

Probing conformational changes in the I-like domain and the cysteine-rich repeat of human $\beta 3$ integrins following disulfide bond disruption by cysteine mutations: identification of cysteine 598 involved in $\alpha \text{IIb} \beta 3$ activation

Ping CHEN, Chantal MELCHIOR, Nicolaas H.C. BRONS, *Nicole SCHLEGEL, **Jacques

CAEN and Nelly KIEFFER

Laboratoire Franco-Luxembourgeois de Recherche Biomédicale (CNRS/CRP-Santé), Centre Universitaire, Luxembourg; *Service d'Hématologie Biologique, Hôpital Robert Debré, Paris France; and **Académie des Sciences, Institut de France, Paris, France

Corresponding author :

Dr Nelly KIEFFER
LFL – University Center
162A, avenue de la Faiencerie
L-1511 Luxembourg

Phone : (352) 466644-440
Fax : (352) 466644-442
Email : kieffer@cu.lu

Abstract

We have investigated receptor function and epitope expression of recombinant α Ib β 3 mutated at cysteines C¹⁷⁷ or C²⁷³ in the I-like domain, as well as C⁵⁹⁸, located in the fourth repeat of the membrane proximal cysteine rich region and mutated in a Glanzmanns thrombasthenia type II patient. The β 3 mutants β 3_{C177A}, β 3_{C273A} and β 3_{C598Y} exhibited a decreased electrophoretic mobility in SDS-PAGE under non-reducing conditions, confirming the disruption of the respective disulfide loops. Despite reduced surface expression, the α Ib β 3_{C177A}, α Ib β 3_{C273A} and α Ib β 3_{C598Y} receptors mediated cell adhesion to immobilized fibrinogen and translocated into focal adhesion plaques. The β 3_{C598Y} mutation, but not the β 3_{C177A} or β 3_{C273A} mutations, induced spontaneous binding of the ligand mimetic monoclonal antibody PAC-1, while the β 3_{C177A} and β 3_{C273A} mutants exhibited reduced complex stability in the absence of Ca²⁺. Epitope mapping of function blocking monoclonal antibodies (mAb) allowed the identification of two distinct subgroups : mAbs A2A9, p12-46, 10E5 and P256 did not interact with α Ib β 3_{C273A} and bound only weakly to α Ib β 3_{C177A}, while mAbs AP2, LM609 and 7E3 bound normally to mutant α Ib β 3_{C273A}, but interacted only weakly with mutant α Ib β 3_{C177A}. Furthermore, a cryptic epitope recognized by mAb 4D10G3 and not exposed on wild type α Ib β 3 became accessible only on mutant α Ib β 3_{C177A} and was mapped to the 60 kDa chymotrysin fragment of β 3. Finally, the LIBS (ligand-induced binding site) epitopes AP5, D3, LIBS1 and LIBS2 were spontaneously expressed on all three mutants

independent of RGDS or DTT treatment. Our results provide evidence that disruption of a single cysteine disulfide bond in the cysteine rich repeat domain, but not in the I-like domain, activates integrin α I**IIb** β 3. In contrast, disruption of each of the disulfide bonds in the two long insertions of the I-like domain predicted to be in close contact with the α subunit β -propeller domain, affects the stability of the α I**IIb** β 3 heterodimer and inhibits complex-specific mAb binding, without affecting the RGD binding capacity of the MIDAS-like domain.

Running title :

Cysteine 598 involved in α IIb β 3 receptor activation

Introduction

Integrins are heterodimeric cell surface receptors that mediate cell-cell or cell-matrix interactions and regulate numerous aspects of cell behaviour such as cell motility, proliferation, differentiation and apoptosis (1). During the process of receptor-ligand interaction, integrins function as allosteric receptors able to switch from a low to a high affinity ligand binding state (2). This molecular switch is thought to rely on conformational changes of the ectodomain of the receptor, brought about by intracellular signaling pathways in connection with integrin cytoplasmic tails and known as inside-out signaling (2,3). Conformational changes that coincide with receptor activation have been monitored by fluorescence energy transfer studies (4), as well as activation-dependent monoclonal antibodies (5). Ligand binding to activated α IIB β 3 itself induces additional conformational changes linked to the exposure of neoantigenic sites termed ligand-induced binding sites (LIBS), that are present on the occupied receptor but not on the resting receptor (6-9). Certain of these anti-LIBS antibodies also mimic physiologic inside-out signaling by increasing the ligand binding affinity of α IIB β 3 (7,9).

Although the molecular basis for the conformational changes associated with integrin receptor function modulation is still elusive, evidence has recently been provided that sulfhydryls may contribute to receptor function and that thiol bond reshuffling could represent a possible mechanism responsible for the conformational changes necessary for integrin activation (10).

Blocking of free sulfhydryls on the platelet surface inhibits platelet adhesion to collagen, fibronectin and fibrinogen (11), platelet aggregation following ADP stimulation (10), as well as adhesion of Mn^{2+} or antibody activated platelets (11), suggesting that free sulfhydryls play an important role in the process of integrin-ligand interaction, and that sulfhydryl blockers could act downstream of the conversion of the integrin high affinity state, possibly on the process of ligand binding itself. On the other hand, mild reducing agents, such as dithiothreitol, can increase the ligand binding function of $\alpha IIb\beta 3$ as well as other integrins, a method commonly used to activate recombinant integrins in transfected nucleated cells (12-17). Also, disruption of the long-range Cys⁵-Cys⁴³⁵ disulfide bond in the $\beta 3$ subunit by site directed mutagenesis or by mild proteolytic cleavage results in an increased affinity of $\alpha IIb\beta 3$ for fibrinogen (18-21). Evidence has also been provided that mutational introduction of disulfide bonds into the $\alpha L\beta 2$ integrin I domain locks this receptor in an open, ligand binding or closed non-binding conformation (22,23). And finally, Yan and Smith have recently demonstrated that a small number of cysteines, located within the EGF-like cysteine rich repeats (CRR) of the $\beta 3$ subunit, are unpaired and exhibit the properties of a redox site that could be involved in integrin activation (10). This $\alpha IIb\beta 3$ activation appears to be controlled by a protein-disulfide isomerase activity (PDI) (24,25), recently shown to be an intrinsic activity of integrin $\alpha IIb\beta 3$ (26). These data suggest that disulfide bond reshuffling in the $\beta 3$ subunit could be a potential mechanism involved in $\alpha IIb\beta 3$ receptor activation and ligand binding.

In this study, we have investigated the structural and functional relevance of the two disulfide

bonds, Cys¹⁷⁷-Cys¹⁸⁴ and C²³³-C²⁷³, present in two long insertions supposedly located on the same side of the I-like domain and facing the α subunit β propeller domain (27), as well as a third disulfide bond involving C⁵⁹⁸ linked to C⁵⁸⁸ and located in the fourth repeat of the C-terminal CRR of β 3. Our results provide evidence that disruption of a single cysteine disulfide bond in the cysteine rich repeat domain, but not in the I-like domain, activates integrin α IIb β 3. In contrast, disruption of each of the disulfide bonds in the two long insertions of the I-like domain affects the stability of the α IIb β 3 heterodimer and inhibits complex-specific mAb binding, without modifying the RGD binding capacity of the MIDAS-like domain.

