Critical cysteine residues for regulation of integrin αIIbβ3 are clustered in the epidermal growth factor domains of the β3 subunit

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Chemical or enzymic reduction/oxidation of integrin cysteine residues (e.g. by reducing agents and protein disulphide isomerase) may be a mechanism for regulating integrin function. It has also been proposed that unique cysteine residues in the integrin β3 subunit are involved in the regulation of αIIbβ3. In the present study, we studied systematically the role of disulphide bonds in β3 on the ligand-binding function of αIIbβ3 by mutating individual cysteine residues of β3 to serine. We found that the disulphide bonds that are critical for αIIbβ3 regulation are clustered within the EGF (epidermal growth factor) domains. Interestingly, disrupting only a single disulphide bond in the EGF domains was enough to activate αIIbβ3 fully. In contrast, only two (of 13) disulphide bonds tested outside the EGF domains activated αIIbβ3. These results suggest that the disulphide bonds in the EGF domains should be intact to keep αIIbβ3 in an inactive state, and that there is no unique cysteine residue in the EGF domain critical for regulating the receptor. The cysteine residues in the EGF domains are potential targets for chemical or enzymic reduction.

Key words: αIIβ3 integrin, disulphide bond, epidermal growth factor domain (EGF domain), fibrinogen, integrin activation, mutagenesis.

INTRODUCTION

Integrins are a family of cell-adhesion receptors that mediate cell–extracellular matrix interactions and cell–cell interactions [1]. It has been proposed that signalling from inside the cells regulates the ligand-binding affinity of integrins by designated inside-out signalling (reviewed in [2]). It has also been proposed that integrins may be regulated by chemical and enzymic oxidation/reduction (e.g. by dithiothreitol, NO or protein disulphide isomerase). Integrin αIIbβ3 (glycoprotein IIb–IIIa, CD41/CD61), a platelet fibrinogen receptor, plays a critical role in primary haemostasis by mediating interactions between platelets and fibrinogen [3]. It has been reported that dithiothreitol induces specific binding of purified αIIbβ3 to fibrinogen, and exposes a ligand-induced binding site (LIBS) on both αIIb and β3 [4]. These effects appear to be the result of a direct action of dithiothreitol on αIIbβ3. It has been reported that the redox potential of blood regulates the activation of αIIbβ3 and that disulphide bond cleavage with sulphydryl generation in β3 is involved in activation of this receptor [5]. Thus evidence is accumulating that reduction/oxidation of disulphide linkages may regulate αIIbβ3.

The crystal structure of αvβ3, another fibrinogen receptor with the common β3 subunit, shows that the β3 subunit has at least two EGF (epidermal growth factor)-like domains (EGF-3 and -4) and probably has two additional EGF domains (EGF-1 and -2) at the stalk region of β3 [6]. The β3 subunit contains 56 cysteine residues that are highly conserved among β subunits and that are involved in disulphide bond formation [7]. It has recently been shown that there is a difference in conformation between the active and inactive forms of integrin αIIbβ3 [8], which may come from the difference in the number and positions of unpaired cysteine residues [9]. It has been proposed that a select group of unpaired cysteine residues exhibit the properties of a redox site involved in integrin activation. In a recent model [10], the conformational transition from resting to active integrin proceeds through a series of changes in the position and number of disulphide bonds; the positions of the cysteine residues involved in this process have not been clearly defined.

It has been reported that the natural $Cys^{598} \rightarrow Tyr$ mutation [11] and the $Cys^{560} \rightarrow Arg$ mutation [12] of the β3 subunit activates αIIbβ3. Also disrupting the Cys^{435}–Cys^{5} disulphide bond [13], or disrupting the Cys^{663}–Cys^{687} disulphide bond [14] constitutively activates αIIbβ3. It has also been reported that disruption of a disulphide bond between Cys^{406} and Cys^{655} does not affect αIIbβ3 ligand binding [15]. It has not been established, however, whether or not the effect of disrupted disulphide bonds is specific to their positions.

In the present paper, we tested systematically the role of disulphide bonds in β3 in the regulation of αIIbβ3 by mutating individual cysteine residues (29 in total). We found that the cysteine mutations that activate αIIbβ3 are clustered within the EGF domains of β3. In contrast, mutating cysteine residues outside the EGF domains did not activate αIIbβ3, with few exceptions. These results suggest that disulphide bonds in the EGF domains may be critically involved in integrin regulation and are potential targets for chemical or enzymic reduction.

