad, Hercules, CA, U.S.A.). ITC titrations were performed as described above. In the second procedure, CIB was decalcified by three rounds of precipitation with 3% trichloroacetic acid and refolded as described in detail by Haiech et al. [18]. Each round of precipitation was followed by resuspension of CIB in 1 M Chelex-treated non-neutralized Tris base except after the final precipitation when CIB was resuspended in 1 M Hepes/NaOH, pH 7.5. CIB was then dialysed extensively against either the modified ITC buffer, which contained Ca²⁺ chelators as described above, or ITC buffer, which contained 5 mM Ca²⁺, and analysed for peptide-binding properties by ITC as above.

Preparation of washed platelets

Whole blood was drawn by venipuncture from healthy aspirin-free donors and collected in ACD (83 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid, pH 4.5; 1:7, v/v). Platelet-rich plasma was isolated by centrifugation at 250 g for 20 min, and prostaglandin E₁ (100 nM) or prostaglandin I₂ (50 ng/ml) was added. The platelet-rich plasma was centrifuged for 15 min at 850 g, and the platelets were resuspended in Hepes-buffered saline (10 mM Hepes, pH 7.4, 138 mM NaCl, 12 mM NaHCO₃, 10 mM KCl, 5.5 mM glucose, 0.35% BSA, 2 units/ml heparin and 1 unit/ml apyrase). Resuspended platelets were washed three times in buffer A without heparin but with prostaglandin E₁ or prostaglandin I₂ added prior to each centrifugation (850 g, 10 min). The washed platelets were finally resuspended in buffer A without BSA or inhibitors and the concentration was adjusted to (0.5–1.0) × 10⁹ platelets/ml. Platelets incubated for ≈ 30 min at 37 °C before use.

Platelet activation and cytoskeleton isolation

Washed platelets were stimulated with human thrombin (1 unit/ml) under non-stirring or stirring conditions [1200 rev./min in a Chronolog aggregometer (Havertown, PA, U.S.A.)] at 37 °C. Stimulation of platelets with other agonists was performed under stirring conditions only. Platelet cytoskeletons and membrane skeletons were isolated as described by Fox et al. [19], except that the low-speed centrifugation to isolate the cytoskeleton was for 6 min. Briefly, activated platelets were lysed (1:1, v/v) by addition of 2 × lysis buffer (100 mM Tris/HCl, pH 7.5, 2% Triton X-100, 10 mM EGTA, 100 mM benzamidine, 2 mg/ml leupeptin and 1 mM Pefabloc SC). The lysate was

centrifuged at $\approx 15000 g$ for 6 min to obtain the actin cytoskeleton. In some experiments the supernatant was centrifuged at $100000 g$ (Beckman TLA 100.2 rotor) for 2.5 h to obtain the membrane skeleton. The supernatant following this centrifugation was designated the Triton X-100-soluble fraction. All fractions were solubilized in Laemmli sample buffer, separated by SDS/PAGE [20], and electrotransferred to Immobilon-P membranes [21]. Membranes were incubated in blocking buffer [5% non-fat dried milk (Bio-Rad) in Tris-buffered saline (20 mM Tris/HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween-20] for 1–2 h and stained with UN2 or SEW8 in blocking buffer for 1 h. The membranes were incubated with sheep anti-mouse Fc γ chain-specific IgG (Jackson) or donkey anti-rabbit IgG (Amersham) conjugated with horseradish peroxidase and developed using enhanced chemiluminescence (ECL) as per the manufacturers' instructions. Immunoblots were analysed on a GS670 densitometer (Bio-Rad).

