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Calcium Integrin Binding Protein Associates with Integrins $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ Independent of β_3 Activation Motifs

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ABSTRACT

The Calcium Integrin Binding protein (CIB) has been identified as interacting specifically with the cytoplasmic tail of the integrin α_{IIb} domain to induce receptor activation and integrin $\alpha_{IIb}\beta_3$ mediated cell adhesion to extracellular proteins. In K562 cells stably expressing mutated integrin $\alpha_V\beta_3$, or chimeric $\alpha_V\beta_3$ carrying α_{IIb} cytoplasmic tail, we report that the interaction of CIB with β_3 integrins is not $\alpha_{IIb}\beta_3$ specific but binds α_{IIb} as well as α_V cytoplasmic tail domains. A double mutation of two proline residues to alanine residues in the α_{IIb} cytoplasmic domain, previously shown to disturb its conformation, inhibits chimeric $\alpha_V/\alpha_{IIb}\beta_3$-CIB interaction. This demonstrates that α_{IIb} cytoplasmic domain loop-like conformation is required for interaction with CIB. Moreover, mutations of β_3 cytoplasmic domain residues Tyr-747 and/or Tyr-759 to phenylalanine residues (Y747F, Y759F, and Y747,759F) as well as residues Ser-752 to proline or alanine (S752P and S752A), do not affect the $\alpha_{IIb}\beta_3$ or $\alpha_V\beta_3$ interaction with CIB. Since tyrosine residues Tyr-747 and/or Tyr-759 are the sites of tyrosine phosphorylation of β_3 subunit, these results suggest that the β_3 integrin-CIB interaction occurs through a β_3-phosphorylation independent mechanism. Likewise, ablation of conformation-dependent affinity change in β_3 Ser752Pro mutation had no affect on CIB-β_3 interaction. In summary, our results demonstrate that the α_{IIb}-subunit integrin and CIB interaction is non-exclusive and requires the loop-like α_{IIb}-cytoplasmic domain conformation. An interaction of CIB with α_V-containing integrins provides an additional role for this molecule in keeping with its expression outside of platelets.

Keywords: Leukocyte; Integrin; Cytoskeleton; Hematopoietic; Activation; Signaling

1. Introduction

The Calcium Integrin Binding (CIB) protein is a 22-kDa intracellular member of the family of regulatory calcium-binding proteins that includes calmodulin, calcineurin B and recoverin. It was originally detected through the use of yeast two-hybrid systems as interacting specifically with the integrin α_{IIb}-cytoplasmic tail in the presence of calcium [1]. Various methods have subsequently confirmed this interaction *in vitro* [2-4] and *in vivo* [5]. This CIB-α_{IIb} interaction has been shown to promote the affinity of the $\alpha_{IIb}\beta_3$ integrin to fibrinogen [5]. Moreover, it has been shown that the association of CIB with integrin $\alpha_{IIb}\beta_3$ during outside-in signaling is required for platelet spreading on fibrinogen [6] through regulation of focal adhesion kinase (FAK) activation [7]. Furthermore, it was reported that CIB binds to the GTPase Rac3 and that co-expression of active Rac3 and CIB in Chinese hamster ovary (CHO) cells also expressing $\alpha_{IIb}\beta_3$ results in enhanced $\alpha_{IIb}\beta_3$-mediated cell adhesion and spreading on fibrinogen [8]. In addition to its interaction with the $\alpha_{IIb}\beta_3$ integrin, FAK and Rac3 regulatory functions, CIB may have other functions because it interacts with several diverse cytosolic proteins involved in neuronal function (FnK and Snk kinase) [9] and Alzheimer's disease (Presenilin 2) [10].

A previous model based on homology modeling and nuclear magnetic resonance (NMR) studies suggests that the N-terminal domain of CIB containing positively charged amino residues could interact with the acidic C-terminal tail of α_{IIb} whereas the C-terminal domain of CIB could bind the α_{IIb} membrane proximal α-helix structure carrying the highly conserved GFFKR motif [4]. In yeast two-hybrid assays using fragments of CIB as bait or in intrinsic tryptophan fluorescence assays, the C-terminus of CIB serves as a site of interaction of CIB with the α_{IIb} cytoplasmic tail [3]. This study localized the CIB binding site on α_{IIb} to within a 15-amino acid residue stretch including residues within the membrane-splanning region as well as several residues within the membrane proximal region of the α_{IIb} cytoplasmic domain (from L983 to R997). Because the membrane proximal GFFKR motif of α_{IIb} is highly conserved among α in-

