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Function and conformation analyses of an aspartate substitution of the invariant glycine in the integrin β I domain α 1- α 1' helix

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ABSTRACT

We showed that the α L β 2 integrin with the non-functional mutation G150D cannot be induced with Mg/EGTA to express the mAb KIM127 epitope, which reports the leg-extended conformation. We extended the study to the α IIb β 3, an integrin without an α I domain. The equivalent mutation, i.e. G161D, also resulted in an expressible, but non-adhesive α IIb β 3 integrin. An NMR study of synthetic peptides spanning the α 1- α 1' helix of the β 3 I domain shows that both wild-type and mutant peptides are α -helical. However, whereas in the wild-type peptide this helix is continuous, the mutant presents a discontinuity, or kink, precisely at the site of mutation G161D. Our results suggest that the mutation may lock integrin heterodimers in a bent conformation that prevents integrin activation via conformational extension.

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1. Introduction

Integrin heterodimers are type-1 transmembrane glycoproteins which mediate firm adhesion between cells and between cells and the extracellular matrix. Each integrin is a non-covalently associated heterodimer of one α and one β subunit [1]. The integrins can be classified into two types depending on the presence of an I domain (α I) in α subunit head region. In human, nine out of 18 α subunits contain the α I domain. When present, the α I is the major ligand binding domain. When absent, ligands will bind to a site at the interface formed by the β -propeller domain of α subunit and I domain of β subunit (β I) [2].

Integrins at the cell surface undergo drastic structural rearrangements from the resting state to the fully activated state upon stimulation, as elucidated by evidence from electron microscopy [3–5] and X-ray crystallography [6–10]. The integrins assume a compact bent form in their resting state which has low binding affinity to ligands. The intermediate state, with medium binding affinity to ligands, is by way of a leg extension. Finally, the swing-out of the hybrid domain of integrin β subunit with respect to the headpiece converts the integrin to the fully activated state with high binding affinity to ligands [11].

Abbreviations: EGTA, ethylene glycol tetra-acetic acid; WT, wild-type; NOE, Nuclear Overhauser Effect; I-EGF, Integrin-Epidermal Growth Factor; HEK, Human Embryonic Kidney; Asp, Aspartic Acid; Gly, Glycine.

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Extensive mutagenesis studies have shown the contribution of various regions of the integrin molecule to the structural rearrangements associated with activation. Of relevance to this study, the α 1 and α 7 helices of the β I domain had shown to contribute to regulate the integrin activation. The swing-out of the hybrid domain is triggered by a downward movement of the α 7 helix and a corresponding upward movement of α 1 helix of β I domain [5]. More specifically, the α 1 helix of the β I domain undergoes a transformation from a split α 1 helix (referred to as the α 1 and α 1' helices) to a continuous helix upon activation [12]. Mutation at the disrupted region of the α 1 helix had been shown to abolish the binding of the α IIb β 3 integrin to the ligand mimic antibody IgM PAC-1 [13].

We have previously reported the in vitro functional analysis of a G150D mutation in the integrin β 2 subunit in a patient with leukocyte adhesion deficiency 1 (LAD-1) [14]. This missense mutation did not affect the cell surface expression of the α L β 2, α M β 2 and α X β 2 integrins, but their ligand binding activities were totally abolished under the stimulations with the activating mAb KIM185, in the presence of Mg/EGTA, or Mn²⁺. Structural analysis of the wild-type (with Gly-150) and mutant (with Asp-150) α 1 peptides by NMR showed that the mutant peptide could not assume a continuous helical structure as compared to the wild-type peptide. In this study, we analyzed the effect of the equivalent mutation in the α IIb β 3 integrin to see if it would affect the integrin without an α I domain in the same way. Furthermore, we showed that the loss of adhesion property of the α L β 2 integrin caused by the G150D mutation may be related to its failure to undergo leg extension upon Mg/EGTA activation.