RESULTS

Tissue expression of CIB

Since α IIb β 3 expression is largely restricted to platelets and megakaryocytes, we asked whether CIB expression is restricted to haematopoietic cells or more widely distributed. Probing of a multiple-tissue Northern blot with CIB cDNA indicated a single species of mRNA (0.9 kb) in all tissues examined, especially pancreas and heart (Figure 1, top panel). Reprobing the Northern blot with β -actin cDNA confirmed that differences in CIB distribution could not be accounted for by differences in loading (results not shown). We determined previously that CIB protein is expressed in platelets [11]. Using platelets as a positive control, we found by Western blotting that CIB protein is also expressed in cell lines of megakaryocytic or erythrocytic lineages, i.e. DAMI, Meg 01, K562 and HEL, and in the non-haematopoietic T47D breast epithelial carcinoma cell line (Figure 1, bottom panel). Previous lack of detection of message or protein in some of these sources may have been due to less-optimized conditions [11]. Thus mRNA encoding CIB and CIB protein appear to be widely distributed.

ITC

We reported previously that CIB bound to the α IIb cytoplasmic domain, as detected in the yeast two-hybrid system, and to the intact α IIb β 3 integrin in an ELISA assay [11]. To gain more quantitative information on this interaction, we examined CIB binding to several α IIb cytoplasmic-domain peptides by ITC. ITC detects the interaction of molecules in solution, without separation of bound from unbound molecules as in some more classical binding assays, thus allowing more accurate measurement of affinity, stoichiometry and other physical characteristics of binding [22].

CIB binding was measured to an α IIb cytoplasmic-domain peptide with a C-terminal glutamine (peptide Q), as predicted by MS of platelet α IIb, which suggests a post-translational (E $\rightarrow$ Q) modification [15]. Results from ITC indicated that CIB binding to this α IIb cytoplasmic-domain peptide was strongly favoured; a K_d of 700 nM and a stoichiometry of 1:1 (peptide/CIB) were obtained. These values were confirmed by an independent technique; changes in intrinsic fluorescence of the α IIb peptide upon binding to CIB in solution indicated a similar K_d and stoichiometry (D. D. Shock, L. V. Parise and J. Sondek, unpublished work). As measured by ITC, CIB also bound to an α IIb peptide with a C-terminal glutamic acid (peptide E), with slightly lower affinity (K_d , 2 μ M; results not shown). The

Figure 1 Expression of CIB in various human tissues and cell lines

(Top panel) A multiple-tissue Northern blot of 10 μ g of total mRNA from the indicated human tissues were probed with 32 P-labelled CIB cDNA as described in the Experimental section. (Bottom panel) Western blot of cell lysates. Platelets, as a positive control, or cell lines were lysed in 1% Triton X-100 in PBS. Samples (25 μ g) of the whole lysates were separated by SDS/PAGE, transferred on to PVDF membranes and immunostained with the anti-CIB mAb, UN2, as described in the Experimental section. Note that the left and right blots are from separate experiments; relative amounts of CIB can be compared within each blot but not between blots. The 22-kDa CIB appears to migrate at ≈ 25 kDa due to the aberrant migration of the prestained molecular-mass markers.

interaction of CIB with the α IIb peptide is driven predominantly by entropic factors and appears largely hydrophobic (see the Discussion section). This is consistent with our inability to co-immunoprecipitate CIB with α IIb β 3 from detergent-lysed platelets (results not shown), since detergents would be expected to disrupt hydrophobic interactions.

The specificity of this interaction is supported by the observation that, under similar conditions, the α IIb cytoplasmic-domain peptide did not interact with the CIB homologue, purified bovine brain CaM (Figure 2B, $\circ$). Specificity was further confirmed by the observation that CIB also did not interact with a truncated α IIb peptide consisting of residues 986–1002 (Figure 2B, $\blacktriangledown$). This result suggests further that one or more of the hydrophobic amino acids at the N-terminus of the cytoplasmic domain and/or the charged residues at the C-terminus are required for CIB binding.