Materials and Methods

Monoclonal antibodies

The mAb LM609 (anti- $\alpha v\beta 3$) was purchased from Chemicon International (Temecula, Ca) and the mAb PAC-1 (activation dependent, $\alpha IIb\beta 3$ complex specific) from Becton Dickinson (San Jose, CA). The following mAbs were generous gifts: S1.3 and 4D10G3 (D.R. Phillips, COR Therapeutics, South San Francisco, CA); LIBS1 and LIBS2 (M.H. Ginsberg, Scripps Research Institute, La Jolla, CA); D3 (L.K. Jennings, University of Tennessee, Memphis, TN, USA), A2A9 (S.J. Shattil, Scripps Research Institute, La Jolla, CA); 7E3 and 10E5 (B.S. Coller, Mount Sinai Hospital, New York); 16N7C2 (M. Hoylaerts, Center for Molecular and Vascular Biology, Leuven, Belgium); P37 (J. Gonzalez-Rodriguez, Instituto Quimica Fisica, Madrid, Spain); AP2 (T. Kunicki, Scripps Research Institute, La Jolla, CA); P256 (C. Bachelot, INSERM U428, Faculté des Sciences Pharmaceutiques, Paris France); pl2-46 and pl2-73 (C. Kaplan, Institut de Transfusion Sanguine, Paris, France); 4F8 (Biocytex, Marseille, France); Y2/51 (D. Mason, John Radcliffe Hospital, Oxford, UK.) SZ21 and SZ22 (C.G. Ruan, Jiangsu Institute of Haematology, Suzhou, China).

Construction and transfection of mutant $\beta 3$ integrin cDNA

Three distinct mutations C¹⁷⁷A, C²⁷³A, and C⁵⁹⁸Y, were introduced into the $\beta 3$ wt cDNA subcloned into the pcDNA 3.1(-) zeo vector by PCR-based mutagenesis. Briefly, primers were used that contained the mutation to be introduced into the $\beta 3$ cDNA as well as 5 and 3 end restriction sites allowing convenient insertion of each amplified cassette into the pcDNA3.1(-) zeo- $\beta 3$ vector from which the wild type sequence had been removed. The presence of each mutation was verified by DNA sequencing of the mutant $\beta 3$ cDNA. Wild type and mutant $\beta 3$ integrin cDNA constructs were transfected into CHO cells expressing recombinant human α IIB using the lipofectamine method (Life Technologies, Inc.) as previously described (28). Positive colonies were isolated by cylinder cloning and further subcloned by limiting dilution.

Flow cytometry analysis

Cell surface expression of recombinant α IIB $\beta 3$ was analysed by flow cytometry with a panel of anti- α IIB, anti- $\beta 3$ and complex-specific anti- α IIB $\beta 3$ monoclonal antibodies as previously described (28). Cells were detached from culture plates with EDTA buffer, pH 7.4 and washed twice with serum free IMDM. The cells (5×10^5 /ml) were incubated for 30 min at 4°C with saturating amounts of specific primary antibodies, washed and further incubated for 20 min on ice with FITC conjugated secondary antibodies, and then analysed on an Epics Elite ESP flow cytometer. For PAC-1 binding, the cells were resuspended in HEPES buffer (137 mM NaCl, 5 mM KCl, 50 mM HEPES, 1 mg/ml glucose, pH7.4) and incubated for 20 min at room temperature in the presence or absence of the α IIB $\beta 3$ -activating mAb D3. PAC-1 (3.5 μ g) was next added and incubated for 45 min at room temperature. After two further washings with HEPES buffer, the

cells were resuspended in 100 μ l of phycoerythrin-conjugated goat anti-mouse IgM, diluted 1/100 in HEPES buffer, and incubated for 30 min on ice. Cells were then washed twice with HEPES buffer and resuspended in 400 μ l of HEPES buffer containing 7ADD prior to flow cytometry analysis. PAC-1 binding (FL2) was analyzed on the gated subset of single, live cells. For LIBS epitope analysis, the cells were first incubated with or without DTT or RGDS at room temperature for 30 min. Anti-LIBS antibodies were then added and incubated on ice for another 45 min. Bound IgG was detected with FITC-labeled anti-mouse IgG and analysed by flow cytometry. For comparative data analysis between cell clones, β 3 expression was normalized for each cell clone with mAb P37 and the mean fluorescence measured with the different mAbs expressed as % of the mean fluorescence obtained with P37.

Western blot and immunoprecipitation

For Western blot analysis, the cells were detached, washed with PBS and lysed for 30 min at 4°C in Triton X-100 buffer (1% TritonX-100, 20 mM Tris-Cl, 150 mM NaCl, pH7.4, 1 mM CaCl₂, 1 mM MgCl₂, 50 μ M AEBSF, 10 μ g/ml E64, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin). Lysates were precleared by centrifugation at 10,000 rpm for 10 min at 4°C and the protein concentration determined. For immunoprecipitation experiments, cell lysate (500 μ g protein) was first incubated overnight at 4°C with P37 or 4F8 (anti- β 3) and then incubated with protein A-Sepharose beads (50 μ l of a 50% suspension in lysis buffer) for another 2 hrs. The beads were washed with lysis buffer 6 times and then boiled in 30 μ l of SDS sample buffer (2% SDS, 10% of glycerol, 25 μ g/ml bromophenol blue in 15.625 mM Tris-Cl, pH6.8). For analysis of chymotrypsin treated platelets, washed platelets were incubated for 1 h at 37°C with 0.5 mg/ml

of α -chymotrypsin, washed and then solubilized. CHO cell lysate (50 μ g protein), platelet lysate (5 μ g protein) or immunoprecipitates were resolved under non-reducing or reducing conditions by 7.8% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond C nitrocellulose membrane. The membrane was blocked in blotting buffer (20 mM Tris-Cl, pH7.4, 150 mM NaCl, 5% dry milk, 0.1% Tween-20) overnight at 4°C and probed for 2 hrs at room temperature with primary antibodies directed against the α IIb or β 3 subunits. After several 20 min washes in blotting buffer, the membrane was incubated with secondary goat anti-mouse IgG conjugated to horseradish peroxidase, then washed in TBS (20 mM Tris-HCl, pH7.4, 137 mM NaCl) and developed using the chemiluminescence ECL kit (Amersham, UK) according to the manufacturer's instructions.

Cell adhesion on immobilized fibrinogen and immunofluorescence staining of focal adhesion plaques.

Washed cells were plated onto glass coverslips, precoated with 20 μ g/ml fibrinogen, and allowed to spread at 37°C. The cells were then briefly rinsed with PBS and fixed for 15 min at 4°C with 2% paraformaldehyde in PBS containing 3% sucrose, pH 7.4. After several washes, the cells were permeabilized for 15 min with labeling buffer (PBS pH 7.4, 0.5% Triton X-100, 0.5% BSA) at room temperature, and incubated for 45 min at room temperature with mAb P37 (anti- β 3) or the complex-specific anti- α IIb β 3 mAb p12-73. After several washes, FITC-labeled goat-anti-mouse IgG was added and incubated for 30 min at room temperature. For co-staining of actin stress fibers, rhodamine-phalloidin was included in the final incubation. The coverslips were finally washed three times with labeling buffer, mounted in Mowiol 40-88/DABCO and

examined with a Leica-DMRB fluorescence microscope using a 63 × oil immersion objective.

