

UC Davis

UC Davis Previously Published Works

Title

Critical cysteine residues for regulation of integrin alpha IIb beta 3 are clustered in the epidermal growth factor domains of the beta 3 subunit

Permalink

<https://escholarship.org/uc/item/64v413k4>

Journal

Biochemical Journal, 378

ISSN

0264-6021

Authors

Kamata, T
Ambo, H
Puzon-McLaughlin, W
et al.

Publication Date

2004-03-01

Peer reviewed

Critical cysteine residues for regulation of integrin α IIb β 3 are clustered in the epidermal growth factor domains of the β 3 subunit

Tetsuji KAMATA*, Hironobu AMBO†, Wilma PUZON-McLAUGHLIN*, Kenneth Khiem TIEU*, Makoto HANDA†, Yasuo IKEDA† and Yoshikazu TAKADA*¹

*Department of Cell Biology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, U.S.A., †Department of Internal Medicine, Keio University School of Medicine, Shinano-machi, Shinjuku, Tokyo, Japan

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 β 3 subunit are involved in the regulation of α IIb β 3. In the present study, we studied systematically the role of disulphide bonds in β 3 on the ligand-binding function of α IIb β 3 by mutating individual cysteine residues of β 3 to serine. We found that the disulphide bonds that are critical for α IIb β 3 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 α IIb β 3 fully. In contrast, only two (of 13) disulphide bonds tested outside the EGF domains activated α IIb β 3. These results suggest that the disulphide bonds in the EGF domains should be intact to keep α IIb β 3 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: α IIb β 3 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 α IIb β 3 (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 α IIb β 3 to fibrinogen, and exposes a ligand-induced binding site (LIBS) on both α IIb and β 3 [4]. These effects appear to be the result of a direct action of dithiothreitol on α IIb β 3. It has been reported that the redox potential of blood regulates the activation of α IIb β 3 and that disulphide bond cleavage with sulphhydryl generation in β 3 is involved in activation of this receptor [5]. Thus evidence is accumulating that reduction/oxidation of disulphide linkages may regulate α IIb β 3.

The crystal structure of α v β 3, another fibrinogen receptor with the common β 3 subunit, shows that the β 3 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 β 3 [6]. The β 3 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 α IIb β 3 [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 Cys⁵⁹⁸ → Tyr mutation [11] and the Cys⁵⁶⁰ → Arg mutation [12] of the β 3 subunit activates α IIb β 3. Also disrupting the Cys⁴³⁵–Cys⁵ disulphide bond [13], or disrupting the Cys⁶⁶³–Cys⁶⁸⁷ disulphide bond [14] constitutively activates α IIb β 3. It has also been reported that disruption of a disulphide bond between Cys⁴⁰⁶ and Cys⁶⁵⁵ does not affect α IIb β 3 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 β 3 in the regulation of α IIb β 3 by mutating individual cysteine residues (29 in total). We found that the cysteine mutations that activate α IIb β 3 are clustered within the EGF domains of β 3. In contrast, mutating cysteine residues outside the EGF domains did not activate α IIb β 3, 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. Yläne (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.

¹ To whom correspondence should be addressed, at the present address: Department of Dermatology, University of California at Davis Medical Center, Research III, Suite 3300, 4645 2nd Avenue, Sacramento, CA 95817, U.S.A. (e-mail ytakada@ucdavis.edu).

