The platelet integrin αIIbβ3 has an endogenous thiol isomerase activity

Running title: Endogenous thiol isomerase activity in integrins

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SUMMARY

Integrins are cysteine-rich heterodimeric cell-surface adhesion molecules that alter their affinity for ligands in response to cellular activation. The molecular mechanisms involved in this activation of integrins are not understood. Treatment with the thiol-reducing agent, dithiothreitol can induce an activation-like state in many integrins suggesting that cysteine-cysteine dithiol bonds are important for the receptor’s tertiary structure and may be involved in activation-induced conformational changes. Here we demonstrate that the platelet-specific integrin, αIIbβ3, contains an endogenous thiol isomerase activity, predicted from the presence of the tetrapeptide motif, CXXC, in each of the cysteine-rich-repeats (CRRs) of the β3 polypeptide. This motif comprises the active site in enzymes involved in disulphide exchange reactions, including protein disulphide isomerase (PDI; EC 5.3.4.1) and thioredoxin. Intrinsic thiol isomerase activity is also observed in the related integrin, αvβ3, which shares a common β-subunit. Thiol-isomerase activity within αIIbβ3 is time-dependent and saturable, and is inhibited by the PDI inhibitor, bacitracin. Furthermore, this activity is calcium-sensitive and is regulated in the EDTA-stabilized conformation of the integrin. This novel demonstration of an enzymatic activity associated with an integrin subunit suggests that altered thiol bonding within the integrin or its substrates may be locally modified during αIIbβ3 activation.
INTRODUCTION

Integrins are cell-surface, calcium-dependent, heterodimeric adhesion molecules that play a critical role in cell-cell and cell-substrate adhesion. In cells at rest, integrins are present in a latent or resting conformation. Following cellular activation, they undergo conformational changes to become high affinity receptors for their specific ligand(s). The ‘switch’ mechanism whereby integrins are converted from their resting conformation is critically important to their cellular function. However, the mechanisms underlying these conformational changes have not yet been deduced.

The conformational changes in the platelet-specific integrin, αIIbβ3, are the composite result of at least two processes. Firstly, intracellular signals converge on the cytoplasmic tails of the integrin conveying the intention to activate. Secondly, the extracellular domains, which constitute >95% of the molecules, respond with an increased affinity for ligand and an altered display of antibody epitopes suggestive of altered protein folding. We have shown that the conserved α-subunit cytoplasmic sequence, KVGFFKR is critical for the intracellular-mediated activation of the platelet integrin[1]. The precise role played by this peptide sequence remains uncharacterized. However, Vinogradova et al [2], have recently proposed a structural basis for this effect which proposes a protein-protein interaction with the integrin cytoplasmic tails. Deletion or mutation of this cytoplasmic sequence from the αIIb subunit were found to increase the ligand binding affinity of the mutant receptor expressed in Chinese hamster ovary cells[3, 4]. In contrast, ligand binding to the ectodomains of integrins has also been demonstrated to affect intracellular recognition sites on the cytoplasmic tails of integrins in a process known as outside-in signalling[5]. These studies provide evidence for transmembrane modulation of integrin conformation although the mechanisms mediating these changes are not understood.
The subunits of integrins are rich in cysteine residues. The pairing of these residues in disulfide bonds, particularly in the platelet specific integrin \( \alpha \text{IIb}\beta 3 \), have been shown to be important in the structural integrity of the receptor[6, 7]. Chemical reduction of the thiol bonds by the reducing agent dithiothreitol induces an active conformation in \( \alpha \text{IIb}\beta 3 \)[8, 9], although the physiological significance of this observation is not understood. Selective mutation of critical cysteines in the platelet integrin \( \alpha \text{IIb}\beta 3 \) results in the induction of a constitutively active form of the receptor lending further support to the proposal that altered dithiol bonding is implicated in integrin activation[10]. Dithiol reduction also causes activation of other integrins[11, 12].

Analysis of the integrin cysteine patterns reveals that there are nine CXXC repeats in each \( \beta \)-integrin subunit. This sequence has previously been identified in the active site motif in the bacterial thiol reducing enzyme, thioredoxin and in the mammalian thiol isomerase, protein disulfide isomerase (PDI). A glycine residue is most often found as the first amino-acid position of the repeat. Other proteins that possess this sequence include the chaperone molecules Erp61 and Erp72[13], the gonadotropins, lutropin and follitropin[14, 15] and fibronectin[16]. All these proteins have demonstrable endogenous PDI-like activity. Von Willebrand factor also has numerous CGXC sequences but no thiol isomerase activity was shown[17]. In the integrin \( \beta \) subunit, the specific CGXC sequence is repeated four times; once in each of the regions identified as the cysteine-rich repeat regions (CRRs)[18]. We, therefore, investigated purified integrin \( \alpha \text{IIb}\beta 3 \) for the presence of endogenous thiol isomerase activity. In addition, we evaluated its biochemical and pharmacological profile in in-vitro assays and demonstrated that this enzymatic activity is a common feature of integrins.
Experimental Procedures.