Results

Effect of cysteine mutations on α IIb β 3 expression in CHO cells

Cysteine residues are highly conserved among integrin β subunits and are important for the correct folding of integrin subunits during protein synthesis, subunit-subunit heterodimerization as well as receptor conformation and function. Here we have investigated the structural and functional role of two cysteine loops within the I-like domain (C¹⁷⁷-C¹⁸⁴, C²³³-C²⁷³) of the β 3 subunit. In order to disrupt these cysteine loops, C¹⁷⁷ and C²⁷³ were mutated into alanine. In addition, we also investigated the effect of the naturally occurring C⁵⁹⁸Y mutation located in the CRR domain and initially identified in a thrombasthenic type II patient (29). The mutated β 3 subunits were stably transfected into CHO cells expressing an endogenous pool of the human α IIb subunit, and zeomycin-resistant cell clones were selected and analysed for cell surface

exposure of the mutant α IIb β 3 receptors. The anti- β 3 mAb P37 was chosen for flow cytometry analysis since it interacts with a linear epitope close to the N-terminus of β 3, that is not affected by conformational changes induced by disulfide bond disruption of cysteine residues (30). As shown by flow cytometry in figure 1, all three mutants were expressed on the cell surface, although the mean fluorescence intensity was reduced, corresponding to about 30 % (β 3C^{177A}), 40 % (β 3C^{273A}) and 10 % (β 3C^{598Y}) of wild type α IIb β 3 expression. Immunochemical analysis of the mutated receptors by SDS-PAGE and Western blot revealed a lower than normal electrophoretic mobility of β 3 under non-reducing conditions, while their mobility was identical to wild type β 3 under reducing conditions, illustrating that each cysteine mutation did indeed induce a conformational change of the β 3 subunit through disulfide bond disruption. The ratio of immature versus mature α IIb was increased for all mutants as compared to wild type α IIb, a result commonly observed with mutations affecting processing and cell surface exposure of recombinant α IIb β 3 receptors (Figure 2).

The cysteine mutations do not prevent CHO cell adhesion on fibrinogen.

To determine whether the mutants were able to mediate CHO cell adhesion to immobilized fibrinogen, cells expressing α IIb β 3 were allowed to attach and spread on human fibrinogen-coated microtiter plates as described in Materials and Methods. As shown in Figure 3, cells expressing wild type α IIb β 3 readily attached and spread on fibrinogen in contrast to mock-transfected CHO cells. Interestingly, all mutants were able to attach and spread on fibrinogen, although spreading was slower for the cell clones with reduced surface expression of the transfected mutant receptor (data not shown). Immunofluorescent staining of the mutant

receptors in cells adherent on fibrinogen revealed that all mutants were able to translocate into focal contacts located at the tips of phalloidin-labeled actin stress fibers. (Figure 3). Tyrosine phosphorylation of FAK kinase did also occur, however, to a lesser extent than that observed for wild type α IIB β 3 (data not shown).

The β 3 C¹⁷⁷A and C²⁷³A mutations, but not the C⁵⁹⁸Y mutation, affect the binding of complex specific blocking anti- α IIB β 3 antibodies to CHO cells.

Recent data have provided evidence that the sequence within the C¹⁷⁷-C¹⁸⁴ loop is recognized by several ligand-mimetic or complex specific and blocking anti- α IIB β 3 mAbs. To determine the structural role of the C¹⁷⁷-C¹⁸⁴ and C²³²-C²⁷³ loops in epitope expression, we tested the reactivity of a series of complex-specific and blocking anti- α IIB β 3 monoclonal antibodies. Due to the different expression levels of α IIB β 3 in the transfected cell clones, we used the anti- β 3 mAb P37 to normalize α IIB β 3 expression. As shown in figure 4, binding of β 3 subunit specific mAbs P37, Y2/51, 4F8, 16N7C2 and SZ21 was not affected by the C¹⁷⁷A, C²⁷³A and C⁵⁹⁸Y mutations. In contrast, binding of the complex specific and blocking anti- α IIB β 3 mAbs A2A9, PI2-46 and 10E5 was greatly reduced for the mutations C¹⁷⁷A and C²⁷³A, while these antibodies bound normally to the β 3 subunit with the C⁵⁹⁸Y mutation. An interesting result was observed with mAbs AP2 and 7E3, as the epitopes recognized by these antibodies were disrupted by the C¹⁷⁷A but not the C²⁷³A mutation. Since transfected CHO cells express an endogenous hamster α v subunit that associates with the human β 3 subunit, we also monitored the binding of the complex-specific anti- α v β 3 mAb LM609. Although surface expression of the chimeric

$\alpha\nu\beta 3$ receptor was quite low, specific binding of the LM609 mAb could be observed for the wild type $\alpha\nu\beta 3$ and the $\alpha\nu\beta 3_{C273A}$ receptors, while the binding to the $\alpha\nu\beta 3_{C177A}$ receptor was completely blocked. Finally, binding of subunit-specific or complex specific mAbs to the $\alpha I I b \beta 3_{C598Y}$ receptor was normal.

The defective binding of complex-specific monoclonal antibodies to the $\alpha I I b \beta 3_{C177A}$ receptor prompted us to investigate the stability of the $\alpha\beta$ heterodimer by immunoprecipitation experiments. As shown in figure 5, under standard cell lysis conditions, all three mutant heterodimers could be immunoprecipitated with the $\beta 3$ subunit specific mAb P37. However, when $\alpha I I b \beta 3_{C177A}$ cells were lysed with a buffer devoid of Ca^{2+} , mAb P37 was unable to coprecipitate the $\alpha I I b \beta 3_{C177A}$ complex, while the $\alpha I I b \beta 3_{wt}$ complex was readily detectable. A similar result was also observed for mutant $\alpha I I b \beta 3_{C273A}$ (data not shown). These results suggest that following disruption of the $C^{177}-C^{184}$ or $C^{233}-C^{273}$ loops, $\alpha\beta$ subunit heterodimerization can still occur but has an absolute requirement for divalent cations.

The $\beta 3$ mutants spontaneously expose LIBS epitopes in the absence of receptor activation or ligand binding.

Since the mutated cysteines investigated here are located within the I-like domain involved in receptor-ligand interaction, we investigated the ability of the mutant $\alpha I I b \beta 3$ receptors to interact with RGDS, by monitoring the expression of the LIBS epitopes D3, LIBS1, LIBS2 and AP5. As

shown in figure 6, RGDS binding to CHO cells expressing wild type α IIB β 3 increased the exposure of all LIBS epitopes, with a maximal binding observed for mAb D3. Surprisingly, all mutants exhibited constitutive binding of the four LIBS antibodies tested, independent of RGDS or mAb D3 stimulation. However, maximal spontaneous LIBS epitope exposure was only observed for mutants α IIB β 3_{C177A} and α IIB β 3_{C598Y}, while LIBS epitope exposure on α IIB β 3_{C273A} could be further increased following RGDS treatment. Since the LIBS epitopes AP5, D3 and LIBS2 have been localized in the β 3 subunit to residues 1-6, 422-490, and 602-609 respectively, our results suggest that disruption of each of the investigated disulfide loops induces long range conformational changes extending from the N-terminal to the C-terminal membrane proximal domain of the β 3 ectodomain.

De novo exposure on β 3_{C177A} of a cryptic epitope recognized by mAb 4D10G3.

In an effort to further monitor conformational changes of the β 3 subunit induced by disulfide bond disruption, we tested the binding of a number of available anti- β 3 monoclonal antibodies. One of these, mAb 4D10G3, known to react with SDS-denatured β 3 but not with native β 3, selectively bound to CHO cells expressing the α IIB β 3_{C177A} receptor, but did not bind to cells expressing either the wild type or the other mutant receptors. In order to localize the 4D10G3 epitope within the β 3 subunit, we performed Western blot analysis using chymotrypsin-digested platelet α IIB β 3. As shown in figure 7, 4D10G3 identified the 105 and 60 kDa bands of chymotrypsin digested β 3. This latter the 60 kDa band has been shown to comprise two fragments linked by the C⁵-C⁴³⁵ disulfide bond, and spanning residues 1-101 and 349 762,

respectively (30).