Construction and transfection of cDNAs for human $\beta 3$ mutants

Wild-type human $\beta 3$ 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 $\beta 3$ 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 $\mu\text{g}/\text{ml}$ 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 $\alpha\text{IIb}\beta 3$ 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 $1 \times$ Tyrode solution), pH 7.4, supplemented with 2 mM CaCl_2 and 2 mM MgCl_2 . Cells were then incubated with 150 $\mu\text{g}/\text{ml}$ FITC-labelled fibrinogen in the presence of 10 $\mu\text{g}/\text{ml}$ 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 $\alpha\text{IIb}\beta 3$ expression (PE staining) in FACScan. Relative fibrinogen binding was calculated as $(F_{\text{PT}} - F_{\text{mIgG}})/(F_{\text{wPT}} - F_{\text{w_mIgG}})$, 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, F_{wPT} is the median fluorescence intensity of fibrinogen binding to cells expressing wild-type $\alpha\text{IIb}\beta 3$ in the presence of PT25-2, and $F_{\text{w_mIgG}}$ is the median fluorescence intensity of fibrinogen binding to cells expressing wild-type $\alpha\text{IIb}\beta 3$ in the presence of normal mouse IgG. Relative $\alpha\text{IIb}\beta 3$ 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 $\alpha\text{IIb}\beta 3$. The activation index was calculated as $100 \times (F_o - F_r)/(F_o \text{ PT} - F_r \text{ PT})$, 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], $F_o \text{ PT}$ is the median fluorescence intensity of fibrinogen binding in the presence of 10 $\mu\text{g}/\text{ml}$ PT25-2, and $F_r \text{ PT}$ is the median fluorescence intensity of fibrinogen binding in the presence of 10 $\mu\text{g}/\text{ml}$ PT25-2 and GRGDS peptide.

RESULTS AND DISCUSSION

We reported previously that the $\text{Cys}^{560} \rightarrow \text{Phe}$ mutation in the EGF domains of $\beta 3$ [24] is associated with the bleeding tendency in

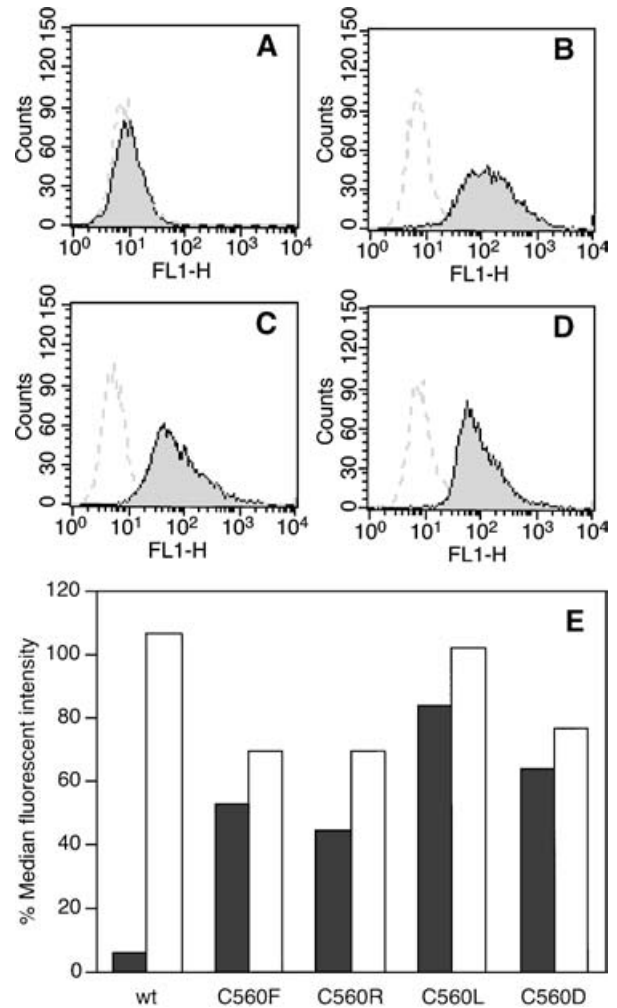


Figure 1 Effects of the $\beta 3$ $\text{Cys}^{560} \rightarrow \text{Phe}$ mutation on binding of soluble fibrinogen to $\alpha\text{IIb}\beta 3$ (A–D) and effects of mutating Cys^{560} to other amino acid residues on the activation status of $\alpha\text{IIb}\beta 3$ (E)

CHO cells transiently expressing wild-type (A, B) or the $\text{Cys}^{560} \rightarrow \text{Phe}$ mutant (C, D) of $\alpha\text{IIb}\beta 3$ 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 $\alpha\text{IIb}\beta 3$ -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 $\alpha\text{IIb}\beta 3$. Wild-type (wt) or mutant (one-letter amino acid codes are used) $\beta 3$ was transiently transfected together with wild-type αIIb into CHO cells. FITC-fibrinogen binding to $\alpha\text{IIb}\beta 3$ -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 $\alpha\text{IIb}\beta 3$ (results not shown).