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tegrin subunits, the specificity of the CIB-α_{IIb} interaction suggests the presence of a supplementary determinant involved in this exclusive relationship. The α_{IIb} and β_3 subunit cytoplasmic domains form α-helices and exhibit two-stranded coiled-coil structures in which there are several electrostatic and hydrophobic interactions [11,12]. The inhibition of these interactions is emerging as the molecular mechanism leading to the receptor activation and, through competition with α_{IIb} subunit in binding the β_3 subunit; CIB may contribute to this process. The α_{IIb} subunit cytoplasmic domain is organized in a "closed" conformation where the highly conserved N-terminal and membrane proximal GFFKR motif form an α-helix followed by a turn, and the acidic C-terminal loop interacts with the N-terminal helix. The substitutions of proline residues 998 and 999 with alanine residues generate an "open" conformation where the interactions between the N-terminal helix and C-terminal moiety are abolished, inducing receptor activation [13].

In the present study using K562 cells expressing mutated integrins, we report that, in addition to the N-terminal membrane proximal GFFKR motif, the acidic C-terminal of the α_{IIb} subunit cytoplasmic domain as well as the α_{IIb} "closed" conformation are important for the α_{IIb}-CIB interaction. Furthermore, we show for the first time that CIB also interacts with the α_V cytoplasmic domain. Moreover, mutations of tyrosine or serine residues on the β_3 subunit cytoplasmic domain, previously shown to prevent β_3 tyrosine phosphorylation or receptor activation, failed to prevent interaction with CIB. Together these data indicate that in K562 cells, in addition to the N-terminal GFFKR motif, the binding of CIB to the cytoplasmic domain is α_{IIb} cytoplasmic domain conformation dependent and is not $\alpha_{IIb}\beta_3$ specific, providing a basis for understanding CIB-integrin interaction *in vivo* and providing an additional role for CIB in non-platelet cell types.

2. Materials and Methods

2.1. Construction of Chimeric and Mutant Integrins

The human erythroleukemic cell line K562 transfected with cDNA encoding various mutated β_3 or truncated α_V integrin subunits were derived and maintained as previously described [14]. In addition, K562 cells were transfected with cDNA encoding full-length β_3 and with cDNA encoding either truncated or double substituted proline residues to alanine residues of the α_{IIb} cytoplasmic domain fused with the α_V extracellular moiety. Transfected cells were selected for expression by fluorescent cell sorting flow cytometry using mAb to α_V and β_3 as previously described [14]. The recombinants α_V/α_{IIb}, in which α_V cytoplasmic sequence has been replaced with the corresponding α_{IIb} sequence were prepared in the following way: human wild type α_{IIb} cDNA (a kind gift of Dr Ulhas P. Naik of the University of Delaware) was subjected to PCR using the IHY009 primer 5'CGAGGGGA**GGTTACC**GTGTGGACACAGCTG3' introducing a new *BstE*II site (underlined) at the 2933 position and the IHY010 primer 5'GGC**GGATCCATCA**CTCCCCCTCTTCATC3' introducing a new *Bam-H*I site (underlined) at the 3210 position. After *BstE*II-*Bam*HI digestion, the PCR generated product was ligated into pcDNA3.1 zeo (-) α_V digested with *BstE*II-*BamH*I, creating pcDNA3.1 zeo (-) α_V/CTα_{IIb} construct. Similarly, using α_{IIb} cDNA as template and the same forward and reverse primers; 5'CCTCT**GGATCC**TCTTCTT**A**CAGGGGTGGC3', 5'CATCT**GGATCC**AGGGGTGGCCG**TTA**CCGCTTG3' and 5'CCGGA**TCC**GCTTGAAGAAGCC**TT**GACGTTCC3' 5'GGCCGGA**TCC**GCTTGAAGAA**TTA**GACCTTCC3', we generated PCR products with new stop codons and BamHI sites at 3087 and 3096, 3078 and 3090, 3063 and 3077 positions respectively. As shown previously, using α_{IIb} cDNA as template and IHY009 with 5'GCGGAACCGG**G**CA**G**CCCTGGAAG- AAGATG3' and IHY010 with 5'CATCTTCTTCCAGGG**C**TG**C**CCGGTTCCGC3' in a first round PCR we made PCR products which were then used as a template for nested PCR using IHY009 and IHY010 primers. These final products were digested with BstEII and BamHI and ligated in to pcDNA3.1 zeo (-) α_V/CTα_{IIb} digested with the same enzymes. These products, with β_3 cDNA, were stably expressed in K562 cells to generate Kα_V/α_{IIb}WTβ_3, Kα_V/α_{IIb}999β_3, Kα_V/α_{IIb}995β_3, Kα_V/α_{IIb}990β_3 and Kα_V/α_{IIb}PP998/999AAβ_3 mutants expressing $\alpha_V/\alpha_{IIb}\beta_3$ integrin receptors having the extracellular α_V domain fused to the α_{IIb} transmembrane and cytoplasmic lacking the 9, 13 or 18 last amino acids or with the proline residues 998 and 999 mutated to alanine residues respectively. The mutations were assessed and confirmed for each mutant by DNA sequence analysis of the recombinant cDNA using the forward IHY009 primer in automated DNA sequencing analysis ABI 3100 Genetic Analyzer (SUNY Upstate Medical University, NY) and are depicted in **Figure 1**.