EXPERIMENTAL

Monoclonal antibodies (mAbs)

PL98DF6 [16] was from Dr J. Ylänne (Biocenter Oulu and Department of Biochemistry, University of Oulu, Oulu, Finland). PT25-2 was generated as described previously [17].

Abbreviations used: CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; mAb, monoclonal antibody; PE, phycoerythrin.

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Construction and transfection of cDNAs for human β3 mutants

Wild-type human β3 cDNA was subcloned into pBJ-1 vector, and mutation was introduced [18]. The presence of mutation was verified by DNA sequencing. Wild-type and mutant β3 cDNA constructs in pBJ-1 vector (20 μg) were transfected by electroporation into CHO (Chinese-hamster ovary) cells (1×10^7 cells) [19], together with wild-type αIIb in pBJ-1 vector. Transfected cells were maintained in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10 % (v/v) foetal calf serum at 37 °C in 6 % CO_2 for 2 days. Then the cells were detached with 3.5 mM EDTA and used for assays.

Flow cytometry

Cells were washed once with DMEM and were then resuspended in the same medium. Cell suspension (50 μl) was incubated with an equal volume of primary mAb (1:250 dilution of ascites or 10 μg/ml of purified mAb) on ice for 30 min. After washing with DMEM, cells were incubated with FITC-conjugated anti-mouse IgG (Biosource, Camarillo, CA, U.S.A.) for 30 min on ice.

Fibrinogen binding

Human fibrinogen (Enzyme Research Laboratories, South Bend, IN, U.S.A.) was labelled with FITC as described previously [20,21]. Fibrinogen binding to cells transiently expressing αIIbβ3 was determined as described previously in [22] with some modifications. Briefly, cells were first incubated with PL98DF6 followed by phycoerythrin (PE)-conjugated anti-mouse IgG (Biosource). Cells were washed with modified Tyrode–Hepes buffer (5 mM Hepes, 5 mM glucose, 0.2 mg/ml BSA and 1× Tyrode solution), pH 7.4, supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Cells were then incubated with 150 μg/ml FITC-labelled fibrinogen in the presence of 10 μg/ml control mouse IgG or PT25-2 in the same buffer for 30 min. After removing unbound fibrinogen, cells were resuspended in Hepes-buffered saline, pH 7.4, supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Binding of fibrinogen (FITC staining) was analysed on a gated subset of cells highly positive for αIIbβ3 expression (PE staining) in FACScan. Relative fibrinogen binding was calculated as $(F_{PT} - F_{mIgG})/(Fw_{PT} - Fw_{mIgG})$, where F_{PT} is the median fluorescence intensity of fibrinogen binding in the presence of PT25-2, F_{mIgG} is the median fluorescence intensity of fibrinogen binding in the presence of normal mouse IgG, Fw_{PT} is the median fluorescence intensity of fibrinogen binding to cells expressing wild-type αIIbβ3 in the presence of PT25-2, and Fw_{mIgG} is the median fluorescence intensity of fibrinogen binding to cells expressing wild-type αIIbβ3 in the presence of normal mouse IgG. Relative αIIbβ3 expression is a ratio of the median fluorescence intensity of PL98DF6 binding to the gated population to the median fluorescence intensity of PL98DF6 binding to the gated population expressing wild-type αIIbβ3. The activation index was calculated as $100 \times (F_o - F_r)/(F_o\ PT - F_r\ PT)$, where F_o is the median fluorescence intensity of fibrinogen binding, F_r is the median fluorescent intensity of fibrinogen binding in the presence of 1 mM GRGDS peptide [22,23], F_o PT is the median fluorescence intensity of fibrinogen binding in the presence of 10 μg/ml PT25-2, and F_r PT is the median fluorescence intensity of fibrinogen binding in the presence of 10 μg/ml PT25-2 and GRGDS peptide.

RESULTS AND DISCUSSION

We reported previously that the $Cys^{560} \rightarrow$ Phe mutation in the EGF domains of β3 [24] is associated with the bleeding tendency in Glanzmann's thrombasthenia. It has been reported that the similar $Cys^{560} \rightarrow$ Arg mutation activates αIIbβ3 [12]. To study the effect of the $Cys^{560} \rightarrow$ Phe mutation on αIIbβ3 function, we transiently expressed the mutant on the surface of CHO cells together with wild-type αIIb. We examined the ability of the αIIb/β3 $Cys^{560} \rightarrow$ Phe mutant to bind to soluble FITC-labelled fibrinogen with or without an anti-αIIbβ3 mAb PT25-2, which specifically activates αIIbβ3. Wild-type αIIbβ3 in CHO cells does not bind to ligand unless it is activated [19,25]. We selected cells that express wild-type or mutant αIIbβ3 at high levels, and tested their ability to bind to soluble fibrinogen. The αIIb/β3 $Cys^{560} \rightarrow$ Phe mutant bound to soluble fibrinogen without activation (Figures 1A–1D), suggesting that the mutation induced constitutive activation of