2. Material and methods

2.1. Antibodies and reagents

The hybridoma of mAb MHM23 ($\beta 2$ integrin heterodimer specific) [15] was a gift from Prof. AJ McMichael (John Radcliffe Hospital, UK). The hybridoma of conformation reporter mAb KIM127 [16] was kindly provided by Dr. MK Robinson (UCB, Cell-Tech, UK). mAbs were purified from respective hybridoma supernatant using Hi-Trap protein G/A columns (GE Healthcare Life Sciences). mAb 7E3 ($\beta 3$ functional blocker) was obtained from Prof. Barry Collier (Rockefeller University, NY, USA). mAb HIP8 (anti- $\alpha 11b$ mAb) was purchased from eBioscience. FITC-conjugated sheep anti-mouse IgG and fibrinogen from human plasma were both purchased from Sigma.

2.2. Plasmids

The pcDNA3.0 expression plasmids carrying αL , $\alpha 11b$, $\beta 2$ and $\beta 3$ cDNA were constructed as previously described [17]. All mutants were created using SDM (site-directed mutagenesis) method provided by Stratagen. All mutations were verified by sequencing (Axil Scientific, Singapore). The numbering of amino acids of integrin subunits started at the initiation methionine.

2.3. Cell culture and transfection

HEK293T (Human embryonic kidney 293T) cells were bought from ATCC. The cells were maintained in high-glucose DMEM (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin (Hyclone) at 37 °C incubator with 5% CO₂ in air. HEK293T were transiently transfected with relevant expression plasmids by PEI (polyethylenimine), as described previously [18].

2.4. Immunoprecipitation

HEK293T transfected with the $\alpha L\beta 2$ integrin or its mutants were labeled with 0.5 mg/ml EZ-link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) on cells surface. Labeled cells were collected and incubated with quadruplet conditions: mAb MHM23; mAb KIM127 with or without $\alpha L\beta 2$ activator (Mg/EGTA: 5 mM MgCl₂ plus 1.5 mM EGTA); and irrelevant mAb LPM19c for 30 min at 37 °C. After that, cells were washed and lysed in lysis buffer (10 mM Tris, 150 mM NaCl, 0.15 mM CaCl₂, 0.5 mM MgCl₂, 1% (v/v) Nonidet P-40, pH 8.0) for 20 min on ice. Cell lysates were incubated with 25% protein-A Sepharose (GE healthcare) beads which were pre-coated with rabbit anti-mouse IgG (Sigma) for 3 h at 4 °C. Beads were washed for 3 times. Bound proteins were eluted and loaded on SDS acrylamide gel under reducing condition before electroblotting onto PVDF membrane. Blot was incubated with streptavidin conjugated HRP (GE healthcare) at RT for 1 h followed by ECL detection method.

2.5. Flow cytometry

Integrin expression at cell surface was described previously [19]. Briefly, transfected cells were incubated with $\alpha 11b$ specific mAb HIP8 on ice for 1 h before labeling with FITC-conjugated IgG. Negative control was acquired by using an irrelevant primary mAb (αL specific). Stained cells were collected by BD FACSCalibur (BD Biosciences, USA), followed by data plotting using Flowjo (Tree Star).

2.6. Cell adhesion assay

150 ng fibrinogen in bicarbonate buffer (50 mM, pH 9.2) was coated onto each 96-well followed by incubating at 37 °C for 3–4 h. 1% (w/v) BSA was used to block the non-specific sites at 37 °C for 0.5 h. Coated plate was washed twice before adding cells. Transfected 293T were labeled by 1 μ g/ml BCECF (Invitrogen) at 37 °C for 20 min, followed by transferring to coated plate and incubating at 37 °C for 30 min with or without activator (0.5 mM Mn²⁺) or blocker (mAb 7E3). The fluorescence signal which indicates the ligand-bound cells of each well was measured using a FL600 plate reader (Bio-Tek instruments, Winooski, USA).

2.7. NMR data collection and structure calculation

23-mer peptides of wildtype and mutant were synthesized by Peptide Synthesis Core Facility (SBS, NTU). NMR were performed at 298 K using an Avance II 700 NMR spectrometer with cryogenic probe as previously described [14]. Briefly, peptide samples were dissolved in 0.5 mM glucose in PBS at pH 7.4. TOCSY (total correlation spectroscopy) and NOESY (nuclear overhauser effect spectroscopy) experiments were performed to obtain sequence-specific resonances. NOE distance restraints obtained from NOESY spectra were used for structure calculation. A summary of structure statistics based on 20 structures can be found in the Supplementary Table 1.