Materials-Ribonuclease A (RNase) and protein disulfide isomerase (PDI) were obtained from CalBiochem-Novabiochem (CA). αvβ3 and α5β1 were supplied by Chemicon (CA). Sephadex G25 column, bacitracin, guanidine hydrochloride, ethylenediaminetetra acetic acid (EDTA), dithiothreitol (DTT), cytidine 2’,3’cyclic monophosphate (cCMP), 3-(n-morpholino) propanesulfonic acid (MOPS) and trizma base were all supplied by Sigma. CD41a (an antibody to αIIbβ3) was obtained from Immunotech, Marseilles, France and anti-PDI from Stressgen Biotechnology Corporation CA. Goat anti-mouse Alexa-488 conjugated IgG and goat anti-rabbit Alexa 546 conjugated IgG were obtained from Molecular Probes, Leiden, Netherlands.

Purification of αIIbβ3-This was purified from outdated units of human platelets as described previously[19] and stored at -80°C in a buffer containing 20mM Tris, 1mM CaCl2 and 0.1% Triton-X-100. Samples were concentrated to approximately 4mg/ml by filtration through centrex filters (Schleicher and Schuell, 10kD MWCO).

Preparation of rdRNase-30mg of purified RNase was reduced and denatured (rdRNase) at room temperature for 18 hours in the presence of 0.15M dithiothreitol and 6M guanidine HCl in 0.1M Tris, pH 8.6 before desalting on a sephadex G25 column equilibrated with 0.01N HCl. The concentration of the reduced and denatured (rd)RNase fractions were determined from its extinction coefficient 9200m⁻¹cm⁻¹ at 275nm. Fractions were stored under argon at -80°C for up to 14 days before use.

 Reactivation of reduced and denatured RNase- The reduced enzyme at a final concentration of 30µM was diluted into 0.1M Tris-HCl pH 7.4 with 1mM EDTA containing either 5µM bovine protein disulfide isomerase, purified GPIIb/IIIa at the concentrations indicated, 5µM ovalbumin
or no isomerase. The extent of reactivation of rdRNase was monitored by removing aliquots at various time points and measuring recovered RNase activity, as described by Boniface et al. A typical assay consisted of 1.4µM RNase and 0.44mM cytidine 2',3' cyclic monophosphate in 0.1M 2-(N-morphlino)propanesulfonic acid (MOPS) pH 7.0. Changes in absorbance at 284nm were monitored in Unicam split beam spectrophotometer. Activity was expressed as a percentage of a native RNase control.

**Determination of Enzymatic Parameters of integrins**- To determine these parameters, the RNase refolding assay described above was used. Concentrations of reduced denatured RNase ranging from 7.5 to 75 µM was used in the absence of isomerase or in the presence of protein disulfide isomerase (1.5µM) or αIIbβ3 (1.5µM). The amount of active RNase was determined by measuring the initial rate of recovery of activity over 1 minute at 284 nm after a 48 hour incubation. Plots of substrate concentration versus initial velocity were fit to Michaelis-Menten equation using Deltagraph to determine $V_{\text{max}}$ and $K_{m}$. The uncatalysed initial rates, determined in the absence of a source of isomerase activity, were subtracted from the catalysed rates determined in the presence of PDI or integrin. A standard curve was generated from the initial rates of various concentrations (0-4µM) of native RNase. A value for µM of RNase regenerated per minute for Vmax was then determined from the slope of this curve.

**Immunoprecipitation**- 2µg/ml of either purified αIIbβ3 or protein disulfide isomerase in 50mM Tris pH 7.4 with 150mM NaCl and 1% Triton-X 100 was precipitated with a rabbit polyclonal anti human PDI antibody (a kind gift of Dr. Eric Quemeneur, Centre d’Etudes Saclay, Gif-sur-Yvette, France). Precipitates were harvested with protein G beads. Samples were separated on 12.5% SDS-PAGE and transferred to PVDF. Membrane was probed with 1:1000 dilution of anti-
PDI antibody (rabbit anti-protein disulfide isomerase polyclonal antibody from Stressgen Biotech Corporation) followed by 1:1500 dilution of Protein A peroxidase. Membrane was developed with ECL.

Confocal Microscopy-Glass slides pre-coated with poly-L-lysine (Sigma) or slides coated with fibrinogen (20µg/ml) for 2 hours were blocked with 1% BSA in tris buffered saline (TBS, pH 7.4) for 1.5 hours in a humidified staining tray. Gel filtered platelets (GFP), prepared as described previously [20] were diluted to $2.0 \times 10^5$/ml and allowed to adhere to the poly-L-lysine coated slides for 15 minutes and to the fibrinogen-coated slides for 30 minutes. Slides were then washed with TBS and fixed in ice-cold methanol (7 minutes). Slides were blocked with 1% BSA for 20 minutes. Primary antibodies (1:200 dilution for monoclonal anti-αIIbβ3 complex specific and 1:50 for polyclonal anti-protein disulfide isomerase) were incubated for 45 minutes. 1:400 dilution of goat anti-mouse Alexa-488 conjugated IgG and goat anti-rabbit Alexa 546 conjugated IgG in TBS were incubated for 10 minutes. Slides were washed and mounted in fluorescent mounting medium (Dako, Carpinteria, CA) before imaging on a Zeiss Axioplan 2 confocal microscope. All experiments were carried out at room temperature.

