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The integrin αL leg region controls the Mg/EGTA mediated activation of LFA-1

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Abstract

We have shown that Mg/EGTA (5 mM Mg\(^{2+}\) and 1.5 mM EGTA) could effectively promote the adhesion of integrin αLβ2 to its ligand ICAM-1 but could not promote that of the αMβ2 to denatured BSA. In order to determine the structural differences between αL and αM that specifically contribute to Mg/EGTA sensitivity, a series of αL/αM chimeras were constructed. Our results showed that αLβ2 with αM calf-1 domain completely lost the response to Mg/EGTA activation. In the reverse experiment, αMβ2 would require the presence of both the αL calf-1 and calf-2 domain to initiate the Mg/EGTA sensitivity.
Keywords

Divalent cation
Integrin activation
Domain swapping
Cell Adhesion

Abbreviations

EGTA, Ethylene glycol tetra-acetic acid; LFA-1, Leukocyte function associated antigen-1 (αLβ2); Mac-1, Macrophage antigen-1 (αMβ2); ICAM, Intercellular adhesion molecule; BSA, Bovine serum albumin; WT, Wild-type.

Highlights

- Addressed the difference of Mg/EGTA sensitivity in the activation between αLβ2 and αMβ2, by domain swopping.
- The leg region, rather than the headpiece causes the Mg/EGTA sensitivity.
- αL calf-1 domain is responsible for Mg/EGTA regulated activation.
- The presence of both calf-1 and calf-2 domains of αL restored the Mg/EGTA sensitivity of αMβ2.
1. Introduction

Integrins are a family of membrane glycoproteins that serve as bi-directional signaling molecules between the interior of the cell and its extracellular environment. Integrins are heterodimers, each member is consisted of one \( \alpha \) subunit and one \( \beta \) subunit, which associate with each other non-covalently \([1]\). In human, there are 24 integrin heterodimers formed by selective combinations from 18 different \( \alpha \) subunits and 8 \( \beta \) subunits. Of specific interest to us are the members of the \( \beta_2 \) integrin family. Four \( \alpha \) subunits, namely \( \alpha_L \), \( \alpha_M \), \( \alpha_X \), and \( \alpha_D \), combine with the common \( \beta_2 \) subunit to form a closed set of \( \alpha_L\beta_2 \), \( \alpha_M\beta_2 \), \( \alpha_X\beta_2 \), and \( \alpha_D\beta_2 \) integrins. They are expressed exclusively on leukocytes. Specifically, \( \alpha_L\beta_2 \), \( \alpha_M\beta_2 \), and \( \alpha_X\beta_2 \) are expressed on peripheral blood leukocytes and the regulation of their adhesion properties has been studied extensively \([2,3,4,5]\). Generally, these integrin molecules are at a resting state, i.e. incapable to mediate cell adhesion to their ligands.

Integrin activation is regulated bi-directionally. The inside-out signal can be from another receptor on the cell, such as the CD14 on monocytes and CD40 on B cells \([4]\). The signal is transmitted to the cytoplasmic tail of the \( \beta_2 \) subunit by intracellular proteins such as talin and kindlin \([6]\). Upon such interaction, the ectodomain of the integrin changes from a folded compact conformation to an extended and open conformation. Functionally, the open integrin can adhere to ligand with high affinity. Activation can also be induced from outside via ligand binding. The signal can be manifested to cellular activities such as cell spreading and cell migration. The \( \alpha \) subunits of the \( \beta_2 \) integrin family belong to the subset that has an inserted I-domain, which we shall refer to as the \( \alpha_I \) domain. For these integrins, the \( \alpha_I \) domain is the major ligand binding site \([7]\).
In the laboratory, the transition of integrin activity states can be studied by the manipulation of extracellular conditions: either by antibodies that promote the conformational change associated with activation, or by the alteration of the concentrations of divalent cations. \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) are capable in regulating the adhesion between leukocyte integrins and ligands, among which \( \text{Ca}^{2+} \) generally inhibits the ligand-receptor binding, whereas \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) generally promote the adhesion [8,9,10]. Divalent cation binding sites have been identified in the headpiece of the integrin heterodimer, i.e. in the \( \beta \)-propeller of the \( \alpha \) subunits, the \( \beta I \) domain of the \( \beta \) subunits, and also on the \( \alpha I \) domain for those \( \alpha \) subunits with this domain. As of today, the structure of the ectodomain of three integrin heterodimers had been solved by X-ray crystallography; they are the \( \alpha V\beta_3 \) [11], \( \alpha IIb\beta_3 \) [12] and \( \alpha X\beta_2 \) [13] integrins. For \( \alpha IIb \) and \( \alpha V \), a \( \text{Ca}^{2+} \) binding site has also been found in the genu region between the thigh and the calf-1 domain. However, this \( \text{Ca}^{2+} \) binding site was not identified in the \( \alpha X \) subunit.