2.2. Cell Transfection and Flow Cytometry Analysis

K562 cells, an erythroleukemic, promonocyte cell line were grown in Iscove's Modified Eagle's Medium (IMDM) plus 10% fetal calf serum (FCS). About 1×10^7 cells were washed twice with IMDM, resuspended in 500 µl of HEPES Buffered Saline containing 25 µg of each purified recombinant α_V/α_{IIb} subunit cDNA (in pcDNA3.1 expression vector carrying a zeocin resistance gene) and

β_3 integrin cDNA (in pRc/RSV expression vector carrying a geneticin resistance gene). After incubation on ice for 10 min, cells were electroporated twice at 225 volts, 500 μF capacitance and 500 ohms and then resuspended in 10 ml of IMDM plus 10% fetal calf and incubated for 24 hrs. The cells were transferred into 10 ml of the same medium supplemented with 1.2 μg/ml of geneticin, incubated for 10 days and then in the same medium described above and also containing 1mg/ml zeocin. Transfected cells and populations of transfectants expressing high levels of integrins were obtained by fluorescence cell sorting using a mouse monoclonal anti-β_3 subunit antibody (mAb 7G2). The expression level of the recombinant receptors was maintained by periodic sorting with magnetic beads linked to an anti-β_3 subunit (mAb AP3) and anti-α_V subunit (mAb L230) and monitored by flow cytometry using the same antibody. Flow cytometry was carried out using a Coulter Epics XL flow cytometer (Coulter, Miami, FL).

2.3. Analysis of β_3 Integrin-CIB Interaction

K562 cells (10×10^6) expressing equivalent amounts of transfected integrins were washed twice and resuspended in 300 μl of IMDM containing 100 μM sodium vanadate (Na_3VO_4) with 2.0 mM MnCl for 15 min and lysed in 500 μl phosphate buffered saline (PBS) containing 0.5% NP40, 200 mM $CaCl_2$, 150 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2.0 mM Na_3VO_4. The lysates were centrifuged at 15,000 rpm for 10 minutes at 4˚C and then precleared with gelatin-Sepharose for 1 hour at 4˚C. Precleared lysates were then immunoprecipitated with goat anti-mouse-Sepharose beads (ICN, Cosa Mesa, CA) coated with either anti-β_3 (mAb 7G2) and anti-α_V (mAb 3F12) or anti-CIB (mAb UN2-NH) monoclonal antibodies for 4 h at 4ºC. Immunoprecipitates were washed and separated on 7.5% (for β_3 detection) or 12% (for CIB detection) SDS-PAGE. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes. Equivalence of immunoprecipitated β_3-integrin or CIB was verified by probing immunoprecipitated products with anti-β_3 (mAb 7G2) or anti-CIB (mAb UN2-NH) monoclonal antibodies and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) and compared with prestained molecular weight markers (Rainbow, Amersham Biosciences).

2.4. Antibodies and Reagents

The monoclonal anti-β_3 integrin antibody 7G2 used in immunoprecipitation and β_3 western blotting was a gift from Dr. Eric J. Brown of UCSF. Monoclonal antibodies AP3 (anti-β_3), M148 (anti-α_{IIb}) and L230 (anti-α_V) have been used to assess the surface expression on transfected K562 cells as previously described [15]. The monoclonal anti-CIB antibody UN2-NH was a gift from Dr. Ulhas P. Naik of the University of Delaware. All other reagents unless otherwise noted were purchased from Sigma.