Effect of $\beta 3$ C¹⁷⁷A, C²⁷³A and C⁵⁹⁸Y mutations on PAC-1 binding.

Previous studies have shown that α IIb β 3 expressed in CHO cells exists in a low-affinity/avidity state and does not bind soluble fibrinogen or the ligand mimetic antibody, PAC-1, unless the receptor is activated by chymotrypsin digestion, mild reduction with DTT, or by incubation with an activating antibody, such as D3. To further investigate the effect of the cysteine mutations on α IIb β 3 activation, we investigated the binding of mAb PAC-1 before or after mAb D3 stimulation of CHO cells expressing α IIb β 3 wt, α IIb β 3_{C177A}, α IIb β 3_{C273A} and α IIb β 3_{C598Y}. Of major interest, in the absence of receptor activation, PAC-1 spontaneously bound to CHO α IIb β 3_{C598Y} cells, but did not bind to CHO α IIb β 3 wt or CHO α IIb β 3_{C177A} and α IIb β 3_{C273A} cells unless they were stimulated with mAb D3 (Figure 8). PAC-1 binding was specific since it could be completely inhibited by RGDS (data not shown). These results provide evidence that cysteine disulfide bond disruption in the CRR domain, but not in the I-like domain, induces α IIb β 3 receptor activation

Discussion

The $\beta 3$ integrin subunit contains 56 cysteine residues that are highly conserved in all β subunits and are essentially located in the two cysteine rich domains, the N-terminal PSI domain and the

C-terminal CRR domain, while the β subunit I-like domain has only two intradomain disulfide bonds. A long range C⁵-C⁴³⁵ loop that brings the PSI domain into close contact with the CRR domain, appears to be of particular importance since disruption of this loop through cysteine mutation induces a major conformational change leading to receptor activation (20). In contrast, mutational disruption of the C⁴⁰⁶-C⁶⁵⁵ loop of the second long range thiol bond appears to have no effect on α IIb β 3 receptor function (44). So far, no structural or functional role has been attributed to cysteine residues present in the I-like domain or the CRR domain. However, with the recent demonstration that each CRR domain of integrin β subunits has two highly conserved cysteine rich motives, with motive I containing the active site tetrapeptide CXXC of the ubiquitous thiol modifying enzymes PDI and thioredoxin, and with the evidence that integrin α IIb β 3 exhibits an endogenous thiol isomerase activity (26), it has become apparent that cysteine residues in the CRR domain could play a major role in receptor activation.

Here we have investigated the structural and functional relevance of the two unique disulfide bonds present in the I-like domain, C¹⁷⁷-C¹⁸⁴ and C²³²-C²⁷³, as well as a disulfide bond, C⁵⁸⁸-C⁵⁹⁸, located in the fourth repeat of the CRR domain and disrupted in a Glanzmann type II patient due to a C⁵⁹⁸Y substitution. The C¹⁷⁷-C¹⁸⁴ and C²³²-C²⁷³ loops are of particular interest since they are located in two long insertions not present in α -subunit I-domains. According to the quaternary structural model of the β 2 subunit I-like domain developed by Zang et al (36), (figure 9), or the structural model of Tuckwell and Humphries (37), one inserted loop is tied down to the back of the I-like domain by a disulfide bond corresponding to C²³²-C²⁷³ in

the $\beta 3$ subunit. The second corresponds to the C^{177} - C^{184} loop defined in $\beta 1$ and $\beta 3$ integrins and located near the top of the I-like domain. The two loops are located on the same side of the I-like domain, and have been reported to face the ligand binding domain in the proposed β propeller domain of the α IIB subunit (38). The $\beta 3$ 177 CYDMKTTC 184 sequence has previously been shown to be critical for ligand binding and ligand specificity and is called the specificity determining loop (39). More importantly, the sequence within this C^{177} - C^{184} loop appears to be part of the discontinuous antigenic sites recognized by some ligand-mimetic monoclonal antibodies, and amino-acid substitutions in this sequence prevent ligand-mimetic monoclonal antibody as well as fibrinogen binding (38). On the other hand, the C^{232} - C^{273} bond, which does not exist in the $\beta 4$ integrin subunit, is in close proximity to the hexapeptide 275 VGSDNH 280 in the $\beta 3$ subunit, that confers species restricted heterodimer assembly to α IIB $\beta 3$ (40). And finally, C^{598} is located in the conserved motive II of the fourth CRR and is part of a small tryptic fragment of the fourth CRR (residues 581-600), that has recently been identified to contain unpaired cysteines (10).

By mutating the cysteine residues C^{177} or C^{273} into alanine, and C^{598} into tyrosine, we disrupted each of the three cysteine loops by creating free sulfhydryls. Indeed, the expressed $\beta 3A^{177}$, $\beta 3A^{273}$ and $\beta 3Y^{598}$ mutants, when studied under nonreducing conditions by SDS-PAGE, exhibited a slower than normal electrophoretic mobility as compared to wild type $\beta 3$, a result consistent with that observed for other $\beta 3$ cysteine mutations, such as $C^{457}Y$ (41) or Cys ^{542}R

(42). It is noteworthy that this difference in electrophoretic mobility was not observed for the purified resting (AS-1) and active α Ib β 3 (AS-2) receptors, reported to differ in the number and position of unpaired cysteines (10). The three mutations investigated here had a clear inhibitory effect on α Ib β 3 biosynthesis, processing and cell surface exposure. However, these mutations did not prevent α Ib β 3-mediated cell spreading on immobilized fibrinogen or translocation of the mutant receptors into focal adhesion plaques. Similar results have been reported for other β 3 mutants, such as β 3_{L196P} (43), β 3_{C345A} (20) or β 3_{C655Y} (19,44). These data tend to support the notion that α Ib β 3-mediated cell spreading on immobilized fibrinogen, that relies essentially on the fibrinogen γ chain dodecapeptide interaction with the α Ib subunit, is not affected by single amino acid substitutions in the β 3 subunit.

Previous data have provided evidence that residues within the C¹⁷⁷-C¹⁸⁴ loop are critical for ligand specificity (39) and α Ib β 3 ligand binding (38). Replacement in the β 3 subunit of residues 177-184 with corresponding sequence of the β 1 subunit changed the ligand specificity of α v β 3, by blocking α v β 3 binding to multiple ligands and to mAb LM609, while the reciprocal mutation conferred α v β 1 the binding specificity for von Willebrand factor, fibrinogen and vitronectin (39). This C¹⁷⁷-C¹⁸⁴ sequence has also been shown to be part of the discontinuous binding sites recognized by some ligand-mimetic mAbs, and amino-acid substitutions in this sequence blocked fibrinogen binding as well as ligand mimetic mAbs binding such as OP-G2 or the function blocking mAbs A2A9 and 7E3 (38). Here we show a distinct effect of the C¹⁷⁷A and C²⁷³A mutation on complex-specific and blocking monoclonal antibody binding to α Ib β 3. While

none of the C¹⁷⁷A or C²⁷³A mutations had an effect on β 3 subunit specific mAb binding, such as P37, Y251, 4F8, or SZ21, they both inhibited the binding of the complex specific and blocking mAbs A2A9, 10E5, pL2-46, P256. In contrast, only the C¹⁷⁷A mutation affected AP2, 7E3 and LM609 binding, while the C²⁷³A mutation had no inhibitory effect. Our data demonstrate that disruption of the C¹⁷⁷-C¹⁸⁴ loop affects the epitopes for AP2 and LM609, that have been localized to the E¹⁷¹-E¹⁷⁴ sequence close to the C¹⁷⁷-C¹⁸⁴ loop, as well as the epitope for 7E3 located within the C¹⁷⁷-C¹⁸⁴ loop. In contrast, the epitopes for the complex specific antibodies such as A2A9 appear to be more complex since disruption of either the C¹⁷⁷-C¹⁸⁴ or the C²³²-C²⁷⁷ loop inhibited antibody recognition.