3. Results

3.1. G150D mutation in $\beta 2$ prevents integrin $\alpha L\beta 2$ activation via conformational extension

We have previously shown that the mutation of G150D, found in a patient with LAD-1, had no effect on the expression of the $\beta 2$ integrins on transfectants, but the cells were not able to adhere to ligand coated surfaces in the presence of the activating agents i.e. Mg/EGTA or the antibody KIM185 [14]. Here we made use of the reporter antibody KIM127, which was previously shown to bind to the extended form of $\alpha L\beta 2$ integrin [20,21]. In the presence of Mg/EGTA, KIM127 precipitated the wild-type but not $\alpha L\beta 2$ -G150D. As a positive control, the constitutively active mutant $\alpha L\beta 2$ -N351S [20] was precipitated by KIM127 even in the absence of Mg/EGTA (Fig. 1).

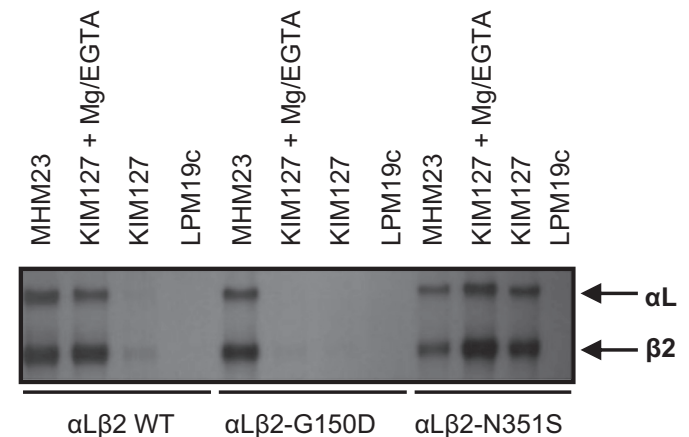


Fig. 1. Immunoprecipitation of biotin labeled $\alpha L\beta 2$ integrin on transfected HEK293T cells by reporter mAb KIM127. The irrelevant mAb LPM19c (anti- αM) and heterodimer specific mAb MHM23 were used as negative and positive control, respectively.

3.2. G161D mutation in $\beta 3$ abolished the fibrinogen-binding property of integrin $\alpha \text{IIb}\beta 3$

Integrin $\alpha \text{L}\beta 2$ contains an αI domain. To test the functional generality of this substitution, we also examined the effect of the corresponding mutation G161D in $\beta 3$ on the ligand-binding properties of integrin $\alpha \text{IIb}\beta 3$ which does not contain an αI domain. HEK293T cells were transfected with wild-type αIIb and wild-type $\beta 3$ or $\beta 3$ -G161D expression plasmids. The G161D mutation did not affect expression of the $\alpha \text{IIb}\beta 3$ integrin, but the transfectants were not able to adhere to fibrinogen coated surfaces even in the presence of 0.5 mM MnCl_2 (Fig. 2).

3.3. The Asp disrupted the helical structure of $\beta 3$ I domain $\alpha 1$ - $\alpha 1'$ helix

We next synthesized two peptides (23-mer) spanning the entire $\alpha 1$ and $\alpha 1'$ helix of the $\beta 3$ integrin subunit, one representing the wild type (Gly-161) and the other being mutant G161D. The structural conformations of the two peptides were analyzed by NMR. Overlays of the 20 lowest energy conformations of the two peptides are shown in Supplementary Fig. 1. While the wild-type peptide is represented by a continuous α -helix in all models, the mutant peptide exhibits two α -helical stretches separated by a