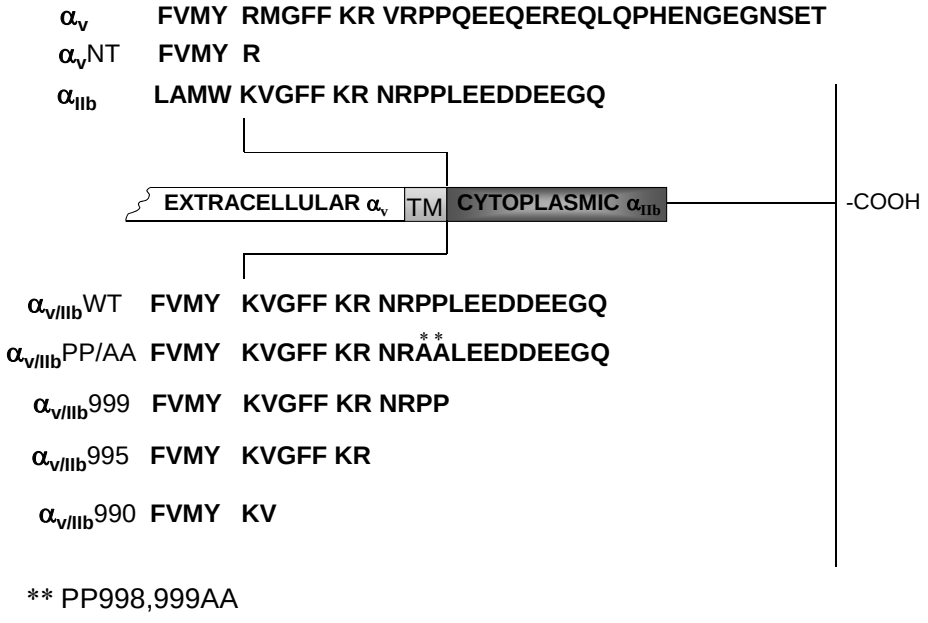

Figure 1. Schematic of chimeric, mutant, and truncated integrin construction. A comparison of the cytoplasmic domain sequences of α_V and α_{IIb} integrins is shown along with the sites of chimeric splicing, point mutations, and cytoplasmic truncations. cDNA modifications were performed as described in Methods and verified by DNA sequencing. A line indicates the conserved charged residue (K or R) of each integrin at the boundary of the transmembrane domain (TM), immediately preceding the cytoplasmic tail.

3. Results and Discussion

3.1. Expression of Mutant and Chimeric Integrins in K562 Cells

We have previously demonstrated that the tyrosine residues 747 and the 759 of the integrin β_3 cytoplasmic domain as well as the integrin α_V cytoplasmic tail are involved in the phosphorylation of β_3 integrins [14,15]. Likewise, others have shown that serine residue 752 is involved in β_3 integrin activation [16] and in β_3 integrin-mediated FAK phosphorylation [17]. Moreover, CIB is considered to bind specifically to the $\alpha_{IIb}\beta_3$ integrin through interaction with α_{IIb} cytoplasmic domain [1]. β_3 phosphorylation and the conformational change associated with increased affinity are critical to β_3 function. Further, while CIB association is reportedly specific to α_{IIb}, CIB expression is not restricted to platelets.