Inhibitor Studies-Bacitracin (0-100µM) was added to each reaction mix at 0 hours. Examination of the reactivation of reduced and denatured RNase in the presence of this inhibitor was carried out as described above. The results are expressed as % of maximal recovery of the refolding activity of rdRNase observed in the absence of bacitracin.
RESULTS

Analysis of the integrin cysteine patterns reveals that there are four highly conserved CRRs in each β-integrin subunit[18]. In Fig. 1, we show the alignment of the second cysteine-rich-repeat from human β3, with the same region from other β-integrins ranging from sponge and coral to mammalian sequences. There is 44-63% sequence identity within this region increasing to 100% for the β3 subunits. The cross-species homology index is further increased when conservative amino-acid substitutions are accounted for, giving a value of 56-83% homology. The cysteine residues are 100% conserved. Similar homology scores are obtained when the first, third or fourth CRRs are aligned. The four CRRs of β3 subunits from human, mouse and dog are 90-94% identical. The alignments also reveal the presence of two motifs in each repeat: motif I has the sequence ‘GXCXCXCCX’ and is separated by 5-12 amino acids from motif II which has the sequence ‘GXXCXC’ (Fig 1b). In all integrins, the first CRR is imperfect with its first cysteine replaced by an F,M,L or Y. Similarly, the fourth CRR is incomplete in all integrins, lacking the second motif but having a similar GXXCXXX in β1, β3, and β5-8 integrins. β8-integrins are an exception to the integrin family and have very corrupted CRRs, the first CRR containing a CXXC, but not the flanking residues of motif I. It lacks motif II. The second CRR also lacks motif II. The third CRR lacks C2 in motif I but has a normal motif II. The fourth CRR, like all other integrins, lacks a true motif II. Motif I contains the active site sequence (CXXC) of the ubiquitous thiol modifying enzymes, protein disulphide isomerase (PDI) and thioredoxin. Each β-integrin subunit contains nine repeats of this CXXC tetrapeptide sequence; two in the amino terminal region, one in each of the four CRRs, and two
in the modified motif II of the fourth CRR. The final CXXC is immediately C-terminal to the fourth CRR.

Other proteins that possess this motif include the insulin-like growth factor-binding protein, IGFBP-3[21], chaperone molecules Erp61 and Erp72[13], the gonadotropic hormones lutropin and follitropin[14, 15], fibronectin[16] and vWF [17]. Their active sites are compared to the active site sequences of PDI and thioreredoxin in Fig. 1c. All these proteins, with the exception of vWF, have demonstrable endogenous thiol isomerase activity. Moreover, the four CXXC repeats found in the four CRRs of the β-integrins have a glycine (G) residue in their most N-terminal ‘X’ position in common with PDI and many of the other proteins known to have endogenous thiol isomerase activity[13-16, 21]. We therefore assessed the platelet integrin, αIIbβ3, for the presence of endogenous thiol isomerase activity.

Platelet αIIbβ3 was purified as described by Phillips et al[19]. Coomassie staining of reducing SDS-PAGE gels shows the presence of pure protein bands corresponding to the β3 subunit and the heavy chain of alpha IIb at 95kD and 115kD, respectively. The light chain of αIIb is observed at 25kD. There is no evidence for any additional co-purifying or contaminating proteins in this preparation (Fig. 2a). The ability of the purified αIIbβ3 to express thiol isomerase activity was determined in an assay described by Pigiet et al[22]. Purified αIIbβ3 (15µM) was shown to have equivalent thiol isomerase capacity to PDI (5µM). In contrast, ovalbumin, a cysteine-rich protein that lacks a CXXC motif, had no isomerase activity above baseline levels when compared to an uncatalysed reaction (Fig. 2b). This activity in αIIbβ3 was shown to be saturable and both time- and concentration-dependent (Fig. 2c). To ascertain that the activity
observed in these assays was not due to contaminating or co-purifying platelet PDI, the purified integrin preparation was probed for the presence of PDI immune-reactive proteins. Platelet PDI has a molecular weight of 57kD and is readily seen in immunoblot analysis of platelet lysates. Only one isoform of PDI is identified in human platelets as determined by immuno blotting. Similarly, platelet cDNA scanning with degenerate primers to conserved regions of published PDI shows evidence for only one platelet isoform of PDI in agreement with Chen et al,[23](data not shown). PDI is not apparent in coomassie stained gels or in western blots of purified αIIbβ3, nor could it be immunoprecipitated from large volumes of the purified integrin (Fig 2d). From these studies, we conclude that our integrin preparation is devoid of contaminating platelet PDI. Furthermore, in indirect immnuofluoresence studies it was found that, regardless of the activation state of the platelet, PDI did not colocalise with αIIbβ3 (Fig. 3). Activated platelets, which spread on a fibrinogen-coated surface, or resting platelets, immobilized on poly-L-lysine, stain for both αIIbβ3 and for PDI. However, these proteins do not co-localize at any stage as shown in the overlay images (Fig3D, H) where colocalization would be detected by the appearance of yellow pixels.