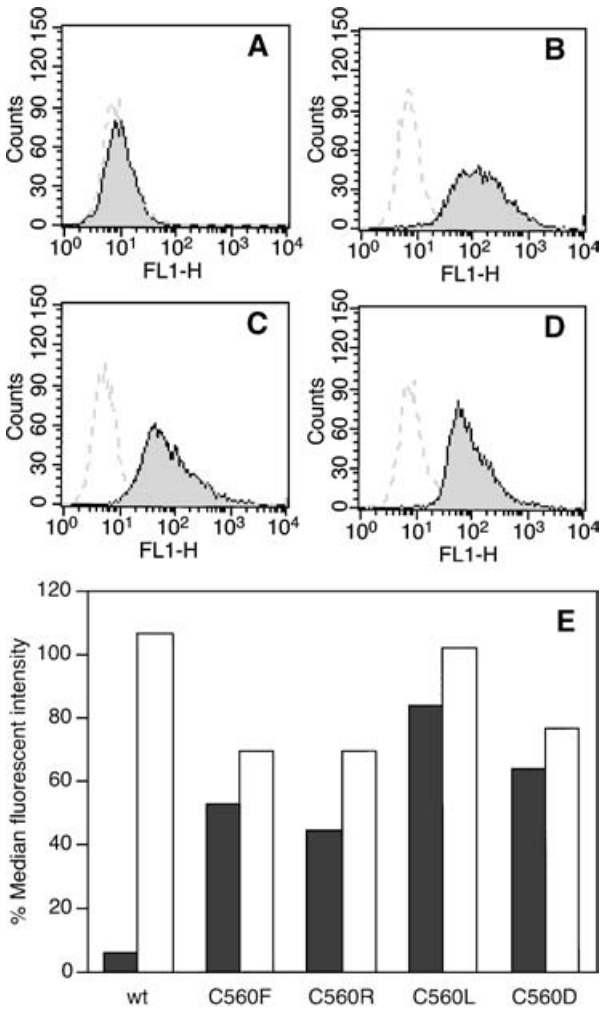

Figure 1 Effects of the β3 $Cys^{560} \rightarrow$ Phe mutation on binding of soluble fibrinogen to αIIbβ3 (A–D) and effects of mutating Cys^{560} to other amino acid residues on the activation status of αIIbβ3 (E)

CHO cells transiently expressing wild-type (**A**, **B**) or the $C^{560} \rightarrow$ Phe mutant (**C**, **D**) of αIIbβ3 were stained with PL98DF6 (anti-αIIb mAb), followed by PE-conjugated anti-mouse IgG. After washing, cells were incubated with FITC–fibrinogen in the presence of control mouse IgG (**A**, **C**) or activating mAb PT25-2 (**B**, **D**). The solid histograms represent FITC-fibrinogen binding (FL1) to αIIbβ3-positive cells (FL2 > 100). The blank histograms represent FITC-fibrinogen binding in the presence of 1 mM GRGDS peptide. (**E**) The effects of mutating Cys^{560} to several other amino acid residues on the activation status of αIIbβ3. Wild-type (wt) or mutant (one-letter amino acid codes are used) β3 was transiently transfected together with wild-type αIIb into CHO cells. FITC–fibrinogen binding to αIIbβ3-positive cells was examined in the presence of PT25-2 (open bars) and of control mouse IgG (closed bars). Mutating Cys^{560} to glycine, alanine, serine methionine, arginine, asparagine, tyrosine, tryptophan and histidine also constitutively activated αIIbβ3 (results not shown).