Our data also show that disruption of the C¹⁷⁷-C¹⁸⁴ or the C²³³-C²⁷³ loop, but not of the C⁵⁸⁸-C⁵⁹⁸ loop, decreased the stability of the $\alpha\beta$ heterodimer, and that these mutant complexes, in contrast to the wild type complex, dissociated in the absence of Ca²⁺, but could be stabilized following the addition of Ca²⁺. A similar sensitivity to Ca²⁺ ions and decreased stability of the α IIB β 3 complex has been observed in several GT patients with known single amino acid mutations such as R²¹⁴W (45,46), D¹¹⁹Y (47,48) and S¹⁶²L (49). Our results suggest that the structural integrity of the two insertions of the I-like domain, in close contact with the α subunit β propeller domain, is important for stable $\alpha\beta$ heterodimerization of α IIB β 3. These data are in accordance with previous studies showing that β 3 subunit residues 217-298 and 324-366 are involved in α/β heterodimerization (50). Also, amino acids in β 3 necessary for the selective

α Ib β 3 subunit compatibility have been identified in a short sequence encompassing residues 275-280 (40,51).

An interesting finding is the selective exposure on the mutant α Ib β 3_{C177A} receptor of an epitope identified by monoclonal antibody 4D10G3. This antibody does not bind to resting or activated platelets nor to the native recombinant α Ib β 3 receptor exposed on the cell surface, but interacts with SDS-denatured β 3, suggesting that in the native receptor, the epitope is cryptic and not accessible for 4D10G3 mAb recognition. Surface exposure of this epitope, that we have mapped to the non-reduced 60 kDa chymotrypsin fragment of the β 3 subunit, comprising the disulfide linked peptides spanning residues 1-101 and 349-762, demonstrates that cryptic sequence of the β 3 subunit not contained within the I-like domain is exposed following disruption of the C¹⁷⁷-C¹⁸⁴ loop. These data suggest that disruption of this C¹⁷⁷-C¹⁸⁴ loop does not only affect the structure of the I-like domain, but influences also the N-terminus and the central domain of the β 3 subunit. We therefore investigated LIBS epitope expression following disruption of the three cysteine bonds. Quite surprisingly, in the absence of receptor activation, all three cell clones spontaneously bound the LIBS mAbs AP5, D3, LIBS1 and LIBS2, suggesting that LIBS epitope mapping on β 3 only poorly relates to specific β 3 subunit conformational changes induced by selective mutations.

A number of naturally occurring cysteine mutations in the β 3 subunit have been identified in Glanzmanns thrombasthenia patients, that are essentially located in the CRR domain, such as

C⁴⁵⁷Y (41), C⁵⁰⁶Y (52), C⁵⁰⁸Y (53), C⁵⁴²Y (42), C⁵⁶⁰R (54), and the C⁵⁹⁸Y initially identified by Schlegel *et al.* (29) and investigated here. All mutations, except C⁵⁰⁸Y, were responsible for reduced β 3 surface expression in platelets, a result confirmed for most of these mutations following transfection of the mutant receptor in mammalian cells. Some of the β 3 mutants (C⁴⁵⁷Y, C⁵⁴²Y) exhibited a slower than normal migration in SDS-PAGE, similar to that observed for β 3C⁵⁹⁸Y, demonstrating that these cysteines are engaged in a disulfide bond. Interestingly, C⁴⁵⁷ and C⁵⁰⁶ correspond to the N-terminal and C-terminal residues of the highly conserved tetrapeptide CGXC in motive I of the first and second CRR, respectively. On the other hand, C⁵⁶⁰ and C⁵⁹⁸ are each part of a CRR motive II. It is noteworthy that previously reported mutations of C⁴⁵⁷ (41) or C⁵⁴² (42) located in CRR motives I had no effect on receptor function, while mutations of C⁵⁹⁸ shown here and C⁵⁶⁰ reported by Ruiz *et al.* (54) both induced receptor activation. Interestingly, C⁵⁹⁸ is comprised within the small tryptic β 3 fragment (residues 581-600) identified by Yan and Smith and shown to contain unpaired cysteines (10). Thus, the fact that the C⁵⁹⁸Y mutation induced spontaneous PAC-1 binding suggests that C⁵⁹⁸ is potentially involved in disulfide bond reshuffling, necessary for receptor activation. Finally, since the C⁵⁶⁰R and C⁵⁹⁸Y mutations have been identified in the integrin β 3 subunit of patients with defective platelet aggregation, the thrombasthenic phenotype of the platelets of these patients can only be explained by the low expression level of the mutant α IIB β 3 receptor.

In summary, our results provide evidence that disruption of a single cysteine disulfide bond in

the cysteine rich repeat domain, but not in the I-like domain, activates integrin α Ib β 3. In contrast, disruption of each of the disulfide bonds in the two long insertions of the I-like domain predicted to be in close contact with the α subunit β -propeller domain, affects the stability of the α Ib β 3 heterodimer, without modifying the RGD binding capacity of the MIDAS-like domain.

References

1. Schwarz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu Rev Cell Dev Biol* (11), 549-599
2. Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J. A., Shattil, S. J., and Ginsberg, M. H. (1996) *J Biol Chem* **271**(12), 6571-4.
3. Yamada, K. M., and Miyamoto, S. (1995) *Curr Opin Cell Biol* **7**(5), 681-9.
4. Sims, P. J., Ginsberg, M. H., Plow, E. F., and Shattil, S. J. (1991) *J. Biol. Chem.* **266**(12), 7345-7352
5. Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F. (1985) *J. Biol. Chem.* **260**,

11107-11114

6. Frelinger, A. L., Lam, S. C., Plow, E. F., Smith, M. A., Loftus, J. C., and Ginsberg, M. H. (1988) *J Biol Chem* **263**(25), 12397-12402
7. Frelinger, A. L., 3rd, Du, X. P., Plow, E. F., and Ginsberg, M. H. (1991) *J Biol Chem* **266**(26), 17106-11.
8. Frelinger, A. L., 3rd, Cohen, I., Plow, E. F., Smith, M. A., Roberts, J., Lam, S. C., and Ginsberg, M. H. (1990) *J Biol Chem* **265**(11), 6346-52.
9. Kouns, W. C., Wall, C. D., White, M. M., Fox, C. F., and Jennings, L. K. (1990) *J Biol Chem* **265**(33), 20594-20601
10. Yan, B., Hu, D. D., Knowles, S. K., and Smith, J. W. (2000) *J Biol Chem* **275**(10), 7249-60.
11. Lahav, J., Gofer-Dadosh, N., Luboshitz, J., Hess, O., and Shaklai, M. (2000) *FEBS Lett* **475**(2), 89-92.
12. Zucker, M. B., and Masiello, N. C. (1984) *Thromb Haemost* **51**(1), 119-124
13. Kouns, W. C., Steiner, B., Kunicki, T. J., Moog, S., Jutzi, J., Jennings, L. K., Cazenave, J. P., and Lanza, F. (1994) *Blood* **84**(4), 1108-1115
14. Davis, G. E., and Camarillo, C. W. (1995) *Exp Cell Res* **216**(1), 113-123
15. Peerschke, E. I. (1995) *Thromb Haemost* **73**(5), 862-867
16. Edwards, B. S., Curry, M. S., Southon, E. A., Chong, A. S., and Graf, L. H. (1995) *Blood* **1995** **86**(6), 2288-2301
17. Gofer-Dadosh, N., Klepfish, A., Schmilowitz, H., Shaklai, M., and Lahav, J. (1997) *Biochem Biophys Res Commun* **232**(3), 724-727