A direct comparison of the abilities of αIIbβ3 and protein disulphide isomerase to catalyse the reactivation of rdRNase was made by determining values for the kcat, and Km of each protein from Michaelis-Menten plots of kinetic data (Fig. 4 and Table 1). Both proteins showed typical equilibrium kinetics. The affinity (Km) of the integrin for its substrate (RNase) is less for αIIbβ3 than for PDI although the catalytic capacity of the enzymes (kcat) is equivalent. The value reported here for the Km of PDI (24.7µM) is identical to that reported by Langanbach et al, in their comparative study of the intrinsic isomerase activity of PDI and fibronectin[16]. In contrast,
however, to the thiol isomerase activity of fibronectin, we show that the catalytic capacity ($k_{cat}$) of $\alpha$IIb$\beta$3 is equivalent to that of PDI ($2.17 \times 10^{-4}$ moles. min$^{-1}$/µM enzyme and $2.45 \times 10^{-4}$ moles. min$^{-1}$/µM enzyme respectively). Thus, the intrinsic thiol isomerase activity within $\alpha$IIb$\beta$3 is potent and has high capacitance, equal to that observed in PDI. The $k_{cat}$ values obtained in this study are different to those obtained by Langanbach et al. This may be explained by the fact that we took into consideration the contribution of spontaneous refolding of rdRNase and subtracted this away from the catalysed reaction in our studies.

To examine the pharmacological profile of this activity, we looked at the effects of bacitracin, a known inhibitor of protein disulphide isomerase[24]. Bacitracin dose dependently inhibited the isomerase activity of both PDI and $\alpha$IIb$\beta$3 (Fig. 5). The IC$_{50}$ value is 11µM for $\alpha$IIb$\beta$3 and 4µM for PDI. Maximal inhibition of $\alpha$IIb$\beta$3 activity is observed at 100µM bacitracin. Moreover, in vitro incubation of platelets with bacitracin results in a dose-dependent abolition of platelet aggregation to all platelet agonists and an inhibition of fibrinogen binding (data not shown). Thus, the endogenous thiol isomerase activity of the platelet integrin can be inhibited by the pharmacological agent, bacitracin, resulting in an inhibition of functional responses in the platelet.

The isomerase activity of platelet integrin $\alpha$IIb$\beta$3 can also be regulated by divalent cations. $\alpha$IIb$\beta$3 forms a calcium dependent heterodimer on the platelet surface and both subunits contain calcium-binding motifs. Divalent cations are required for ligand recognition and influence the affinity of interactions with the integrin receptor[25]. Furthermore, extracellular divalent cations regulate integrin function by controlling the integrin ligand binding events[26].
We investigated the effects of calcium on the thiol isomerase activity of αIIbβ3. The observed isomerase activity αIIbβ3 occurs in the presence of the calcium chelator EDTA (1mM). Removal of this EDTA causes the αIIbβ3 to loose the ability to reactivate rdRNase while the enzymatic activity of PDI is unaffected by the presence or absence of EDTA (Fig. 6). These data suggest that the isomerase activity associated with αIIbβ3 is dependent upon an EDTA-stabilised conformation of the integrin. This is supported by the observation that calcium is displaced from the integrin as the complex between receptor and ligand becomes stabilised[27]. Furthermore, EDTA has been demonstrated to induce a high affinity, activated conformation in αIIbβ3[28] and other integrins[29] equivalent to that induced by agonists or ligand mimetics. A similar EDTA-stabilized active conformation is observed in thrombospondin permitting the exposure of an RGD sequence necessary for ligand binding[30].

Finally, in order to determine if this enzymatic activity is a common property of integrins, we examined commercial preparations of purified αvβ3 and α5β1 for the presence of endogenous thiol isomerase activity. Fig 7 shows that the vitronectin receptor, αvβ3 has thiol isomerase activity. The activity of the αvβ3 is greater than that of αIIbβ3. This data suggests that the activity does indeed reside in the common β3-integrin subunit. The leukocyte fibronectin receptor, α5β1, also expresses thiol enzymatic activity but, under the conditions tested, its potency appears less than that of the β3-integrins. It may be that the conditions of time, temperature, pH and Ca++ concentrations optimized for αIIbβ3 are not ideal for α5β1. The distinct potencies of the different integrins may suggest that the enzymatic activity of each
integrin is regulated by their individual $\alpha$-subunits, or that each may operate under discrete optimal conditions not addressed in this study.
DISCUSSION