In separate experiments, CIB binding to the α IIb cytoplasmic-domain peptide was examined after removal of calcium by exhaustive treatment with a series of calcium chelators or

Figure 2 ITC of CIB binding to α IIb peptides

(A) Calorimetric trace of heat released upon titration of the cytoplasmic domain of the post-translationally modified form of α IIb (Ac-LVLAMWKVGFFKRNRPLEEDEEGQ; peptide Q) into calcium-bound CIB. (B) Specificity of the α IIb cytoplasmic-domain peptide binding to CIB. Titration of CIB with peptide Q (■) or a truncated α IIb cytoplasmic-domain peptide (Ac-AMWKVGFFKRNRPLEE; ▼) were fitted to a one-site model. Values obtained for the binding of peptide Q to CIB were as follows: K_D , 700 nM; ΔG , -8.4 kcal/mol; ΔH , -3.3 kcal/mol; and $-T\Delta S$, -5.1 kcal/mol. The stoichiometry of binding was 1:1 (peptide:CIB fitted ratio, N , = 1.15). ○, indicate a titration of bovine CaM with peptide Q in the presence of 5 mM CaCl_2 . These data do not realistically fit to any model and the heats generated were essentially equivalent to the heats of dilution. (C) Inset shows a binding isotherm from α IIb cytoplasmic-domain peptide E binding to calcium-depleted CIB.

following decalcification of CIB at low pH (see the Experimental section). Upon calcium removal by either method, virtually identical results were obtained; the affinity of CIB for the α IIb peptide had decreased by at least two orders of magnitude and the binding curve was no longer sigmoidal (Figure 2C). This binding curve could no longer be accurately fitted to either a 1:1 or several other stoichiometric models of binding. These results demonstrate that CIB interacts with the α IIb peptide in a largely calcium-dependent manner.

Translocation of CIB and α IIb β 3 to the platelet cytoskeleton

We next asked whether CIB translocates to the low-speed Triton X-100-insoluble actin cytoskeleton of activated or aggregated platelets, as do many signalling molecules. The total amount of

Figure 3 Time course of CIB translocation from the Triton X-100-soluble fraction to the insoluble actin cytoskeleton in thrombin-aggregated platelets

Washed platelets were aggregated with 1 unit/ml thrombin under stirring conditions for the times indicated. The platelets were lysed in Triton X-100 and the insoluble actin cytoskeleton (CSK, A) and the soluble fraction (B) were obtained following centrifugation (6 min, 15 000 *g*). Equivalent amounts of each fraction and whole platelet lysate (lane L), representing the total amount of CIB were electrophoresed, transferred on to PVDF membranes, immunostained with the anti-CIB mAb UN2, and detected by ECL as described in the Experimental section. (C) To determine whether CIB translocation depended on fibrinogen-mediated platelet aggregation, stirred platelets were pretreated for 5 min with either 200 μ M RGDW or RGEW as a control, prior to addition of 1 unit/ml thrombin for the times indicated. Cytoskeletons were obtained as above and the blots were stained with UN2. (D) To determine whether the amount of sedimentable actin cytoskeleton was related to the amount of CIB that sedimented, platelets were aggregated by stirring with thrombin for 2 min as above and lysed with Triton X-100 lysis buffer containing (+) or lacking (−) EGTA. EGTA omission allows actin filaments to be severed by gelsolin. Cytoskeletal samples from equivalent numbers of platelets were electrophoresed on either a 10% gel that was directly stained with Coomassie R-250 (left) or a 14% gel that was transferred on to a PVDF membrane and immunostained with mAb UN2 (right).

CIB in the aliquots of platelet lysate used for experiments in Figures 3(A) and 3(B) is shown in lanes labelled L. Before the addition of thrombin, CIB was absent from the Triton X-100-insoluble cytoskeleton (Figure 3A, time 0), but present in the soluble fraction (Figure 3B, time 0). However, when platelets were stirred with thrombin to induce platelet aggregation, there

Figure 4 Comparison of CIB with α IIB cytoskeletal translocation in platelets aggregated by various agonists