18. Niewiarowski, S., Kornecki, E., Hershock, D., Tuszynski, G. P., Bennett, J. S., Soria, C., Soria, J., Dunn, F., Pidard, D., Kieffer, N., and Nurden, A. T. (1985) *J Lab Clin Med* **106**(6), 651-660
19. Valentin, N., Visentin, G. P., and Newman, P. J. (1995) *Blood* **85**(11), 3028-3033
20. Liu, C. Y., Sun, Q. H., Wang, R., Paddock, C. M., and Newman, P. J. (1997) *Blood* **90**(10 Suppl.), 573a
21. Si-Tahar, M., Pidard, D., Balloy, V., Moniatte, M., Kieffer, N., Van Dorsselaer, A., and Chignard, M. (1997) *J Biol Chem* **272**(17), 11636-47.
22. Lu, C., Shimaoka, M., Ferzly, M., Oxvig, C., Takagi, J., and Springer, T. A. (2001) *Proc Natl Acad Sci U S A* **98**(5), 2387-92.
23. Lu, C., Shimaoka, M., Zang, Q., Takagi, J., and Springer, T. A. (2001) *Proc Natl Acad Sci U S A* **98**(5), 2393-8.
24. Essex, D. W., Chen, K., and Swiatkowska, M. (1995) *Blood* **86**(6), 2168-2173
25. Essex, D. W., and Li, M. (1999) *Br J Haematol* **104**(3), 448-54.
26. O'Neill, S., Robinson, A., Deering, A., Ryan, M., Fitzgerald, D. J., and Moran, N. (2000) *J Biol Chem* **275**(47), 36984-90.
27. Huang, C., Zang, Q., Takagi, J., and Springer, T. A. (2000) *J Biol Chem* **275**(28), 21514-24.
28. Schaffner-Reckinger, E., Gouon, V., Melchior, C., Plancon, S., and Kieffer, N. (1998) *J Biol Chem* **273**(20), 12623-32.
29. Schlegel, N., Chen, P., Binard, S., Maisonneuve, L., Rosa, J. P., Kieffer, N., and Caen, J. (1999) *Blood* **94**(10), Suppl. I, 451a

30. Calvete, J. J., Henschen, A., and Gonzalez-Rodriguez, J. (1991) *Biochem J* **274**(Pt 1), 63-71.
31. Faull, R. J., Wang, J., Leavesley, D. I., Puzon, W., Russ, G. R., Vestweber, D., and Takada, Y. (1996) *J Biol Chem* **271**(41), 25099-106.
32. Takagi, J., Isobe, T., Takada, Y., and Saito, Y. (1997) *J Biochem (Tokyo)* **121**(5), 914-21.
33. Bazzoni, G., Shih, D. T., Buck, C. A., and Hemler, M. E. (1995) *J Biol Chem* **270**(43), 25570-7.
34. Kouns, W. C., Newman, P. J., Puckett, K. J., Miller, A. A., Wall, C. D., Fox, C. F., Seyer, J. M., and Jennings, L. K. (1991) *Blood* **1991** **78**(12), 3215-3223
35. Honda, S., Tomiyama, Y., Pelletier, A. J., Annis, D., Honda, Y., Orzechowski, R., Ruggeri, Z., and Kunicki, T. J. (1995) *J Biol Chem* **270**(20), 11947-54.
36. Zang, Q., Lu, C., Huang, C., Takagi, J., and Springer, T. A. (2000) *J Biol Chem* **275**(29), 22202-12.
37. Tuckwell, D. S., and Humphries, M. J. (1997) *FEBS Lett* **400**(3), 297-303.
38. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) *J Biol Chem* **275**(11), 7795-802.
39. Takagi, J., Kamata, T., Meredith, J., Puzon-McLaughlin, W., and Takada, Y. (1997) *J Biol Chem* **272**(32), 19794-19800
40. McKay, B. S., Annis, D. S., Honda, S., Christie, D., and Kunicki, T. J. (1996) *J Biol Chem* **271**(48), 30544-7.
41. Milet, S., Bourre, O., Peyuruchaud, E., Cazez, R., Combri e, Nurden, P., and Nurden, A.

(1997) *Thromb Haemostas* **78**(2 Suppl.), 360a

42. Ruan, J., Schmutz, M., Clemetson, K. J., Cazes, E., Combrie, R., Bourre, F., and Nurden, A. T. (1999) *Br J Haematol* **105**(2), 523-31.
43. Kieffer, N., Morel-Kopp, M.-C., Melchior, C., and Kaplan, C. (1999) *Blood* **94**(10), Suppl. I, 445a
44. Wang, R., Peterson, J., Aster, R. H., and Newman, P. J. (1997) *Blood* **90**(4), 1718-1719
45. Nurden, A. T., Rosa, J. P., Fournier, D., Legrand, C., Didry, D., Parquet, A., and Pidar, D. (1987) *J Clin Invest* **79**(3), 962-969
46. Djaffar, I., and Rosa, J. P. (1993) *Hum Mol Genet* **2**(12), 2179-80.
47. Ginsberg, M. H., Lightsey, A., Kunicki, T. J., Kaufmann, A., Marguerie, G., and Plow, E. F. (1986) *J Clin Invest* **78**:1103-1111 **78**, 1103-1111
48. Loftus, J. C., O'Toole, T. E., Plow, E. F., Glass, A., Frelinger, A. L., 3rd, and Ginsberg, M. H. (1990) *Science* **249**(4971), 915-8.
49. Jackson, D. E., White, M. M., Jennings, L. K., and Newman, P. J. (1998) *Thromb Haemost* **80**(1), 42-8.
50. Calvete, J. J. (1999) *Proc Soc Exp Biol Med* **222**(1), 29-38.
51. Lee, J. O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) *Cell* **80**(4), 631-8.
52. French, D. L., Bussel, J., Li, J., Scudder, L., and Coller, B. S. (1998) *Blood* **92**, Suppl. I, 33a
53. Schlegel, N., Binard, S., Scrobohaci, M. L., Baruchel, A., Horellou, M. H., Jallu, V., and Kaplan, C. (2000) *Blood* **96**(11) Suppl. 255a

54. Ruiz, C., Liu, C., Sun, Q., Sigaud-Fiks, M., Fressinaud, E., Muller, J. Y., Nurden, P., Nurden, A.T., Newman, P., and Valentin, N. (2001) *Thrombosis and Haemostasis* Suppl. July 2001, **OC88**

Acknowledgements

We thank Drs C. Bachelot, B.S. Coller, M. Hoylaerts, M.H. Ginsberg, J. Gonzalez-Rodriguez, L.K. Jennings, C. Kaplan, T. Kunicki, D. Mason, D.R. Phillips, C.G. Ruan and S.J. Shattil for their generous gift of monoclonal antibodies.