The contribution of thiol bonds to the integrin structure and function has been implied by many other groups[7, 31]. In platelet functional assays, it has been shown that treatment with the reducing agent dithiothreitol causes platelet aggregation, fibrinogen binding and αIIbβ3-conformational changes[8, 9, 32]. Similar changes are observed in other integrins[11, 33, 34]. Deletions of some β3-cysteine residues[10], but not others [35], affects the affinity of the αIIbβ3-integrin for its ligand, fibrinogen. In particular, the long-range disulphide bond between C5 and C435 has been shown to be critically involved in the conformational change associated with αIIbβ3 activation [10, 31]. Mutation of either of these cysteines, but not C406 or C655[35], leads to constitutive integrin activation[10] while the long-range thiol bond (C5-C435) is shown to be altered during in-vitro integrin activation[31]. These data therefore suggest a role for disulphide bond rearrangement in conformational changes of αIIbβ3.

The physiological mediator for this disulphide rearrangement could be the thiol isomerase enzyme known as protein disulphide isomerase (PDI). Evidence in favor of its involvement is that PDI is present in platelets[36] and is released from the platelet surface in response to platelet activation[23]. Furthermore, bacitracin, an inhibitor of PDI, can inhibit platelet aggregation[24, 37]. However, using confocal microscopy and co-immunoprecipitation techniques, we can find no evidence for co-localization of platelet PDI with αIIbβ3. The alternative hypothesis is that αIIbβ3 could contain an endogenous thiol isomerase enzymatic activity. There are several reasons to suggest this. Firstly, the active site sequence common to PDI and thioredoxin (CGXC) is repeated four times within the beta subunit of the platelet
integrin while the less specific CXXC sequence is present an additional five times. The CGXC repeat forms part of a larger motif GXCXCGXCXC, present within each of the four CRRs in β3. These repeats correspond with the CRRs identified by Calvete suggesting that this region arose by duplication of an important functional region of the protein. Secondly, this repeat motif is common to all integrin beta subunits identified to date lending support to the theory that it represents an important functional domain. Thirdly, since thiol rearrangement is essential for αIIbβ3-activation and ligand binding, it is necessary to hypothesise some mechanism of the involvement of a thiol isomerase activity with the integrin in the platelet. As stated above, there is no evidence to support the co-localization of PDI with the platelet integrin. Therefore, it is attractive to propose an endogenous thiol isomerase activity. Finally, bacitracin, a pharmacological inhibitor of thiol isomerase activity, inhibits integrin-specific events such as aggregation and fibrinogen binding in in-vitro assays of platelet function (data not shown). While this observation can argue equally for the involvement of platelet PDI in aggregation, similar studies by Mou et al, show that bacitracin inhibits β1 and β7 integrin-mediated adhesive function in B-lymphocytes by a PDI-independent mechanism [38]. In this report, we unambiguously demonstrate the presence of this activity in the platelet integrin. It is as potent catalytically, as bovine PDI at renaturing the artificial substrate, rdRNase. Its activity is time-dependent, dose-dependent and saturable. Like PDI, it is inhibited in a dose-dependent manner by bacitracin, with a similar IC50. In the in-vitro assays of platelet function, the concentrations of bacitracin necessary to achieve inhibition are 10-100 fold greater than is necessary for the inhibition of thiol isomerase activity. The reason for this dosage-disparity is not clear. It may be that the lipid-soluble bacitracin binds to plasma proteins or inserts in lipid membranes so that its effective concentration is reduced in the cellular environment. The doses of bacitracin used in other in-
vitro studies are routinely in the mM range[23, 39]. In our studies, the dose of bacitracin necessary to achieve a complete inhibition of platelet aggregation depends on the agonist used. 1mM bacitracin can inhibit platelet aggregation to weak platelet agonists such as the thromboxane mimetic, U46619, epinephrine and collagen but higher concentrations are required to inhibit aggregations to TRAP, thrombin or ADP (data not shown). Furthermore, bacitracin has no effect on integrin-independent events in activated platelets such as granule secretion and P-selectin expression. Thus, the endogenous thiol isomerase activity of the platelet integrin can be inhibited by the pharmacological agent, bacitracin and this inhibition exerts a functional response, namely the inhibition of fibrinogen binding and platelet aggregation. The inhibition of an endogenous thiol isomerase activity of αIIbβ3 by bacitracin provides a biochemical mechanism to explain this antiplatelet effect. Unlike PDI, however, the catalytic activity of αIIbβ3 is Ca++ sensitive and is maximal in the presence of the calcium chelator EDTA.

The function of the endogenous integrin thiol isomerase activity remains unknown and was not the focus of this study. However, it is attractive to suggest that it plays a physiological role in the activation-induced conformational changes of the integrins. Such a claim is supported by the observation that bacitracin can inhibit both αIIbβ3 activation and platelet aggregation. The regulation of the thiol isomerase activity by Ca++ is also consistent with this role. A recent report has shown that integrin-binding function is coordinated in migrating neutrophils by local, transient cellular calcium concentrations[40].