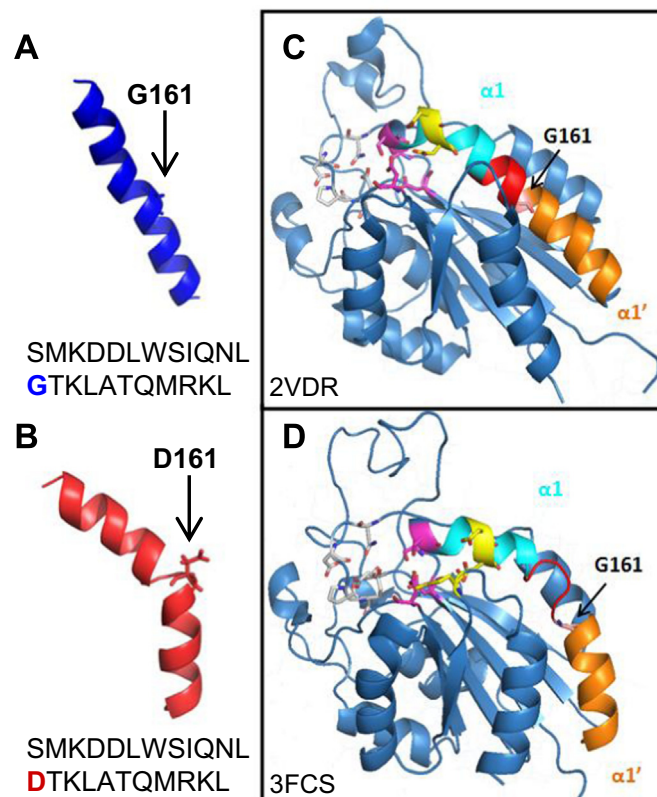


Fig. 3. The NMR structures of wild-type $\beta 3$ peptide spanning the entire $\alpha 1$ and $\alpha 1'$ helices (A) and that of the mutant peptide with G161D (B). The liganded $\beta 3$ I domain structure (C) (extracted from PDB ID: 2VDR) [23], and the un-liganded $\beta 3$ I domain structure (D) (extracted from PDB ID: 3FCS) [7]. Residues coordinating SyMBs were labeled in white, those coordinating MIDAS and ADMIDAS were labeled in magenta and yellow respectively.

kink at the mutated residue Asp. For clarity, only representative structures of each of the two peptides are shown in Fig. 3A and B. The kink of the mutant peptide is supported by several NOEs such as that between H β 2 of Asp-161 and H β ^N of Leu-164 and that between the H β ^N of Asp-161 and H β ^N of Leu-164 (Supplementary Fig. 2A). An equivalent NOE was absent between the H β ^N of Gly-161 and the H β ^N of Leu-164 was absent in the spectra corresponding to the wild-type $\beta 3$ peptide (Supplementary Fig. 2B).

4. Discussion

Herein, we have extended our study on the effect of the G150D mutation in $\beta 2$ on the $\alpha \text{L}\beta 2$ integrin. The mutant $\alpha \text{L}\beta 2$ cannot be immunoprecipitated by the reporter mAb KIM127 in the presence of Mg/EGTA. The epitope of mAb KIM127 was mapped to the I-EGF2/3 domain [22], and is not expressed in the bent resting state but only after activation, e.g. with Mg/EGTA [20]. We have also included the constitutively active mutant with the N351S mutation in $\beta 2$, and it can be immunoprecipitated with KIM127 even in the absence of Mg/EGTA [20].

The glycine (position 150 in $\beta 2$) is conserved in all β integrin subunits. We therefore studied the effect of the equivalent mutation in $\beta 3$ on the $\alpha \text{IIb}\beta 3$ integrin, an integrin without an αI domain. Similar results were obtained, i.e. the G161D mutation in $\beta 3$ supported $\alpha \text{IIb}\beta 3$ expression but not adhesion to fibrinogen coated surfaces. In 2013, Zhang et al. published an extensive study on the unbending of $\alpha 1/\alpha 1'$ as an activation mechanism [13]. A series of mutants at the G161 in the $\beta 3$ integrin, and the resultant $\alpha \text{IIb}\beta 3$ integrins showed mixed binding