Glanzmann's thrombasthenia. It has been reported that the similar $\text{Cys}^{560} \rightarrow \text{Arg}$ mutation activates $\alpha\text{IIb}\beta 3$ [12]. To study the effect of the $\text{Cys}^{560} \rightarrow \text{Phe}$ mutation on $\alpha\text{IIb}\beta 3$ function, we transiently expressed the mutant on the surface of CHO cells together with wild-type αIIb . We examined the ability of the $\alpha\text{IIb}\beta 3$ $\text{Cys}^{560} \rightarrow \text{Phe}$ mutant to bind to soluble FITC-labelled fibrinogen with or without an anti- $\alpha\text{IIb}\beta 3$ mAb PT25-2, which specifically activates $\alpha\text{IIb}\beta 3$. Wild-type $\alpha\text{IIb}\beta 3$ in CHO cells does not bind to ligand unless it is activated [19,25]. We selected cells that express wild-type or mutant $\alpha\text{IIb}\beta 3$ at high levels, and tested their ability to bind to soluble fibrinogen. The $\alpha\text{IIb}\beta 3$ $\text{Cys}^{560} \rightarrow \text{Phe}$ mutant bound to soluble fibrinogen without activation (Figures 1A–1D), suggesting that the mutation induced constitutive activation of

Cys²³–Cys¹³, Cys²⁶–Cys³⁸, Cys⁴⁹–Cys¹⁶, Cys¹⁷⁷–Cys¹⁸⁴,
 Cys²³²–Cys²⁷³, Cys³⁷⁴–Cys³⁸⁶, Cys⁴⁰⁶–Cys⁴²¹, Cys⁴³³–Cys⁴³⁷,
 Cys⁴³⁵–Cys⁵,
 Cys⁴⁶⁰–Cys⁴⁷¹, Cys⁴⁶²–Cys⁴⁵⁷, Cys⁴⁷³–Cys⁴⁸⁶, Cys⁴⁹⁵–Cys⁵⁰³,
 Cys⁵⁰⁸–Cys⁵²¹, Cys⁵²³–Cys⁵²⁸, Cys⁵³⁶–Cys⁵⁴⁴, Cys⁵⁴²–Cys⁵⁴⁷,
 Cys⁵⁴⁹–Cys⁵⁵⁸, Cys⁵⁶⁰–Cys⁵⁸³, Cys⁵⁷⁵–Cys⁵⁸⁶, Cys⁵⁸¹–Cys⁵⁶⁷,
 Cys⁵⁸⁸–Cys⁵⁹⁸, Cys⁶⁰¹–Cys⁶⁰⁴,
 Cys⁶³¹–Cys⁶¹⁷, Cys⁶³⁵–Cys⁶¹⁴, Cys⁶⁸⁷–Cys⁶⁶³

Figure 2 β 3 disulphide bonds tested in the present study

The assignments of the disulphide bonds are based on the crystal structure of α v β 3 [6]. The grey shaded area indicates the EGF domain. The cysteine residues in bold were mutated to serine.

α IIB β 3. GRGDS peptide (Figures 1A–1D), and function-blocking anti- α IIB β 3 mAb, completely blocked fibrinogen binding to the α IIB β 3 Cys⁵⁶⁰ → Phe mutant (results not shown), suggesting that this binding is specific to α IIB β 3. To test whether this constitutive activation is specific to the Cys⁵⁶⁰ → Phe mutation, we mutated Cys⁵⁶⁰ 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 α IIB β 3 (Figure 1E). These results suggest that the constitutive activation of α IIB β 3 by the Cys⁵⁶⁰ → Phe (and probably the Cys⁵⁶⁰ → Arg) mutation is not unique to the Cys⁵⁶⁰ → Phe mutation, and that disruption of the disulphide bond is primarily responsible for the constitutive activation of α IIB β 3.