This work was supported by grants from Centre de Recherche Public-Santé (CRP-Santé,

Luxembourg), CNRS (France), Fondation Luxembourgeoise Contre le Cancer (Luxembourg), and EC Biomed Project BMH4-98-3517. P. Chen is a recipient of a fellowship from the Ministère des Affaires Etrangères, du Commerce Extérieur et de la Coopération, Luxembourg. Data presented were obtained as part of his doctoral thesis to be submitted to the University Paris VII.

The abbreviations used are: CHO: Chinese hamster ovary; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IMDM: Iscove's modified Dulbecco's medium; MIDAS, metal ion-dependent adhesion site; mAb: monoclonal antibody.

Legends to figures

Figure 1: Flow cytometry analysis of α IIb β 3 expression in CHO cells.

CHO- α IIb cells were stably transfected with pcDNA vector encoding full length β 3 wild type or

$\beta 3_{C177A}$, $\beta 3_{C273A}$ and $\beta 3_{C598Y}$ mutants. Surface expression of the recombinant $\alpha IIb\beta 3$ heterodimers was measured by flow cytometry following staining of the cells with the anti- $\beta 3$ mAb P37. The superposed histograms correspond to $\alpha IIb\beta 3$ wild type (bold solid line); $\alpha IIb\beta 3_{C177A}$ (solid line); $\alpha IIb\beta 3_{C598Y}$ (dashed line); $\alpha IIb\beta 3_{C273A}$ (double-dashed line); and mock transfected CHO cells used as a negative control (shaded histogram).

Figure 2: Western blot analysis of recombinant $\beta 3$ integrin mutants expressed in CHO cells.

CHO cell lysates were prepared and protein concentration determined as described under Materials and Methods. Equal amounts of protein from mock- or $\beta 3$ transfected CHO cells (50 ug) were resolved by 7.8% SDS-PAGE under non-reducing and reducing conditions, transferred to nitrocellulose, and immunoblotted with monoclonal antibodies against human integrin $\beta 3$ (P37) and αIIb (SZ22). Lane 1: CHO- $\alpha IIb\beta 3$ cells; lane 2: CHO- $\alpha IIb\beta 3_{C177A}$ cells; lane 3: CHO- $\alpha IIb\beta 3_{C273A}$ cells; lane 4: CHO- $\alpha IIb\beta 3_{C598Y}$ cells; and lane 5: CHO mock transfected cells. The arrow indicates the reduced electrophoretic mobility of mutant $\beta 3$ as compared to $\beta 3$ wild type.

Figure 3: Immunofluorescent costaining of recombinant human $\beta 3$ integrin subunit and stress fibers in transfected CHO cells adherent on fibrinogen-coated glass coverslips.

$\alpha IIb\beta 3$ -transfected CHO cells were allowed to adhere on fibrinogen-coated glass coverslips. After paraformaldehyde fixation and detergent permeabilization, the cells were incubated with anti- $\beta 3$, anti- αIIb and anti- $\alpha IIb\beta 3$ complex specific mAbs and stained with fluorescein-conjugated

goat antimouse IgG and rhodamine-labeled phalloidin. Microphotographs of the same cell visualize α IIB β 3 integrin localization (left panel) and actin stress fiber organization (right panel).

1: CHO- α IIB β 3 wt; 2: CHO- α IIB β 3 C177A; 3: CHO α IIB β 3 C273A and 4: CHO- α IIB β 3 C598Y.

Figure 4: Effect of cysteine mutations on the binding of integrin subunit specific or complex specific monoclonal antibodies to CHO cells expressing recombinant mutant α IIB β 3.

CHO cells expressing wild type α IIB β 3 (black bars) or mutated α IIB β 3 (open bars) were incubated with a panel of anti- α IIB, anti- β 3 and anti- α IIB β 3 complex specific monoclonal antibodies as described in Materials and Methods. Due to the different α IIB β 3 expression levels of the transfectants, data obtained with the panel of monoclonal antibodies were normalized for each cell clone and expressed as % of the fluorescence measured for mAb P37 binding (100%).

1: CHO- α IIB β 3C177A; 2: CHO α IIB β 3C273A and 3: CHO- α IIB β 3C598Y.

Figure 5: Immunoprecipitation and Western blot analysis of recombinant α IIB β 3C177A, α IIB β 3C273A and α IIB β 3C598Y mutants.

Detergent extracts of CHO cell transfectants (500 μ g protein) were immunoprecipitated with the anti- β 3 monoclonal antibody P37. The immunoprecipitates were resolved by 7.8% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose and probed with monoclonal antibodies against human β 3 (SZ21) and α IIB (SZ22). (A) Lane 1: CHO- α IIB β 3 wt; lane 2: CHO- α IIB β 3C177A; lane 3: CHO- α IIB β 3C273A; and lane 4: CHO- α IIB β 3C598Y. (B). Cell

lysates were prepared in the presence (lanes 1, 2 and 3) or absence (lanes 4, 5 and 6) of $\text{Ca}^{2+}/\text{Mg}^{2+}$. Lanes 1 and 4: CHO- $\alpha\text{IIb}\beta 3$; lanes 2 and 5: CHO- $\alpha\text{IIb}\beta 3_{\text{C177A}}$; lanes 3 and 6: mock transfected CHO cells.

Figure 6: Flow cytometry assessment of LIBS epitope exposure on CHO transfectants before and after stimulation by RGDS or DTT.

Detached and washed CHO cells were resuspended in HEPES buffer and preincubated for 30 min at room temperature with RGDS (1 mM) or DTT (5 mM). The anti-LIBS mAbs AP5, D3, LIBS1 and LIBS2 were then added and the cells incubated on ice for 45 min. Antibody binding was examined by flow cytometry, and the relative fluorescence intensity was normalized to $\beta 3$ expression using mAb P37. 1: CHO- $\alpha\text{IIb}\beta 3$; 2: CHO- $\alpha\text{IIb}\beta 3_{\text{C177A}}$; 3: CHO- $\alpha\text{IIb}\beta 3_{\text{C273A}}$ and 4: CHO- $\alpha\text{IIb}\beta 3_{\text{C598Y}}$. Solid bars: without stimulation; open bars: with stimulation.

Figure 7: De novo exposure on $\beta 3_{\text{C177A}}$ of a cryptic epitope recognized by mAb 4D10G3

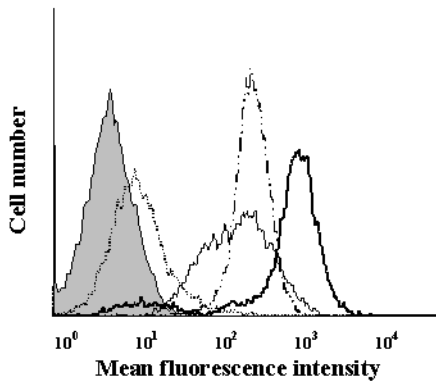
(A) Binding of mAb 4D10G3 to CHO cells expressing wild type or mutant $\alpha\text{IIb}\beta 3$ was analyzed by flow cytometry. The results were normalized with respect to P37 binding. Solid bars: P37; open bars: 4D10G3. (B) Washed platelets were treated with chymotrypsin, lysed and submitted to SDS-PAGE and Western blot. The membrane was then hybridized with mAb 4D10G3. Lane1: α -chymotrypsin digested platelet lysate; lane 2: undigested platelet lysate.