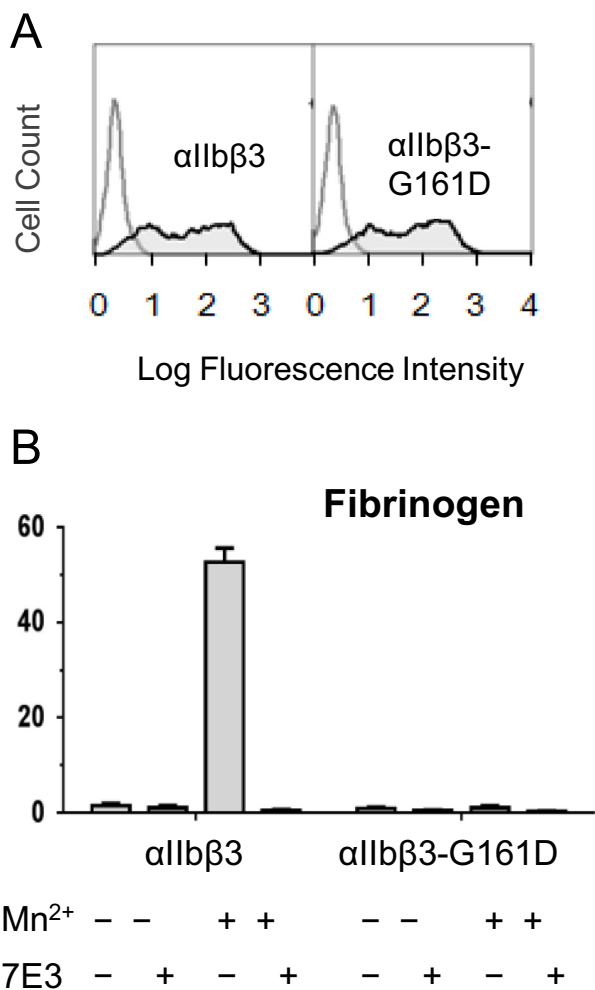


Fig. 2. Expression (A) and fibrinogen adhesion (B) of integrin $\alpha \text{IIb}\beta 3$ -G161D transfectants. (A) Anti- αIIb mAb HIP8 at 10 $\mu\text{g}/\text{ml}$ was used to detect the surface expression of transfected integrins. (B) Mn^{2+} at 0.5 mM was used to activate the binding, $\beta 3$ functional blocking mAb 7E3 was used to establish the adhesion specificity.

properties to the soluble ligand mimetic IgM PAC-1. Interestingly, the binding activities of all the tested mutants that failed to respond to the activation of 0.2 mM Ca^{2+} and 2 mM Mn^{2+} , can be restored by the introduction of the tail-separation mutant F1030A or R1032D in the αIIb subunit. However, none of the constructs contain the G161D mutation. In our hands, we tried to introduce the constitutively active N351S mutation into the G150D mutant in the $\beta 2$ integrin. However, the double mutant cannot be expressed onto the transfectants' cell surface (data not shown).

Based on NMR analyses of $\beta 3$ integrin peptides containing either Gly-161 (wild-type) or Asp-161 (mutant), the former was represented by a continuous α -helix in all structures (Figure 3A and Supplementary Figure 1A), the latter exhibited two α -helical stretches separated by a kink at the mutated residue Asp (Figure 3B and Supplementary Figure 1B). These structures may be compared to those found in the X-ray crystal structures of the $\alpha\text{IIb}\beta 3$ integrin. In the structures of the resting or closed form of $\beta 3$ integrin (Fig. 3D; PDB ID: 3FCS), the $\alpha 1$ and $\alpha 1'$ helices are discontinuous and the Gly-161 is located in between the two α -helical stretches [7]. When the integrin is bound to the ligand peptide LGGAKQRGDV (Fig. 3C; PDB ID: 2VDR), the two helices become one continuous α -helix [23]. Indeed, the same conformation can be induced by three other ligand peptides HHLGGAKQAGDV (PDB ID: 2VDO), LGGAKQAGDV (PDB ID: 2VDP), and HHLGGAKQRGDV (PDB ID: 2VDQ) [23]. Under physiological condition, the $\alpha 1$ and $\alpha 1'$ helices in a full integrin background has the flexibility to make a transition from discontinuous to continuous helix in order to facilitate integrin conformational change from a compact structure to a stretched out one. The mutant peptide, which may be considered in an “unconstrained” condition, cannot assume a continuous α -helix. This leads us to hypothesize that the presence of the Asp in the mutant peptide would lock the integrin in the resting state. This is one of the many features of the current view in the overall conformational changes of integrin activation [12].

Conflict of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.06.013>.

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