Cys^{23}–Cys^{13}, Cys^{26}–Cys^{38}, Cys^{49}–Cys^{16}, Cys^{177}–Cys^{184}, Cys^{232}–Cys^{273}, Cys^{374}–Cys^{386}, Cys^{406}–Cys^{421}, Cys^{433}–Cys^{437}, Cys^{435}–Cys^{5},

Cys^{460}–Cys^{471}, Cys^{462}–Cys^{457}, Cys^{473}–Cys^{486}, Cys^{495}–Cys^{503}, Cys^{508}–Cys^{521}, Cys^{523}–Cys^{528}, Cys^{536}–Cys^{544}, Cys^{542}–Cys^{547}, Cys^{549}–Cys^{558}, Cys^{560}–Cys^{583}, Cys^{575}–Cys^{586}, Cys^{581}–Cys^{567}, Cys^{588}–Cys^{598}, Cys^{601}–Cys^{604},

Cys^{631}–Cys^{617}, Cys^{635}–Cys^{614}, Cys^{687}–Cys^{663}

Figure 2 β3 disulphide bonds tested in the present study

The assignments of the disulphide bonds are based on the crystal structure of αvβ3 [6]. The grey shaded area indicates the EGF domain. The cysteine residues in bold were mutated to serine.

αIIbβ3. GRGDS peptide (Figures 1A–1D), and function-blocking anti-αIIbβ3 mAb, completely blocked fibrinogen binding to the αIIb/β3 Cys^{560} → Phe mutant (results not shown), suggesting that this binding is specific to αIIbβ3. To test whether this constitutive activation is specific to the Cys^{560} → Phe mutation, we mutated Cys^{560} to 12 different amino acids (serine, glycine, leucine, alanine, methionine, aspartic acid, arginine, asparagine, tyrosine, histidine, tryptophan and phenylalanine). All of these mutants constitutively activated αIIbβ3 (Figure 1E). These results suggest that the constitutive activation of αIIbβ3 by the Cys^{560} → Phe (and probably the Cys^{560} → Arg) mutation is not unique to the Cys^{560} → Phe mutation, and that disruption of the disulphide bond is primarily responsible for the constitutive activation of αIIbβ3.

We studied the role of other disulphide bonds in the β3 subunit in regulating ligand binding to αIIbβ3. To do this we mutated the individual cysteine residues of β3 to serine to disrupt individual disulphide bonds according to the assignments of disulphide bonds based on the αvβ3 crystal structure (Figure 2). We transiently expressed the β3 mutants together with wild-type αIIb in CHO cells and tested their ability to bind to soluble fibrinogen as described above. We found that mutating the cysteine residues at positions 435, 460, 462, 473, 495, 508, 521, 523, 536, 542, 549, 560, 575, 581, 588, 601 and 687 induced the binding of soluble fibrinogen to αIIbβ3 without activating mAb PT25-2 (Figure 3A). The anti-αIIbβ3 activating mAb PT25-2 had only a minor effect on fibrinogen binding to constitutively active mutants. This suggests that these mutations constitutively activate αIIbβ3 (with high activation indexes) (Figure 3B). Interestingly, these cysteine residues are clustered within the EGF domains (15 out of 17). These results also suggest that disruption of a single disulphide bond within the EGF domains may lead to constitutive activation of αIIbβ3.

In contrast, we found that mutating the cysteine residues at positions 23, 26, 49, 374, 406, 433, 631 and 635 did not significantly activate αIIbβ3 (with low activation indexes). These β3 mutants bound to soluble fibrinogen similarly to wild-type in the presence of mAb PT25-2, indicating that their ligand-binding functions were intact. These cysteine residues are all located outside the EGF domains. The C^{171} → Ser and C^{184} → Ser mutants did not bind to fibrinogen even when activated with mAb PT25-2, suggesting that mutating Cys^{171} and Cys^{184} inactivated αIIbβ3. A likely reason for this is that these residues are located in the putative fibrinogen-binding sites [26,27].

The present study suggests that the C^{560} → Phe mutation of β3 [24] activates αIIbβ3, like the C^{560} → Arg mutation [12],