We studied the role of other disulphide bonds in the β 3 subunit in regulating ligand binding to α IIB β 3. To do this we mutated the individual cysteine residues of β 3 to serine to disrupt individual disulphide bonds according to the assignments of disulphide bonds based on the α v β 3 crystal structure (Figure 2). We transiently expressed the β 3 mutants together with wild-type α IIB 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 α IIB β 3 without activating mAb PT25-2 (Figure 3A). The anti- α IIB β 3 activating mAb PT25-2 had only a minor effect on fibrinogen binding to constitutively active mutants. This suggests that these mutations constitutively activate α IIB β 3 (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 α IIB β 3.

In contrast, we found that mutating the cysteine residues at positions 23, 26, 49, 374, 406, 433, 631 and 635 did not significantly activate α IIB β 3 (with low activation indexes). These β 3 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 C¹⁷¹ → Ser and C¹⁸⁴ → Ser mutants did not bind to fibrinogen even when activated with mAb PT25-2, suggesting that mutating Cys¹⁷¹ and Cys¹⁸⁴ inactivated α IIB β 3. 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 C⁵⁶⁰ → Phe mutation of β 3 [24] activates α IIB β 3, like the C⁵⁶⁰ → Arg mutation [12],

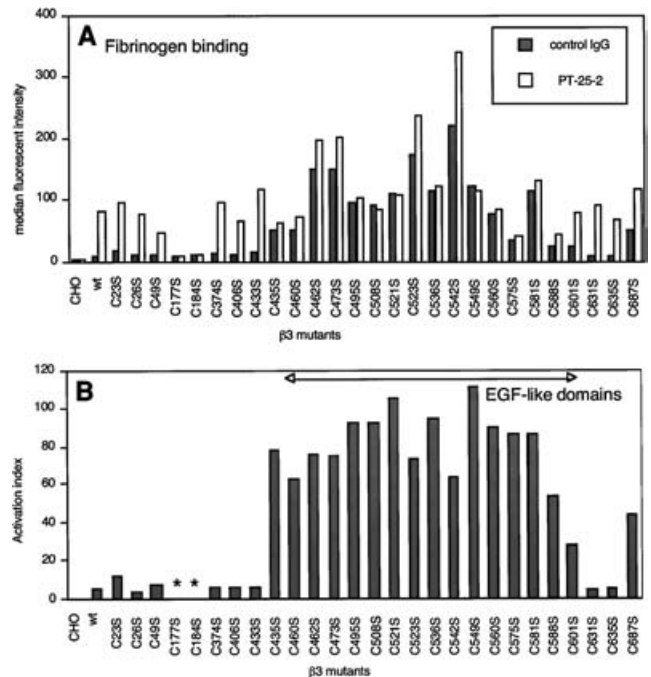


Figure 3 Effects of mutating individual cysteine residues on the activation status of α IIB β 3

Individual β 3 cysteine residues were mutated to serine to disrupt disulphide bonds according to the disulphide bond assignment based on the crystal structure of α v β 3 [6] as shown in Figure 2. Individual β 3 mutants (one-letter amino acid codes are used) were expressed on CHO cells together with wild-type (wt) α IIB as described above. The ability of α IIB β 3-positive cells to bind to soluble fibrinogen was measured in the presence and absence of activating anti- α IIB β 3 mAb PT25-2 (A), and the activation index was calculated (B). The Cys¹⁷⁷ → Ser and Cys¹⁸⁴ → Ser mutants were inactive (shown by *). The Cys²³³ → Ser and Cys²⁷³ → 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 Cys⁵⁶⁰ 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 (Cys³⁹⁸ → Tyr) [11], disrupting the Cys⁴³⁵–Cys⁵ disulphide bond [13] or disrupting the Cys⁶⁶³–Cys⁶⁸⁷ disulphide bond [14], constitutively activates α IIB β 3. It has been reported that disruption of a disulphide bond between Cys⁴⁰⁶ and Cys⁶⁵⁵ does not affect α IIB β 3 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 β 3 and studied their effects on the ligand-binding function of α IIB β 3. 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 α IIB β 3 are clustered within the EGF domains. Mutating cysteine residues outside the EGF domains did not induce activation of α IIB β 3, with few exceptions. These results suggest that disulphide bonds in the EGF domains keep α IIB β 3 in an inactive form, and disruption of a single disulphide bond in the EGF domain is enough to induce activation of α IIB β 3. It is suggested that disulphide bonds in the EGF domains should be completely intact when α IIB β 3 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 $\beta 3$ 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 $\alpha \text{IIb}\beta 3$. Further biochemical studies will be required to test whether the cysteine residues outside the EGF domain of $\beta 3$ are involved in regulation of the integrin.