Washed platelets (5×10^9 /ml) were stimulated with $1 \mu\text{M}$ A23187, $1 \mu\text{M}$ PMA, $2.5 \mu\text{g/ml}$ collagen or control vehicle (0.02% DMSO) for the indicated times. Platelets were lysed, and the actin cytoskeleton (CSK) was sedimented by centrifugation for 6 min at $\approx 15\,000\text{ g}$. The pellets were solubilized in reducing SDS/PAGE sample buffer and subjected to electrophoresis on 4–20% polyacrylamide gradient gels. Proteins were transferred on to PVDF membranes and immunostained with the anti-CIB mAb, UN2 (A), or the anti- α IIB polyclonal antibody, SEW8 (B). Note that the patterns of CIB and α IIB translocation are similar.

was a significant increase in the amount of CIB that co-sedimented with the cytoskeleton (Figure 3A, 10–300 s) and a corresponding loss of CIB from the soluble fraction (Figure 3B, 10–300 s).

To determine whether the redistribution of CIB to the cytoskeleton was a function of ligand binding *per se*, or was dependent on platelet aggregation, platelets were stirred in the presence of thrombin and the RGDW peptide. This peptide was expected to bind to α IIB β 3, block the binding of secreted fibrinogen and block platelet aggregation. The RGDW peptide completely blocked CIB translocation to the cytoskeleton except at the 300-s time point (Figure 3C). However, by this time platelet micro-aggregates were observed. In contrast, CIB translocation to the cytoskeleton was not blocked by a control peptide (RGEW; Figure 3C). We also found that CIB translocation was not observed when platelets were activated by thrombin in the absence of stirring, conditions that result in fibrinogen binding to platelets but without aggregation (results not shown). Collectively, these results suggest that platelet activation and ligand occupancy of α IIB β 3 are insufficient to promote CIB translocation, and that platelet aggregation is required. In separate experiments, we also examined the high-speed platelet-membrane cytoskeleton, but found little CIB present in this fraction, whether obtained from resting or activated platelets (results not shown). Thus CIB translocates to the low-speed Triton X-100-insoluble cytoskeletal fraction in thrombin-aggregated platelets.

If CIB co-sediments with the actin cytoskeleton because of an attachment to it, procedures that decrease the amount of sedimented actin should also decrease the amount of associated CIB. To test this, cytoskeletal fractions were isolated from thrombin-aggregated platelets in the presence of an EGTA-containing lysis buffer as usual, or in a modified lysis buffer that lacked EGTA (Figure 3D). EGTA is normally present to prevent activation of the actin-severing protein, gelsolin, by Ca^{2+} that is released during platelet lysis, whereas in the absence of EGTA, activated gelsolin causes a decrease in actin-filament content of the cytoskeleton [23]. We observed that, in the absence of EGTA, 36% less actin and 57% less CIB sedimented with the Triton X-100-insoluble proteins (Figure 3D). These results suggest that CIB sediments due to an attachment to the cytoskeleton, and not because CIB becomes insoluble for other reasons.

It is well documented that α IIB β 3 translocates to the cytoskeleton upon platelet aggregation [19,24]. We therefore asked whether the translocation of CIB paralleled that of α IIB β 3. This was addressed by comparing a time course of CIB translocation with that of α IIB in platelets that were stirred in the presence of various agonists other than thrombin. Neither CIB nor α IIB β 3 translocated in significant amounts in the absence of an agonist (DMSO; Figure 4). However, in platelets aggregated by the calcium ionophore A23187, or collagen, both CIB (Figure 4A) and α IIB β 3 (Figure 4B) translocated to the cytoskeleton within 1 min, whereas PMA caused a relatively delayed translocation of both CIB and α IIB β 3. The similar patterns of α IIB β 3 and CIB translocation to the cytoskeleton suggest that the translocation of one may depend upon the other.