Similarly, the function of the CRRs is not well understood although it is accepted that they do not play a direct role in ligand recognition[18]. Many activating antibodies bind to the CRR region and induce activation in αIIbβ3[41] and other integrins[29] implying a ‘regulatory
role for the CRRs. In addition, while functional integrin complexes can form when the CRRs are either deleted[42] or mutated, such integrins cannot be regulated between high- and low-affinity states. Only one mutation to date has been characterized that directly involves a cysteine residue in the CRR of αIIbβ3[43]. In this case, the surface expression of the integrin is affected suggesting a role for the CRRs in the assembly, stability or transport of the intact integrin to the platelet surface. In contrast, however, the work of Wippler et al[42], in which the entire CRRs are deleted from αIIbβ3, demonstrates normal levels of integrin expression in Sf9 insect cells implying that the CRRs are not involved in the assembly, stability or surface expression of the integrin complex. A recent study by Yan et al,[31] demonstrates that αIIbβ3 is activated by a series of specific conformational rearrangements in the ectodomain that increase the rate of ligand association. Such changes are focused at a disulfide-bonded knot of β3 adjacent to the CRRs and are independent of signal-induced modifications of the integrin such as phosphorylation. These observations indicate that the CRRs, while not important for integrin assembly, expression or ligand recognition, are critical for the regulation of the integrin affinity state and its inducible capacity to bind ligand.

Other proteins, not belonging to the thiol redox family, have been identified that contain vicinyl cysteine residues. Such proteins include the gonadotropins, lutropin and follitropin[14, 15], retinal cognin[44], fibronectin[16], insulin-like growth factors (IGFs) and IGF binding protein-3[21]. All of these proteins have demonstrable endogenous thiol isomerase activity that has been attributed to the presence of the CXXC motif. The presence of vicinyl cysteines in αIIbβ3 and other integrins strongly suggested, therefore, the presence of an endogenous thiol isomerase activity in this family of adhesion molecules.
The results reported here showing endogenous thiol isomerase activity in integrins are all performed in the presence of 1mM EDTA. In assays where EDTA is omitted, calcium is present at a concentration of 100µM. Integrins are calcium-dependent heterodimers and are therefore dependent on the presence of Ca++ to maintain their dimeric structure. In the presence of 1mM EDTA, the αIIb and β3 chains can be expected to be dissociated. In contrast, at 100µM Ca++, the heterodimer is intact. The lack of thiol isomerase activity in the presence of Ca++ may be explained by an inhibitory effect exerted by the αIIb chain on the enzymatic activity of the β3 subunit. Such a hypothesis is attractive, as the integrin α-subunit has been shown to interact with cytoplasmic signalling proteins in an inducible manner during cellular activation[1, 45-47]. Alternately, local concentrations of Ca++ may exert a direct inhibitory effect on the function of the integrin. In support of this argument, it has been shown recently, that local fluctuating concentrations of intracellular Ca++ can have profound effects on integrin α5β1 affinity for its ligand, fibronectin[40]. Ongoing experiments within our laboratory are aimed at elucidating the mechanisms involved with the regulation of the endogenous isomerase function of the β3 integrin subunit and with determining the native substrates for this enzymatic activity.

The data presented in this report characterizing the endogenous thiol isomerase activity of αIIbβ3 is the first demonstration of an enzymatic activity within an integrin subunit and suggests that altered thiol bonding within the integrin or its substrates may be locally modified during αIIbβ3 activation. Because of the highly conserved nature of the putative isomerase sites within all integrin β-subunits, a similar activity may be predicted in other integrins. Purified vitronectin receptor (αvβ3), which shares a common β-subunit with αIIbβ3 also displays
endogenous thiol isomerase activity suggesting that the enzymatic activity resides in the β3 chain. The potential for disruption of integrin function through specific inhibition of this endogenous isomerase activity offers a new therapeutic concept to supersede existing strategies of competitive antagonism of ligand binding through RGD-mimetic drugs.
References


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The abbreviations used are: CRRs, cysteine rich repeats; DTT, dithiotheitol; PDI, protein disulfide isomerase; rdRNase, reduced and denatured RNase; TRAP, thrombin receptor activating peptide.
Figure legends

Fig 1. **Conservation of CXXC sequences in \( \beta_3 \) cysteine rich repeats (CRRs).** (a) Comparison of the amino acid sequence of the second CRR of the human \( \beta_3 \) integrin subunit with the equivalent region from other \( \beta \)-integrins. The integrins are identified by their Swiss Prot or genbank accession codes. Identical residues are highlighted in yellow. Similar residues are highlighted in grey. (b) (Re)alignment of the four cysteine rich repeats of human \( \beta_3 \) protein. The presumed thiol bonds are indicated by the arrows, taken from Calvete et al., [18]. Identical amino-acids are shown in red text. Motifs I and II are boxed. The numbering sequence is from Swiss Prot P05106. (c) Comparison of the active sites of thioredoxin (TD), protein disulphide isomerase (PDI), endoplasmic-reticular protein-72 (ERP-72), insulin-like growth factor binding protein-1 (IGFBP-1), fibronectin (Fn), follotropin (FSH), von Willebrand factor (vWF) and CRRs I-IV of human \( \alpha \text{II}b\beta_3 \).