The $\alpha \nu \beta 3$ 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 $\alpha \nu$ 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 $\beta 3$ is disrupted.

We thank J. Ylänne for providing valuable reagents. This work is supported by the NIH (National Institutes of Health) grant GM47157 (to Y.T.) and the US Army DAMD17-97-1-7105 grant (to T.K.).

REFERENCES

- Hynes, R. O. (1992) Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**, 11–25
- Shattil, S. J., Kashiwagi, H. and Pampori, N. (1998) Integrin signaling: the platelet paradigm. *Blood* **91**, 2645–2657
- Phillips, D. R., Charo, I. F., Parise, L. V. and Fitzgerald, L. A. (1988) The platelet membrane glycoprotein IIb–IIIa complex. *Blood* **71**, 831–843
- Kouns, W., Steiner, B., Kunicki, T., Moog, S., Jutzi, J., Jennings, L., Cazenave, J. and Lanza, F. (1994) Activation of the fibrinogen binding site on platelets isolated from a patient with the Strasbourg I variant of Glanzmann's thrombasthenia. *Blood* **84**, 1108–1115
- Essex, D. W. and Li, M. (2003) Redox control of platelet aggregation. *Biochemistry* **42**, 129–136
- Xiong, J.-P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Andrzej, J., Goodman, S. L. and Arnaout, M. A. (2001) Crystal structure of the extracellular segment of integrin $\alpha \nu \beta 3$. *Science* **294**, 339–345
- Calvete, J. J., Henschen, A. and Gonzalez-Rodriguez, J. (1991) Assignment of disulphide bonds in human platelet GPIIIa. *Biochem. J.* **274**, 63–71
- Yan, B., Hu, D. D., Knowles, S. K. and Smith, J. W. (2000) Probing chemical and conformational differences in the resting and active conformers of platelet integrin $\alpha \text{IIb}\beta 3$. *J. Biol. Chem.* **275**, 7249–7260
- Yan, B. and Smith, J. W. (2000) A redox site involved in integrin activation. *J. Biol. Chem.* **275**, 39964–39972
- Yan, B. and Smith, J. W. (2001) Mechanism of integrin activation by disulfide bond reduction. *Biochemistry* **40**, 8861–8867
- Chen, P., Melchior, C., Brons, N. H., Schlegel, N., Caen, J. and Kieffer, N. (2001) 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. *J. Biol. Chem.* **276**, 38628–38635
- Ruiz, C., Liu, C. Y., Sun, Q. H., Sigaud-Fiks, M., Fressinaud, E., Muller, J. Y., Nurden, P., Nurden, A. T., Newman, P. J. and Valentin, N. (2001) A point mutation in the cysteine-rich domain of glycoprotein (GP) IIIa results in the expression of a GPIIb–IIIa ($\alpha \text{IIb}\beta 3$) integrin receptor locked in a high-affinity state and a Glanzmann thrombasthenia-like phenotype. *Blood* **98**, 2432–2441
- Sun, Q. H., Liu, C. Y., Wang, R., Paddock, C. and Newman, P. J. (2002) Disruption of the long-range GPIIIa Cys⁵-Cys⁴³⁵ disulfide bond results in the production of constitutively active GPIIb–IIIa ($\alpha \text{IIb}\beta 3$) integrin complexes. *Blood* **100**, 2094–2101
- Butta, N., Arias-Salgado, E. G., Gonzalez-Manchon, C., Ferrer, M., Larrucea, S., Ayuso, M. S. and Parrilla, R. (2003) Disruption of the $\beta 3$ 663–687 disulfide bridge confers constitutive activity to $\beta 3$ integrins. *Blood* **102**, 2491–2497
- Wang, R., Peterson, J., Aster, R. H. and Newman, P. J. (1997) Disruption of a long-range disulfide bond between residues Cys⁴⁰⁶ and Cys⁶⁵⁵ in glycoprotein IIIa does not affect the function of platelet glycoprotein IIb–IIIa. *Blood* **90**, 1718–1719