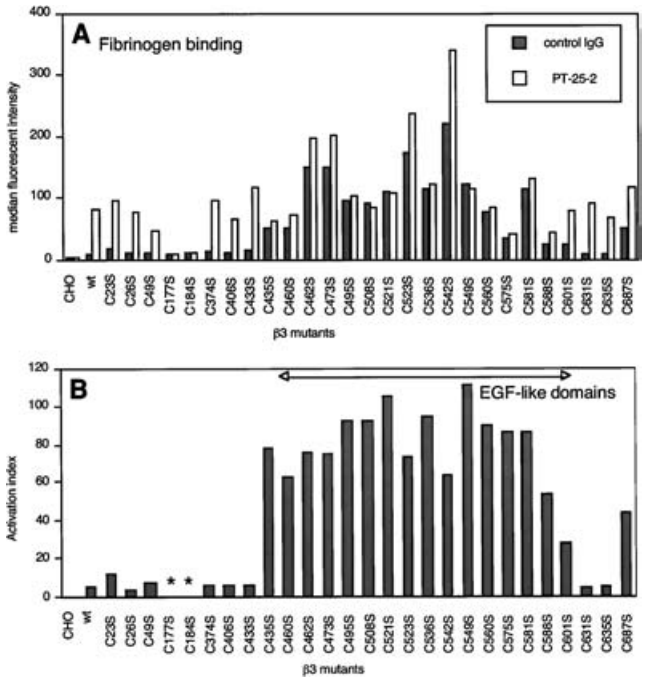

Figure 3 Effects of mutating individual cysteine residues on the activation status of αIIbβ3

Individual β3 cysteine residues were mutated to serine to disrupt disulphide bonds according to the disulphide bond assignment based on the crystal structure of αvβ3 [6] as shown in Figure 2. Individual β3 mutants (one-letter amino acid codes are used) were expressed on CHO cells together with wild-type (wt) αIIb as described above. The ability of αIIbβ3-positive cells to bind to soluble fibrinogen was measured in the presence and absence of activating anti-αIIbβ3 mAb PT25-2 (**A**), and the activation index was calculated (**B**). The Cys^{177} → Ser and Cys^{184} → Ser mutants were inactive (shown by *). The Cys^{233} → Ser and Cys^{273} → Ser mutants were not tested due to poor expression.

and this activation does not depend on the amino acid species to which the cysteine residue is mutated, since mutating Cys^{560} to twelve other different amino acid residues generates the same effects. It is likely that the activation is due to disruption of the disulphide linkage. It has been reported that another natural mutation of a cysteine residue (Cys^{598} → Tyr) [11], disrupting the Cys^{435}–Cys^{5} disulphide bond [13] or disrupting the Cys^{663}–Cys^{687} disulphide bond [14], constitutively activates αIIbβ3. It has been reported that disruption of a disulphide bond between Cys^{406} and Cys^{655} does not affect αIIbβ3 ligand binding [15]. It has not been established, however, whether the effect of disrupting disulphide bonds is specific to their positions. We thus mutated systematically individual cysteine residues of β3 and studied their effects on the ligand-binding function of αIIbβ3. The present results are consistent with the previous reports [11,13–15], and expanded the previous findings further. We have shown that the cysteine residue mutations that activate αIIbβ3 are clustered within the EGF domains. Mutating cysteine residues outside the EGF domains did not induce activation of αIIbβ3, with few exceptions. These results suggest that disulphide bonds in the EGF domains keep αIIbβ3 in an inactive form, and disruption of a single disulphide bond in the EGF domain is enough to induce activation of αIIbβ3. It is suggested that disulphide bonds in the EGF domains should be completely intact when αIIbβ3 is inactive.

It has been proposed that the integrin proceeds through a series of changes in the position and number of disulphide bonds (redox transition) [10]. The positions of unpaired cysteine residues (redox sites) have not been reported, but several cysteine residues in the cysteine-rich EGF domains (at positions 655, 457

and 495) are likely candidates [10]. The results in the present study do not fit well with this model, since the present results suggest that the unpaired cysteine residues cannot be present in the EGF domains of β3 when the receptor is inactive. Interestingly, there is no unique cysteine residue that is critical for regulation of the integrin in the EGF domains. The present study, however, suggests that the inactive form might have unpaired cysteine residues outside the EGF domains. There are several cysteine residues, the mutation of which does not affect fibrinogen binding (positions 23, 26, 49, 374, 406, 433, 631 and 635), that could be unpaired in an inactive form of αIIbβ3. Further biochemical studies will be required to test whether the cysteine residues outside the EGF domain of β3 are involved in regulation of the integrin.

The αvβ3 crystal structure is sharply bent at the stalk region, and it has been proposed that the EGF-3 and -4 domains (and probably the EGF-1 and -2 domains) generate a rigid rod-like module [6]. It has been proposed that the bent form is inactive and the extended form is active [28]. Interestingly the αv stalk region contains three β-sandwich domains, thigh and calf-1 and -2 domains [6], and the two calf domains generate a rigid entity (the calf module) at the C-terminal side of the stalk. Although the disulphide bonds in the EGF domains should be intact when the integrin is in an inactive form, it is unclear how disruption of disulphide bonds in the EGF domains makes the integrin active. It would be interesting to test whether the integrins are bent or extended when one of the disulphide bonds of β3 is disrupted.

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