Fig 2. **Endogenous thiol isomerase activity in purified \( \alpha \text{II}b\beta_3 \).** (a) The \( \alpha \text{II}b\beta_3 \) preparation is free from contaminating PDI. Molecular weight markers (lane 1) and 3\( \mu \)g purified \( \alpha \text{II}b\beta_3 \) (lane 2) are separated on a 7.5\% gel. (b) Endogenous thiol isomerase activity of this purified preparation was determined according to the procedures of Pigiet[22]. Native RNase was reduced and denatured (rdRNase, 30\( \mu \)M), and incubated with 5\( \mu \)M PDI (■), 15\( \mu \)M \( \alpha \text{II}b\beta_3 \) (▼), 5\( \mu \)M ovalbumin (●) or no enzyme (○) in 0.1M Tris-HCl buffer, pH 7.4 containing 1mM EDTA.
Recovery of RNase activity was monitored at the indicated time points. Results are expressed as a percentage of the activity observed in native RNase preparations. Data are the means ±S.D. of n=3 experiments. (c) The endogenous thiol isomerase activity of αIIbβ3 is concentration-dependent. Reactivation of rdRNase by αIIbβ3 at concentrations of 0.5µM (●), 5µM (∇) and 15µM (■). Results are the means ±S.D. of n=3 experiments. (d) To ascertain that there is no contaminating PDI in purified preparations of αIIbβ3, purified PDI (2µg/ml, lane 2) and αIIbβ3 (2µg/ml, lane 3) were immunoprecipitated with a rabbit anti-human protein disulfide isomerase polyclonal antibody (gift of Dr.E. Quemeneur), separated on 12.5% SDS-PAGE and immunoblotted with rabbit anti-protein disulfide isomerase polyclonal antibody from Stressgen. Lane 4 contains 10µg total platelet lysate.

Fig 3. αIIbβ3 and protein disulfide isomerase do not co-localise in adhered platelets. Gel filtered platelets were allowed to adhere to poly-L-lysine (PLL, A-D) or fibrinogen (Fg, E-H) coated slides as described in the "Experimental Procedures" section. These were dual stained for αIIbβ3 using a monoclonal antibody (CD41a; B,F) which is complex specific and for protein disulfide isomerase (anti-PDI; C,G) using a polyclonal antibody along with the appropriately labelled Alexa secondary antibodies. It was observed that resting platelets were obtained on the PLL slides and spread or ‘activated’ platelets on the Fg coated slides using differential interference contrast (DIC) mode (A,E). When these images were overlaid the absence of yellow pixels indicates that αIIbβ3 did not co-localise with protein disulfide isomerase (D,H). Images were made on a Zeiss Axioplan 2 confocal microscope with a 40X oil-immersion lens (1.4n/a). Data shown is representative of n=3 independent experiments.
Fig. 4. **Kinetics of endogenous thiol isomerase activity in purified αIIbβ3.** Native RNase was reduced and denatured (rdRNase; 7.5-75µM), and incubated with 1.5µM PDI (■), or 1.5µM αIIbβ3(●) in 0.1M Tris-HCl buffer, pH7.4, containing 1mM EDTA. Recovery of RNase activity was monitored at 48hrs. Plots of substrate concentration *versus* initial velocity were fit to Michaelis-Menten equation using Deltagraph 4.0 to determine $V_{max}$ and $K_m$. Data are the means of 4 separate experiments. The Lineweaver-Burke transformation of this data is shown in the inset-figure. This data is summarized in Table 1.

Fig 5. **Bacitracin inhibits the endogenous thiol isomerase activity of αIIbβ3 and integrin function.** 5µM each of PDI (■) and αIIbβ3(●) were incubated with the PDI inhibitor, bacitracin, at the doses indicated for 24 hours. The results are expressed as % of maximal recovery of the refolding activity of rdRNase observed in the absence of bacitracin and are shown as mean±S.D. of n=3 experiments.

Fig 6. **The effects of the calcium chelator EDTA on the isomerase activity of αIIbβ3 and PDI.** Refolding of rdRNase was monitored in the absence of a catalyst (uncat) or in the presence of PDI (1.5µM) or αIIbβ3 (1.5µM) for 24 hours. Assays were performed in the presence (dark bars) or absence (shaded bars) of 1mM EDTA. The results are expressed as the change in absorbance of the initial rate of recovered RNase activity over 1 minute (ΔA/min). Data are
shown as mean±s.e.m. of n=4 experiments. Statistical significance was determined using paired Student-t test. ** indicates a p value <0.05.

Fig. 7. **Thiol isomerase activity in various integrins.** Refolding of rdRNase was monitored at a 24 hours in the absence of a catalyst (uncat) or in the presence of PDI (0.5μM), αIIbβ3 (0.5μM), αvβ3 (0.5μM) or α5β1 (0.5μM). The results are expressed as the change in the initial rate of the RNase reaction over 1 minute (ΔA/min) and are shown as are mean±s.e.m. of n=3 experiments.
Table 1. Enzymatic parameters of αIIbβ3 and PDI.