DISCUSSION

CIB was identified recently in the yeast two-hybrid system as a 22-kDa α IIB cytoplasmic-domain-binding protein that contains at least 2 EF-hand motifs and binds Ca^{2+} [11]. It shows the greatest homology with the regulatory molecules calcineurin B and CaM, and KIP2, a molecule of unknown function [25]. In the present study, we found that CIB protein and mRNA have a wide tissue distribution. We have also demonstrated that the binding of CIB to peptides mimicking the α IIB cytoplasmic domain is highly favoured and most likely hydrophobic. Finally, CIB translocates from a detergent-soluble to the Triton X-100-insoluble cytoskeleton in aggregated platelets under conditions that promote translocation of α IIB β 3. These results suggest that CIB may serve as a docking and/or regulatory molecule in a variety of cells, potentially at the cytoplasmic domain of α IIB and in the cytoskeleton in platelets.

In the present study we observed that CIB mRNA and protein are widely distributed. However, unlike CIB, the α IIB β 3 integrin is restricted in distribution, being found only in platelets, megakaryocytes and some transformed cells. CIB may therefore be a widely distributed regulatory molecule with both α IIB β 3 integrin- and non-integrin-related functions.

ITC was used to determine the properties of CIB binding to several α IIB cytoplasmic-domain peptides. In a strictly calcium-

dependent manner, CIB binds to a peptide corresponding to the entire cytoplasmic domain of α IIB with sub-micromolar affinity (K_d , 700 nM) and 1:1 stoichiometry. This interaction is specific, since another EF-hand-containing protein, bovine brain CaM, shows no detectable binding activity towards the α IIB peptide.

While this article was under review, a paper appeared by Vallar et al. [26] claiming, in contrast to our results, that CIB binds with > 10-fold lower affinity and in a Ca^{2+} -independent manner to the α IIB cytoplasmic domain [26]. The higher-affinity interaction that we measured is likely to be more accurate due to the fact that we used full-length CIB, not glutathione S-transferase-CIB, and because this K_d value falls well within a range detected accurately by ITC (10^{-3} – 10^{-9} M) [22]. Moreover, we confirmed our measurements with the independent approach of intrinsic protein fluorescence. Finally, surface plasmon resonance is subject to a large number of pitfalls not intrinsic to ITC [27]. With regard to the Ca^{2+} dependency of CIB binding to the α IIB cytoplasmic domain, the glutathione S-transferase-CIB fusion protein used by Vallar et al. [26] may have been less flexible or conformationally altered such that it was insensitive to changes in Ca^{2+} levels as compared with CIB itself, which we used. In addition, it is well documented that EF-hand-containing proteins such as CIB can bind Ca^{2+} tightly such that simple incubation with EDTA, as indicated by Vallar et al. [26], is often insufficient to remove Ca^{2+} from these proteins [18]. We found that CIB treated for calcium removal with two methods accepted in the EF-hand field (i.e. dialysis for > 24 h against EDTA, EGTA and Chelex, or trichloroacetic acid precipitation and refolding [18]) gave identical results; the affinity of CIB binding to the α IIB cytoplasmic domain was decreased by well over two orders of magnitude following Ca^{2+} removal.

The contacts between model peptides and CaM observed in crystal structures [28] were used to guide the synthesis of a truncated α IIB peptide that would potentially retain binding activity towards CIB. This peptide lacked three N-terminal and six C-terminal amino acids. We found that this peptide had no observable affinity for CIB, suggesting that one or more of the truncated amino acids were critical for high-affinity interaction of α IIB and CIB. However, this finding further substantiates the specificity of the interaction between CIB and the complete α IIB cytoplasmic-domain peptide.

The ITC data also indicate that CIB binding to the α IIB cytoplasmic domain is driven primarily by increased system entropy. The homology of CIB with CaM ($\approx 56\%$) and other EF-hand-domain-containing proteins suggests that CIB may bind effector peptides in a manner similar to EF-hand proteins. Calcium binding causes substantial conformational changes in many EF-hand proteins, leading to the exposure of large hydrophobic surfaces involved in peptide binding [29]. By analogy, large hydrophobic portions of CIB and α IIB cytoplasmic-domain peptide presumably interact, leading to the expulsion of structured water from the peptide-protein interface into bulk solvent. Upon complex formation, displaced water would contribute greatly to overall system entropy, outweighing any entropic costs associated with less-mobile peptide or protein. This would account for the thermodynamics of the reaction in a process classically referred to as the hydrophobic effect [30]. If so, this is consistent with the fact that we have been unable to co-immunoprecipitate CIB with α IIB/ β 3, despite the use of multiple detergents and buffer conditions, since hydrophobic interactions would be disrupted by detergent.