In this study we sought to establish the role of α-subunit structure and β_3 activation motifs in CIB association with β_3 integrins. To that end, K562 cells expressing stable recombinants of α_V or α_{IIb} wild type integrin subunits associated with β_3 subunit bearing either phenylalanine (phe) substitutions of Tyr-747 (K$\alpha_V\beta_3$Y747F, K$\alpha_{IIb}\beta_3$Y747F), Tyr-759 (K$\alpha_V\beta_3$Y759F, K$\alpha_{IIb}\beta_3$Y759F), or both (K$\alpha_V\beta_3$-Y747/759F, K$\alpha_{IIb}\beta_3$Y747/759F), proline (pro) substitution of serine (ser) residue 752 (K$\alpha_V\beta_3$S752P, K$\alpha_{IIb}\beta_3$ S752A) or conservative alanine (ala) substitution of Ser-752 (K$\alpha_V\beta_3$S752A, K$\alpha_{IIb}\beta_3$ S752A). We also used K562 cells expressing stable recombinants of wild type β_3 subunits associated with an α_V subunit where the cytoplasmic domain has been deleted (Kα_VNTβ_3) or fused with α_{IIb} transmembrane and cytoplasmic lacking off the 9 (Kα_V/α_{IIb}999β_3), 13 (Kα_V/α_{IIb}995β_3) or 18 (Kα_V/α_{IIb}990β_3) last amino acids or bearing pro residues 998 and 999 substitution to Ala residues (Kα_V/α_{IIb}PP998/999AAβ_3) as depicted in **Figure 1**. Surface expression of the β_3 integrins was detected on cells transfected with cDNA encoding for mutated α_V, α_{IIb} or β_3. After several rounds of cell sorting, the FACS profiles of recombinants or wild types were similar with the exception of the mutants carrying α_V or α_{IIb} subunits where the deletion includes the membrane-proximal GFFKR motif (**Table 1**). This suggests that there are no obvious differences in the expression stability of the wild-type and various mutants and also confirms the involvement of the membrane-proximal GFFKR motif in efficient cell surface expression which has been shown by several reports to prevent either the heterodimerization of the α and β subunits [18,19] or their cell surface expression [20]. Moreover, when expressed in lymphocytes [20] or K562 cells [21], the deletion of this motif does not induce constitutive activation of the receptor (data not shown) in contrast with previous data of the receptor expressed transiently in CHO cells [22-24]. This suggests that the mechanism of integrin activation is cell type dependent.

Table 1. Expression of integrins in K562 cells.

	Anti-β_3 (AP3)	Anti-α_V (L230)	Anti-α_{IIb} (M148)
Untransfected K562 cells	1.1	1.0	1.3
K$\alpha_V\beta_3$			
WT	13.5	26.4	1.8
Y747F	12.1	18.7	1.2
Y759F	15.2	28.4	0.9
Y747F, Y759F	17.7	31.1	1.1
S752P	12.2	26.6	1.0
S752A	11.2	30.1	1.0
Kα_VNTβ_3	4.1	5.1	0.9
K$\alpha_{IIb}\beta_3$			
WT	11.1	1.3	25.5
Y747F	11.3	1.8	27.1
Y759F	13.2	1.1	20.2
Y747F, Y759F	13.4	1.1	23.7
S752P	11.8	1.3	22.5
S752A	13.4	1.5	26.6
K$\alpha_V/\alpha_{IIb}\beta_3$			
WT	17.7	23.1	1.3
PP998/999AA	13.4	22.0	1.8
999	14.6	19.7	1.2
995	15.1	24.3	0.9
990	4.1	4.3	1.1

K562 cells were stably transfected with cDNA encoding $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$ or chimeric $\alpha_V/\alpha_{IIb}\beta_3$ with α_V, α_{IIb} or β_3 bearing the indicated cytoplasmic mutations. High expressing population were compared with untransfected K562 cells for expression of integrin by flow cytometry using mAb AP3 (anti-β_3), mAb L230 (anti-α_V) and mAb M148 (anti-α_{IIb}), Shown is the mean channel fluorescence from a representative measurement. WT, wild type, given in arbitrary units.

3.2. Interaction of CIB with $\alpha\beta_3$-Integrin Cytoplasmic Domains

We previously showed that upon cell activation by a RGD peptide, the β_3 integrin becomes phosphorylated at Tyr747 in β_3 integrins associated either with α_{IIb} or α_V whereas the phosphorylation at Tyr759 residue occurs only when the β_3 integrin is associated with α_{IIb} suggesting a regulation of this critical biochemical event by the α subunits [15]. Manganese cation (Mn^{2+}) can induce β_3 integrin activation and tyrosine phosphorylation [14]. Previous reports showed that the mutation of Ser752Pro limits β_3 integrin activation [16] and exposes a CIB binding site on α_{IIb} cytoplasmic domain [2]. To determine the involvement of β_3 cytoplasmic tyrosine and serine residues in the interaction of β_3 integrins with CIB, we used CIB blotting to examine immunoprecipitates from Mn^{2+} treated K562 cells expressing either α_V or α_{IIb} wild type subunits associated with wild type β_3 or β_3 bearing Phe substitutions at Tyr747 (Y747F) and Tyr759 (Y759F) both individually and concurrently (Y747,759F) or Pro (S752P) and Ala (S752A) substitution at Ser752. We show that CIB co-immunoprecipitates with all mutated β_3 subunits associated with α_V or α_{IIb} wild type (**Figure 2**). Therefore, in K562 cells, the CIB-β_3 integrin interaction is not $\alpha_{IIb}\beta_3$ specific, suggesting the ability of the α_V cytoplasmic domain to interact with CIB. To confirm this CIB-α_V interaction, we immunoprecipitated lysate from Mn^{2+} treated K562 cells expressing α_V in which the cytoplasmic domain had been deleted (Kα_VNTβ_3) in comparison with cells expressing wild type $\alpha_V\beta_3$ (K$\alpha_V\beta_3$ WT) and $\alpha_{IIb}\beta_3$ (K$\alpha_{IIb}\beta_3$ WT) as control. As shown in **Figure 3(a)**, the deletion of the α_V cytoplasmic domain prevents the co-immunoprecipitation of CIB by anti-β_3 and anti-α_V antibodies, confirming that CIB interacts with a site in the α_V cytoplasmic domain.