<table>
<thead>
<tr>
<th></th>
<th>Km (µM)</th>
<th>kcat (µM min⁻¹/µM enzyme)</th>
<th>kcat /Km (µM⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIIbβ3</td>
<td>36.62</td>
<td>2.17X10⁻⁴</td>
<td>5.93X10⁻⁶</td>
</tr>
<tr>
<td>PDI</td>
<td>24.72</td>
<td>2.45X10⁻⁴</td>
<td>9.91X10⁻⁶</td>
</tr>
</tbody>
</table>

The standard RNase refolding assay was performed using 7.5-75µM rdRNase in the presence of 1.5µM PDI or 1.5µM αIIbβ3. Analysis of Michaelis-Menten data was performed using the curve-fitting programs of DeltaGraph 4.0. Values are derived from the mean data from 4 separate experiments performed in duplicate.
Figure 1

a  

2nd repeat 520-562

P05106 human beta 3  
RGECLGCQCVCHSDFG--KIT--GKYCECDDFSCVRKYGEMCSG
P11835 mouse beta 3  
RGECLGCQCVCHSDFG--KIT--GKYCECDDFSCVRKYGEMCSG
g573375 dog beta 3  
RGECLGCQCVCHSDFG--KIT--GKYCECDDFSCVRKYGEMCSG
P18084 human beta 5  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
Q07441 baboon beta 5  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
P11835 mouse beta 3  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
P18084 human beta 5  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
P26010 human beta 8  
RGCVGCQCLCHSDFG--KLY--GKYCECDDFSCPYKGLSCAG
P26012 human beta 8  
RGCVGCQCLCHSDFG--KLY--GKYCECDDFSCPYKGLSCAG
P05556 human beta 1  
NEDCVGCQVCRKRNINE--YSKFPCECNDNCGDNLNGLGCGG
P12607 xenla beta 1  
NEDCVGCQVCRKRNINE--YSKFPCECNDNCGDNLNGLGCGG
P05107 human beta 2  
LCDCVGCQVCRKRNINE--YSKFPCECNDNCGDNLNGLGCGG
P53714 pig beta 2  
LCDCVGCQVCRKRNINE--YSKFPCECNDNCGDNLNGLGCGG
P11584 drosoph beta  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
AF059607 greenurch  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
g2335165 spon-coral  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG
g3789800 mullosc beta  
RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG

b

Repeat I  
PNHRGNNNGTVECVCRGPWL--GQYCECDSYDRPSQDECSPREVQPSOQ-- 469-523
Repeat II  
------------RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG-- 524-564
Repeat III  
-----------HGQCSCGDCLCDSDWT--GYYCNTRTDTGNCSN--GLLLGCGG-- 565-603
Repeat IV  
------------RGCYGCLCQVCHE--GKYCECDDFSCVRYKGE-MCSG-- 604-660

Motif I  
Motif II

Long Range to cys 713

P07237 PDI  
HAPWCGHCALAP
Q99757 Thioredoxin  
HAPWCGHCALAP
P13667 ERPT2  
YAPWCGHCALAP
P20959 IGFBP-3  
RDAGCGCCALAP
P02751 Fibronectin  
RGWRCNFRPGP
P01225 FSH  
TQCRCNRDQDG
P04275 vWF  
KAPTCLCEVARL
P05106 αIIβ3 CRR I  
GITEFCGVCQGPG
αIIβ3 CRR II  
GECICGCGVCQGPG
αIIβ3 CRR III  
GQECICGCGVCQGPG
αIIβ3 CRR IV  
GQECICGCGVCQGPG

29
Figure 2

(a) 

(b) 

(c) 

(d) 

RNase activity (%) 

RNase activity (%) 

Time (hours) 

RNase activity (%) 

Time (hours)
Figure 3

PLL  Fg

DIC

αIIbβ3

PDI

Overlay
Figure 4

V_{\text{max}} = 5.520587 \times 10^{-2}
K_{\text{m}} = 24.72046

V_{\text{max}} = 6.067326 \times 10^{-2}
K_{\text{m}} = 36.61469

Initial Rate

[\text{rdRNAse}] \mu\text{M}

\frac{1}{\text{rate}} vs. \frac{1}{[\text{rdRNAse}]} \mu\text{M}
Figure 5
Figure 7

![Bar graph showing ΔA/min for different integrin combinations](image-url)

- Uncat
- PDI
- αIIbβ3
- αvβ3
- α5β1

The graph illustrates the ΔA/min values for various integrin combinations, with PDI showing the highest ΔA/min and Uncat showing the lowest.
The platelet integrin αIIbbeta3 has an endogenous thiol isomerase activity
Sarah O’Neill, Aisling Robinson, Adele Deering, Michelle Ryan, Desmond F. Fitzgerald and Niamh Moran

J. Biol. Chem. published online August 14, 2000

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