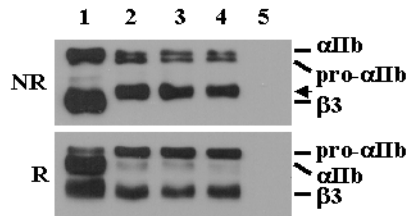
Figure 8: PAC-1 binding to CHO cells expressing α IIIb** β 3wt or mutant α I**IIb** β 3C177A, α I**IIb** β 3C273A and α I**IIb** β 3C598Y**

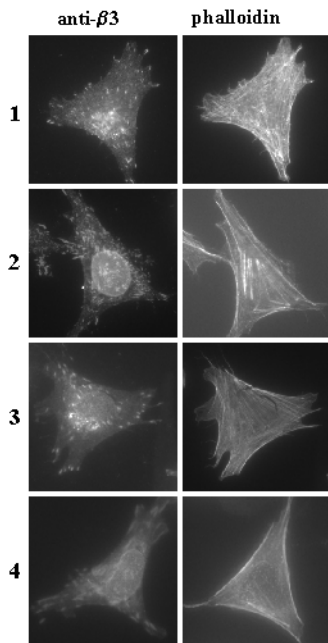
Cells were preincubated for 30 min at 4°C in the presence or absence of the activating mAb D3, washed and further incubated for 45 min at 4°C with mAb PAC-1 . Phycoerythrin-conjugated goat anti-mouse IgM (1/100 dilution) was then added to the cells for 20 min. The cells were washed and analyzed by flow cytometry. The relative fluorescence intensity was normalized to β 3 expression using mAb P37. 1: CHO- α I**IIb** β 3C177A; 2: CHO- α I**IIb** β 3 C273A and 3: CHO- α I**IIb** β 3 C598Y. solid bars: wild type receptor; open bars: mutant receptor.

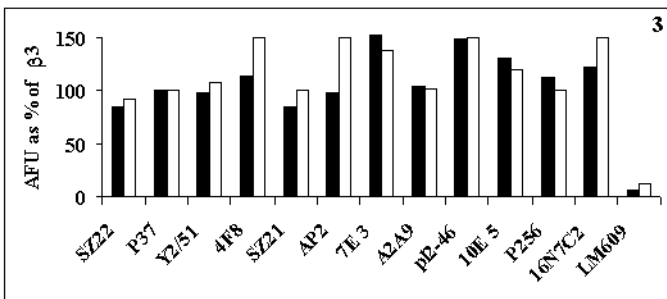
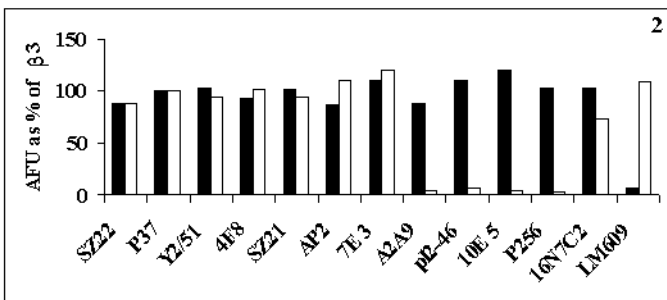
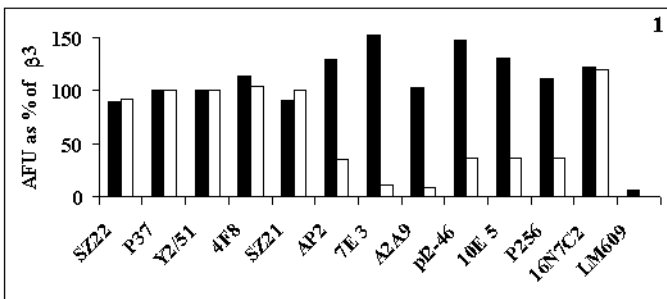
Figure 9: 3D model of the β 3 subunit I-like domain.

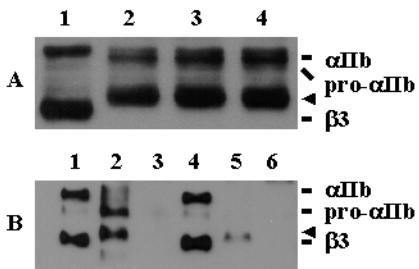
The 3D model of the β 3 subunit I-like domain was adapted from the theoretical structural model of the β 2 I-like domain as published by Zang et al (36). A view of the back face of the I-like domain is shown bearing the α 3, α 4 and α 5 helices. The two long insertions not present in α -subunit I domains are shown in white. The two disulfide bonds, C¹⁷⁷-C¹⁸⁴ and C²³²C²⁷³, located in the insertions are indicated. The metal ion indicates the location of the MIDAS-like domain at the top of the I-like domain.



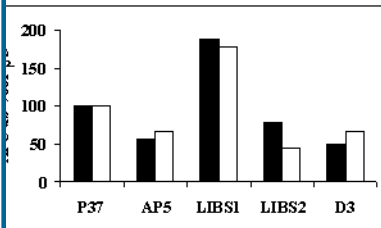
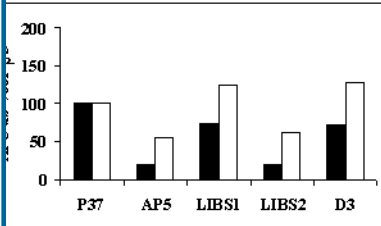
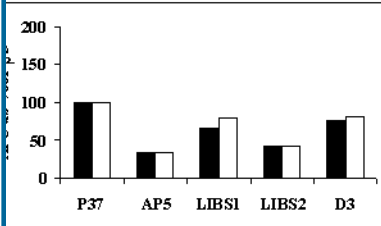
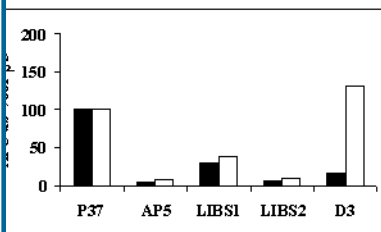




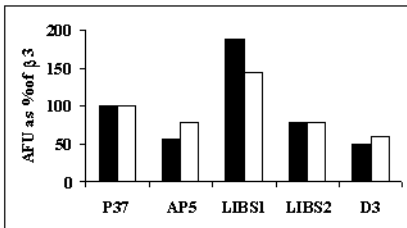
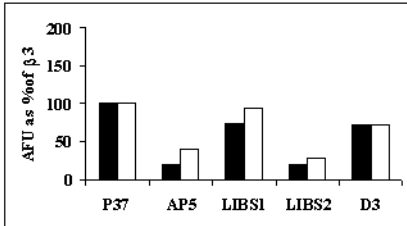
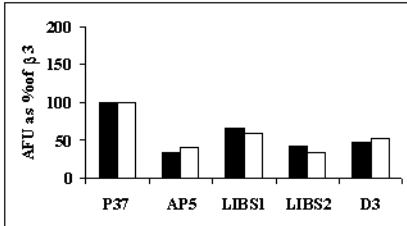
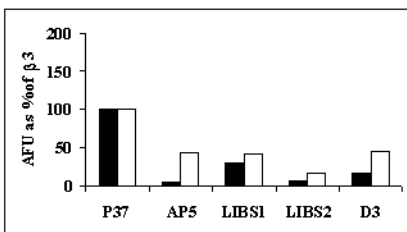




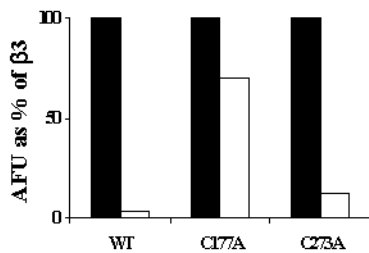
RGDS



DTT



A



B