It was discovered in 1992 that the high concentration of \( \text{Mg}^{2+} \) coupled with the depletion of \( \text{Ca}^{2+} \) by the inclusion of EGTA in the medium would activate LFA-1 from a resting to a ligand binding state [9]. In contrast, it appears that both \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) are required to activate Mac-1 to adhere to iC3b [8]. In this study, we focus to discern the difference of Mg/EGTA mediated activation between LFA-1 and Mac-1 and to identify the domain responsible for this difference.

2. Material and Methods

2.1. Antibodies and reagents
Hybridomas of mAbs were obtained from different sources: MHM24 (anti-αL blocking mAb) and MHM23 (β₂ heterodimer specific mAb) [14] were provided by Professor AJ McMichael (John Radcliff Hospital, Oxford, UK). KIM185 (anti-β₂ activating mAb) [15] was obtained from Dr. MK Robinson (UCB, CellTech, UK). LPM19c (anti-αM blocking mAb) [16] was purchased from ATCC. All mAbs were purified from hybridoma supernatant using Hi-Trap protein G or A columns (Amersham Pharmacia Biotech). Preparation of recombinant human ICAM-1-Fc was described previously [17]. Bovine serum albumin (fraction V, BSA) was bought from Sigma-Aldrich.

2.2 plasmids

The pcDNA3 expression plasmids containing αL, αM and β₂ cDNA were described previously [18]. Each domain swopping plasmid was constructed by a two-step PCR. In the 1<sup>st</sup> step, a pair of 64-70-mer primers containing donor sequences at 5’ tails that were ~30 bases in length and receptor sequences at 3’ tails that were ~35 bases in length was designed. A mega primer containing the domain of interest from the donor in the middle, and flanked by the receptor sequences were synthesized by PCR using Advantage® -GC cDNA Ploymerase Mix kit (ClonTech), followed the manufacturer’s instruction. The mega primer was obtained by gel purification. In the 2<sup>nd</sup> step, the purified mega primer was used to clone the chimera using Pfu Turbo polymerase (Stratagene). Setting of thermo cycling parameters was followed the manufacturer’s instruction with the exception of using a gradually decreased annealing temperature which dropped 1°C every 30 seconds from 76°C to 45°C. Subsequent procedures after PCR were followed by the instruction from the manufacturer. All constructs were verified by sequencing (Axil Scientific, Singapore).

2.3 Cell culture and transfection
Human embryonic kidney 293T (HEK293T) cells were purchased from ATCC, cells were cultured in DMEM (Hyclone) supplemented with 10% (v/v) heat-inactivated FBS (Hyclone), 100 IU/ml penicillin and 100 µg/ml streptomycin (Hyclone) at 37°C in 5% CO₂ humidified incubator. HEK293T cells were transfected with expression plasmids using polyethylenimine (PEI) according to a previously described method [19].

2.4. Flow cytometry

Surface expression detection was described previously [17] using a β₂ heterodimer specific mAb MHM23 followed by FITC-conjugated sheep anti-mouse IgG [20]. Background histograms were obtained using the LPM19c mAb (αM specific) for LFA-1, and MHM24 mAb (αL specific) for Mac-1. Stained cells were collected on a FACS Calibur (BD Biosciences). Data were analyzed using Flowjo (Tree Star).

2.5. Cell adhesion assay

For ICAM-1 adhesion, 100 µl of 5 µg/ml goat anti-human IgG (Fc specific) (Sigma-Aldrich) in bicarbonate buffer (50 mM, pH 9.2) was introduced into each well in 96-well microtiter plates (Polysorb, Nunc Immuno-Plate) and left overnight at 4°C. Non-specific sites were blocked with 130 µl of 0.5% (w/v) BSA in PBS for each well. 50 µl of ICAM-1-Fc in PBS with 0.1% BSA (w/v) at 1 µg/ml was added into each well and left at RT for 2 hours.