- Ylänne, J., Hormia, M., Jarvinen, M., Vartio, T. and Virtanen, I. (1988) Platelet glycoprotein IIb/IIIa complex in cultured cells: localization in focal adhesion sites in spreading HEL cells. *Blood* **72**, 1478–1486
- Tokuhira, M., Handa, M., Kamata, T., Oda, A., Katayama, M., Tomiyama, Y., Murata, M., Kawai, Y., Watanabe, K. and Ikeda, Y. (1996) A novel regulatory epitope defined by a murine monoclonal antibody to the platelet GPIIb–IIIa complex ($\alpha \text{IIb}\beta 3$ integrin). *Thromb. Haemostasis* **76**, 1038–1046
- Deng, W. P. and Nickoloff, J. A. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* **200**, 81–88
- Kamata, T., Irie, A. and Takada, Y. (1996) Critical residues of the αIIb subunit of integrin $\alpha \text{IIb}\beta 3$ (GPIIb–IIIa) for binding to fibrinogen and ligand-mimetic antibodies (PAC-1, OP-G2 and LJ-CP3). *J. Biol. Chem.* **271**, 18610–18615
- Xia, Z., Wong, T., Liu, Q., Kasirer-Friede, A., Brown, E. and Frojmovic, M. M. (1996) Optimally functional fluorescein isothiocyanate-labelled fibrinogen for quantitative studies of binding to activated platelets and platelet aggregation. *Br. J. Haematol.* **93**, 204–214
- Goto, S., Salomon, D. R., Ikeda, Y. and Ruggeri, Z. M. (1995) Characterization of the unique mechanism mediating the shear dependent binding of soluble von Willebrand factor to platelets. *J. Biol. Chem.* **270**, 23352–23361
- Hughes, P., O'Toole, T., Ylänne, J., Shattil, S. and Ginsberg, M. (1995) The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. *J. Biol. Chem.* **270**, 12411–12417
- Hughes, P. E., Shipley, T., O'Toole, T. E. and Shattil, S. (1994) The membrane-proximal region in the $\beta 3$ cytoplasmic domain of integrin $\alpha \text{IIb}\beta 3$ plays a key role in affinity modulation. *Mol. Biol. Cell* **5**, 183a
- Ambo, H., Kamata, T., Handa, M., Taki, M., Kuwajima, M., Kawai, Y., Oda, A., Murata, M., Takada, Y., Watanabe, K. and Ikeda, Y. (1998) Three novel integrin $\beta 3$ subunit missense mutations (H280P, C560F, and G579S) in thrombasthenia, including one (H280P) prevalent in Japanese patients. *Biochem. Biophys. Res. Commun.* **251**, 763–768
- O'Toole, T., Loftus, J., Du, X., Glass, A., Ruggeri, Z., Shattil, S., Plow, E. and Ginsberg, M. (1990) Affinity modulation of the $\alpha \text{IIb}\beta 3$ integrin (platelet GPIIb–IIIa) is an intrinsic property of the receptor. *Cell Regul.* **1**, 883–893
- Takagi, J., Kamata, T., Meredith, J., Puzon-McLaughlin, W. and Takada, Y. (1997) Changing ligand specificities of $\alpha \nu \beta 1$ and $\alpha \nu \beta 3$ integrins by swapping a short diverse sequence of the β subunit. *J. Biol. Chem.* **272**, 19794–19800
- Puzon-McLaughlin, W., Kamata, T. and Takada, Y. (2000) Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding sites in integrin $\alpha \text{IIb}\beta 3$. *J. Biol. Chem.* **275**, 7795–7802
- Takagi, J. and Springer, T. A. (2002) Integrin activation and structural rearrangement. *Immunol. Rev.* **186**, 141–163

Received 6 November 2003; accepted 22 December 2003

Published as BJ Immediate Publication 22 December 2003, DOI 10.1042/BJ20031701