Several reports have shown that CIB can interact with the α_{IIb} cytoplasmic domain by a calcium dependent mechanism and induce the activation of $\alpha_{IIb}\beta_3$ integrin; enabling $\alpha_{IIb}\beta_3$-mediated cell adhesion or spreading on fibrinogen [6,7]. The membrane proximal GFFKR motif of the α_{IIb} cytoplasmic domain has been shown to be a site of this interaction [3]. However, since this motif is highly conserved among α integrin subunits and since CIB is considered to interact specifically with the α_{IIb}

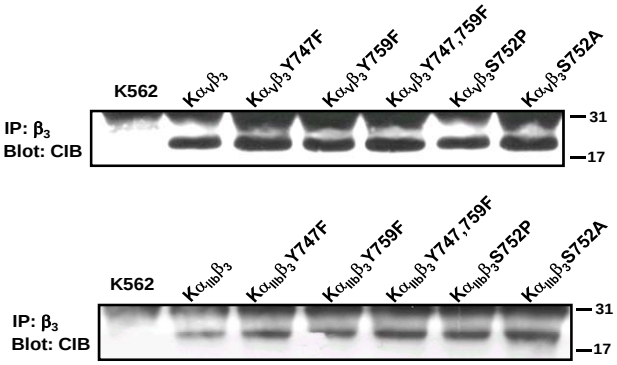

Figure 2. Effect of mutations of β_3 cytoplasmic domains on interaction with CIB. CIB blots of $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ immunoprecipitations from K$\alpha_V\beta_3$Y747F, K$\alpha_{IIb}\beta_3$Y747F, K$\alpha_V\beta_3$Y759F, K$\alpha_{IIb}\beta_3$Y759F, K$\alpha_V\beta_3$Y747/759F, K$\alpha_{IIb}\beta_3$Y747/759F, K$\alpha_V\beta_3$S752P, K$\alpha_{IIb}\beta_3$, K$\alpha_V\beta_3$S752A, K$\alpha_V\beta_3$S752P, and K$\alpha_{IIb}\beta_3$S752A cells. Cell lysates were immunoprecipitated with mAb 7G2 (anti-β_3) and CIB detected within immunoprecipitates by Western blot using mAb UN2-NH (anti-CIB). Shown is a representative of multiple experiments.