For BSA adhesion assay, 100 µl of 100 µg/ml BSA in bicarbonate buffer was coated on each well and the plate was left overnight at 4°C. Non-specific sites were blocked with 130 µl of 0.2% (w/v) polyvinylpyrrolidone (PVP) in PBS for each well. In both cases the wells were washed twice with wash buffer (RPMI1640 with 10 mM HEPES and 5% v/v FBS) before adding cells. Transfected cells were harvested, washed and incubated with 1 µg/ml BCECF (Invitrogen) in wash buffer at 37°C for 20 min. Labeled cells were transferred to each wells
with or without activation reagents (Mg/EGTA: 5 mM MgCl$_2$ and 1.5 mM EGTA; Mn$^{2+}$: 0.5 mM MnCl$_2$; mAb KIM185: 10 µg/ml) and incubated at 37°C for 30 min. To establish specificity, appropriate antibodies were added to block adhesion. Unbound cells were removed by washing twice with wash buffer. Fluorescence signal, which correlates with the number of cells adhering to the ligand-coated well, was measured using a FL600 fluorescence plate reader (Bio-Tek instruments, Winooski, VT).

3. Results

3.1 The effect of Mg/EGTA on αLβ$_2$ and αMβ$_2$ mediated adhesion

Manipulation of divalent cation concentrations, generally Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$, in the cell medium had been found to affect their integrin mediated adhesion to ligands. Mn$^{2+}$, at sub mM level, has been shown to promote αLβ$_2$ and αMβ$_2$ mediated adhesion to their ligands. However, the requirements for Mg$^{2+}$ and Ca$^{2+}$ are different for the two integrins. It was shown that both Mg$^{2+}$ and Ca$^{2+}$ are required for αMβ$_2$ mediated adhesion [8,21] whereas Ca$^{2+}$ is inhibitory to αLβ$_2$ mediated adhesion [9]. This observation is confirmed in an experiment shown in Figure 1. αLβ$_2$ and αMβ$_2$ transfectants were assayed for their adhesion to their respective ligands ICAM-1 and denatured BSA. Adhesion can be promoted for both transfectants with the β$_2$-specific activating mAb KIM185, αLβ$_2$ transfectants, but not the αMβ$_2$ can be activated by Mg/EGTA. Since the two integrins share the common β$_2$ subunit, it is logical to argue that this difference is due to certain structural features that are different in αL and αM subunits.

3.2 αL calf-1 domain is required for Mg/EGTA mediated activation
A series of αL/αM chimera were constructed such that the domains of the leg region of the αM subunit were into the αL background. These chimeras, together with the β2 subunit, were co-transfected into HEK293T cells. Expression of the chimeric integrins was monitored by flow cytometry using the mAb MHM23, and adhesion to ICAM-1 was performed. The results are shown in Figure 2. Chimeras with the calf-1 domain of αM would render the integrin αMβ2-like, in that Mg/EGTA cannot stimulate the adhesion of the transfectants to ICAM-1.

Significantly, the integrin with only the αM calf-1 domain on an αLβ2 background is not responsive to Mg/EGTA stimulation, suggesting that Mg/EGTA response element is located in the calf-1 domain of αL subunit.

3.3 The restoration of Mg/EGTA mediated activation on αMβ2

The converse series of chimera were constructed where domains of the leg region of αL were introduced into the αM background. Mg/EGTA stimulation can be demonstrated if the region C-terminal and inclusive of the calf-1, calf-2 and cytoplasmic segment is from αL. Replacement with just the calf-1 or calf-2 region with those of αL result in integrins that are constitutively active in binding to the αMβ2 ligand denatured BSA. In these cases, Mg/EGTA stimulation cannot be determined. Nevertheless, these results do not contradict those obtained with the series with αL background.

4. Discussion

The requirement of divalent cations for integrin-mediated adhesion has long been investigated [2,10,22,23]. However, the distinct divalent cation requirements of αLβ2 and αMβ2 had not been addressed. Some studies had chose to add to the cell medium with millimolar level of EGTA and MgCl2 as a standard condition for αLβ2-mediated adhesion
[24,25,26,27,28], while millimolar level of MnCl$_2$ was used to promote $\alpha$M$\beta_2$ mediated adhesion [27,29,30]. In this article we have established, Mg/EGTA is not able to activate $\alpha$M$\beta_2$ transfectants to adhere to denatured BSA.

We argued that the functional difference must be conferred by the $\alpha$L and $\alpha$M subunit. We therefore constructed a series of chimeric $\alpha$L/$\alpha$M subunit and pinpoint the domain that is responsible for this functional difference. In the series using $\alpha$L as the background, we found that replacement of the calf-1 domain with that of $\alpha$M would abolish the activation by Mg/EGTA. The reverse series was less clear-cut. Using an $\alpha$M background, we can only demonstrate that the Mg/EGTA sensitivity can be observed if both the calf-1 and calf-2 of $\alpha$L were introduced. Introduction of either calf-1 or calf-2 did not yield any informative results as the integrins with these two $\alpha$L/$\alpha$M chimeras are constitutively active. Thus the effect of Mg/EGTA cannot be determined. This is not a totally unexpected result. Keeping an integrin in a resting state would require the coordinated interactions of its domains. These interactions would be disrupted if segments from another integrin were introduced to replace the corresponding segments. It was demonstrated that if we replaced the leg region of the $\beta_2$ subunit with that of $\beta_1$, the resultant $\alpha$L$\beta_{2/1}$ chimera would be constitutively active [31]. Indeed, the $\alpha$X$\beta_2$ integrin was shown to be constitutively active integrins if the $\alpha$X subunit was from human and $\beta_2$ subunit from chicken [32].