Interestingly, a similar analysis of CaM binding to a peptide from smooth-muscle myosin light-chain kinase indicated the opposite thermodynamic behaviour [31]. CaM bound to the peptide with a large favourable enthalpy and negligible or

unfavourable entropy. This behaviour is consistent with the decreased mobility of both CaM and the peptide upon complex formation. It is also consistent with the numerous van-der-Waals and electrostatic interactions within the complex, as observed in the crystal structure [28]. The differences in thermodynamics of peptide binding to CIB versus CaM probably reflect underlying structural differences. CaM possesses inherent flexibility that allows it to bind numerous peptide effectors with high complementarity and affinity [29]. In an unbound state, CIB may be less mobile than CaM. Upon complex formation, the interface between CIB and α IIB may be less complementary than similar CaM-peptide complexes. This binding behaviour would also suggest that CIB may bind fewer target effectors than CaM.

Consistent with its putative role as a calcium-dependent regulatory protein, the affinity of CIB binding to the α IIB peptide in the absence of calcium was at least two orders of magnitude lower than that of calcium-bound CIB. Moreover, the binding could no longer be fitted to a peptide:CIB ratio of 1:1, 2:1 or several other stoichiometric models of binding. The low-affinity binding that remains after calcium removal could represent binding that occurs only with calcium-stripped CIB, binding that occurs due to a low but residual amount of calcium or, less likely, a calcium-independent component that is also present with calcium-replete CIB, and is probably not physiologically relevant. Thus CIB binding to the α IIB cytoplasmic domain is largely calcium-dependent and potentially modulated by changes in intracellular calcium.

CIB exhibits a dramatic and almost complete translocation to the cytoskeleton of agonist-aggregated platelets, but not of activated platelets without aggregation. These results suggest that CIB would not be expected to translocate to the cytoskeleton of agonist-activated platelets from patients with Glanzmann's thrombasthenia, since these platelets do not aggregate. In normal platelets, a variety of signalling molecules also translocate to the cytoskeletal fraction of aggregated platelets, including phospholipase C [32], Tec [33], kinases such as p160 ROCK (Rho-associated coiled-coil-containing kinase) [34] and phosphatidylinositol 3-kinase [35], and protein phosphatases such as PP1 and PP2a [36]. Translocation of many of these molecules also appears to depend upon α IIB/ β 3 occupancy and platelet aggregation. Following CIB translocation in aggregated platelets, the platelets are lysed in Triton X-100. The fact that this association is maintained in the presence of detergent suggests a non-hydrophobic mechanism of association, unlike that with α IIB/ β 3, which might make it possible for CIB to co-associate with the cytoskeleton and the integrin. Whether CIB serves to carry or attach the integrin to the cytoskeleton or whether CIB is required for this translocation is possible but unknown. It is equally possible that CIB and α IIB/ β 3 translocate to the cytoskeleton without direct physical association with one another. One notion of cytoskeleton function is as a solid state for the surface chemistry of signalling reactions. The presence of CIB in the cytoskeleton suggests that it is available to regulate some of the multiple kinases, phosphatases or other signalling molecules that also become associated with the cytoskeleton. This observation is consistent with deposits in GenBank and the literature indicating the identification of proteins with sequences virtually identical to CIB that bind to serum-inducible kinase or SNK [37] (accession no. U83236) and DNA-dependent protein kinase [38]. However, Western blotting of a CIB immunoprecipitate with an anti-DNA-dependent protein kinase antibody was negative. (J. E. Brittain, U. P. Naik and L. V. Parise, unpublished work). Thus the exact role of CIB in cells awaits the further definition of intracellular proteins that interact with this potential regulatory molecule.

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