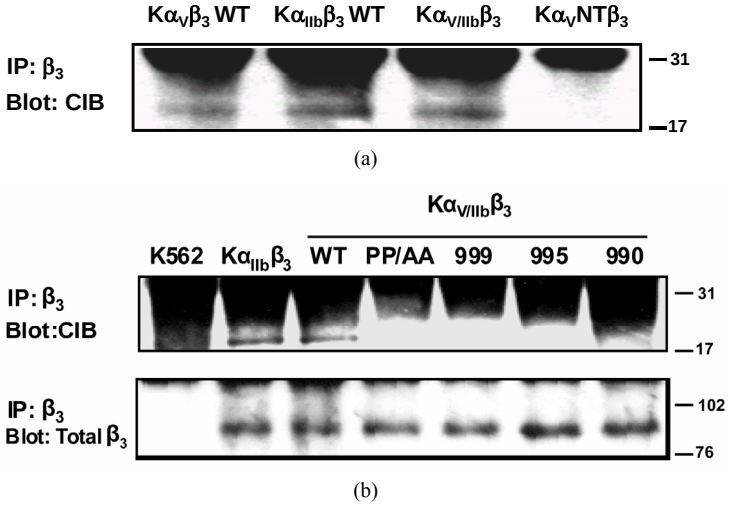

Figure 3. Effect of mutations of α_V and α_{IIb} cytoplasmic domains on interaction with CIB. K562 cells expressing cDNA encoding for $\alpha_{IIb}\beta_3$ or $\alpha_V\beta_3$ wild type, $\alpha_V/\alpha_{IIb}\beta_3$ and α_VNTβ_3 cells (a) or $\alpha_V/\alpha_{IIb}\beta_3$ bearing mutations on α_{IIb} subunit cytoplasmic domain (b) were immunoprecipitated with mAb 7G2 (anti-β_3) and co-precipitated CIB detected by Western blot using mAb UN2-NH (anti-CIB). Total β_3 was determined in a fraction of immunoprecipitates using mAb 7G2 (anti-β_3). Shown is a representative of multiple experiments.

cytoplasmic domain, the CIB-$\alpha_{IIb}\beta_3$ interaction is probably mediated through a supplementary α_{IIb} cytoplasmic domain. As previously suggested by Hwang and Vogel [4], the α_{IIb} C-terminal LEEDDEEGE domain is suited to play this role. The current model of α_{IIb} cytoplasmic domain structure suggests a "closed" conformation where the highly conserved N-terminal membrane-proximal GFFKR motif forms a α-helix followed by a turn, and the C-terminal loop interacts with the N-terminal helix in an intramolecular interaction [11]. The substitution of α_{IIb} cytoplasmic domain residues Pro998 and Pro999 with alanine residues has been shown to convert this structure to an "open" conformation; inducing the $\alpha_{IIb}\beta_3$ activation [13]. To assess the involvement of those different α_{IIb} cytoplasmic domains or their conformation in the α_{IIb}-CIB interaction, we immunoprecipitated β_3 from Mn^{2+} treated K562 cells expressing $\alpha_{IIb}\beta_3$ (K$\alpha_{IIb}\beta_3$) or chimeric $\alpha_V/\alpha_{IIb}\beta_3$ integrin receptors bearing the extracellular α_V domain fused to the α_{IIb} transmembrane and cytoplasmic domain full length (WT) or lacking the final 9 (999), 13 (995) or 18 (990) last amino acids or with the residues Pro998 and Pro999 substituted by Ala residues (PP/AA). Blotting with anti-CIB antibody (UN2-HN) reveals that CIB can interact only with the full-length α_{IIb} cytoplasmic domain with the native conformation. Thus, the deletion of only last 9 last amino acids (LEEDDEEGE) forming the acid C-terminus (999) or with the conserved motif GFFKR prevents the interaction of the α_{IIb} cytoplasmic domain with CIB suggesting that both domains are involved in this interaction. Interestingly, the destruction of the α_{IIb} cytoplasmic domain conformation by the substitution of residues Pro-998 and Pro-999 by Ala residues (PP/AA) also inhibits this interaction (**Figure 3(b)**). Thus, more than the presence of LEEDDEEGE and GFFKR domains, the interaction of CIB with the α_{IIb} cytoplasmic domain requires its native conformation. Using the K562 cells expressing chimeric $\alpha_V/\alpha_{IIb}\beta_3$ integrins with α_{IIb} cytoplasmic domain bearing the deletions or mutations mentioned above, we confirmed these observations by immunoprecipitating the cell lysates with beads coupled to anti-CIB (UN2-NH) and then blotted the transferred proteins with an anti-β_3 antibody. In these reverse immunoprecipitations, CIB association with β_3 required the entire cytoplasmic tail in native conformation (**Figure 4**).

Previous evidence suggests that CIB induces the activation of the $\alpha_{IIb}\beta_3$ integrin by interacting specifically with the α_{IIb} cytoplasmic tail. In the present work, we show that CIB can be co-immunoprecipitated with β_3 integrin associated with α_V or α_{IIb}, suggesting a non-exclusive interaction. This observation has been confirmed by the inhibition of the CIB interaction by the deletion of α_V cytoplasmic domain. The comparison of the α_{IIb} and α_V cytoplasmic tail sequences reveals a few similarities. Both α subunits retain the WKxGFFKR motif that is thought to be CIB binding site [2,4]. In addition, both α subunits contain a PP motif and a very acidic sequence. These two features distinguish α_V and α_{IIb} from all other α subunits. Although the WKxGFFKR motif of α_{IIb} cytoplasmic domain is considered as necessary for the CIB binding, it is unlikely to be the unique factor determining this interaction since this motif is common to all α subunits. We show here that the deletion of the distal domain LEEDDEEGE (mutant 999), previously considered as a potential site for interaction with the N-terminus of CIB [4], prevents the co-immunoprecipitation with the dimeric integrin. As expected, the deletion of the membrane proximal domain GFFKR (mutant 995) also prevents this co-immunoprecipitation. Our data suggest a simultaneous participation of these two α_{IIb} cytoplasmic domains in the interaction with CIB. Moreover, we show here that, even in the presence of these domains, this interaction requires the native α_{IIb} cytoplasmic domain conformation suggesting that the spatial disposition of the α_{IIb} binding sites of CIB is also a determinant of this interaction.

It has been shown that α_{IIb}-CIB interaction is calcium dependent [1] and a model suggests that the α_{IIb} cytoplasmic domain contains a high affinity cation binding site [25]. Additionally, Vinogradova *et al.* have proposed that calcium binding to α_{IIb} cytoplasmic domains is determined by intramolecular interaction between the residues Arg997, Glu1001, Asp1003, and Asp1004 of the native conformation [26]. Since the substitution of residues Pro-998 and Pro-999 by Ala residues (PP/AA) inducing the "open" conformation provokes the destruction of these intramolecular interactions, the inhibition of the CIB-α_{IIb} interaction could be due to inability of the PP/AA mutant to bind calcium cations. The α_{IIb}-β_3 interaction is thought to maintain the receptor in a low affinity state through a transmembrane subunit contact domain and GFFKR juxta-transmembrane region and to be calcium dependent [25]. A structural study suggests that

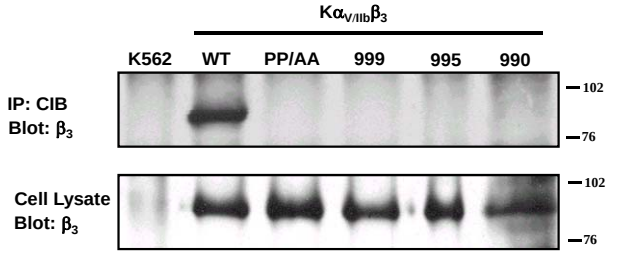

Figure 4. Effect of mutations on α_{IIb} cytoplasmic domains on interaction with CIB. K562 cells expressing cDNA encoding for $\alpha_V/\alpha_{IIb}\beta_3$ bearing indicated mutations or truncations of the α_{IIb} subunit cytoplasmic domain were immunoprecipitated with mAb UN2-NH (anti-CIB) and β_3 detected by Western blotting using mAb 7G2 (anti-β_3). Total β_3 was determined by Western blot of lysates of cells probed with mAb 7G2 (anti-β_3). Shown is a representative of multiple experiments.

the calcium cation is likely to enhance and stabilize the intramolecular interactions of the α_{IIb} cytoplasmic domain but is not essential for the α_{IIb}-β_3 interaction. However calcium stabilizes the $\alpha\beta$ complex in low-affinity states [27] by decreasing the dissociation rate [2].

We previously showed that β_3-tyrosine phosphorylation requires the α_V cytoplasmic domain [14] and can be regulated differentially when the β_3 subunit is associated with α_{IIb} or α_V [15]. This clearly indicates that this critical biochemical process is regulated by the α subunit cytoplasmic domain; probably through steric hindrance determining the access of β_3 subunit cytoplasmic sites for its protein binding partners required for post-ligand events. The incomplete homology between α_{IIb} and α_V cytoplasmic domain suggests two different conformations generating two different steric hindrances. Therefore, the PP/AA mutation of α_{IIb} cytoplasmic domain gives a new tool to explore the influence of the steric access to β_3 cytoplasmic domain interacting proteins. Regardless, the previously reported selective association of CIB with α_{IIb} does not hold true in this hematopoietic cell system and does not offer a mechanism to explain α-subunit regulation of β_3 tyrosine phosphorylation. Naik *et al.*, in the yeast two-hybrid system, using the α_{IIb} cytoplasmic domain as "bait," showed that CIB interacts specifically with the α_{IIb} cytoplasmic domain [1]. Here, we show that this interaction is not α_{IIb} exclusive and suggest that cell environment is a factor determining the α cytoplasmic domain interaction specificity. A recent work suggests that CIB may exist in multiple structural and metal ion-bound states *in vivo*, which may also play a role in its regulation of target proteins such as platelet integrins [28]. A third partner could also be involved since Rac3 and the polo-like kinase Skn have been shown to interact with CIB [8,9].

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