Similar observation was found with the $\alpha$V$\beta_3$ and $\alpha$IIb$\beta_3$ integrins, in which 1 mM Mn$^{2+}$ was able to activate $\alpha$V$\beta_3$, but not $\alpha$IIb$\beta_3$, to bind fibrinogen. Using the similar domain swopping strategy, the domain responsible for Mn$^{2+}$ sensitivity was located to the calf-2 domain of $\alpha$V [33]. The ectodomain structures of both $\alpha$V$\beta_3$ and $\alpha$IIb$\beta_3$ had been solved, but no divalent cation (and such binding site) was found in the calf-2 domain [11,12]. In both the
αV and αIIb subunits, a Ca$^{2+}$ ion was found to be chelated with the residues in the genu and the calf-1 domain [11,12]. However, such Ca$^{2+}$ ion binding site was not found in the αXβ2 integrin [13], although the potential residues can be identified when aligned with those in the αV and αIIb subunits. On detailed analyses of the structure, it was concluded that the backbone structure of αX is sufficiently different such that the geometry is not appropriate to facilitate Ca$^{2+}$ binding [13].

αL is different from αX, which is more closely related to αM. Thus, it is not possible to predict if such a divalent binding site is present in αL. We have attempted to express the various combinations of the domains in leg region of αL and αM (from thigh to calf-2) but with no success. Until such information becomes available, it will remain a puzzle as to why the two integrins αLβ2 and αMβ2 would respond differently to the exposure to Mg/EGTA.

It was widely believed that the divalent cation binding sites at integrin αI domain and βI domain are the sites that account for the divalent cation mediated integrin adhesion. However, mutation on these sites, namely, MIDAS of αI, MIDAS, ADMIDAS and SyMBS of βI usually lead to completely abolished ligand binding or even surface expression [34,35,36,37]. Our results, together which those published by Kamata et al [34] on the αIIbβ3 and αVβ3 integrins, suggest that there may be a remote action of the α subunit leg domain that controls the divalent cation binding sites in the headpiece. Alternatively, there may be other yet to be identified divalent cation binding site in the leg region.

Acknowledgements

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Figure Legends

Fig. 1. Adhesion of integrin $\alpha$L$\beta_2$ (A) and $\alpha$M$\beta_2$ (B) transfectants binding to their respective ligands ICAM-1 and denatured BSA, in the presence of Mg/EGTA. 10 $\mu$g/ml activating mAb KIM185 was included as a positive control.

Fig. 2. Upper panel: Adhesion of wild-type $\alpha$L$\beta_2$ and chimeric $\alpha$LM$\beta_2$ integrins to ICAM-1 with no activating agents (white), in the presence of Mg/EGTA (grey), and the activating mAb KIM185 (black). For each condition, the mAb MHM24, at 10 $\mu$g/ml, was used to block the adhesion and shown on the right. Middle panel: Expression profile of the wild-type and chimeric integrins using the mAb MHM23 ($\beta_2$ heterodimer specific). Background histograms were obtained using the mAb LPM19c ($\alpha$M specific). Lower panel: domain organisation of the $\alpha$ subunits of the chimeric integrins – $\alpha$L domains in white, and $\alpha$M domains in black. A letter “I” is assigned to represent for the $\alpha$I domain, “$\beta$-P” represents for the $\beta$-propeller domain, “T” represents for the thigh domain, “1” represents for the calf-1 domain and “2” represents for the calf-2 domain.

Fig. 3. Upper panel: Adhesion of wild-type $\alpha$M$\beta_2$ and chimeric $\alpha$ML$\beta_2$ integrins to denatured BSA with no activating agents (white), in the presence of Mg/EGTA (grey), and the activating mAb KIM185 (black). For each condition, the mAb LPM19c, at 10 $\mu$g/ml, was used to block the adhesion and shown on the right. Middle panel: Expression profile of the wild-type and chimeric integrins using the mAb MHM23 ($\beta_2$ heterodimer specific). Background histograms were obtained using the mAb MHM24 ($\alpha$L specific). Lower panel: domain organisation of the $\alpha$ subunits of the chimeric integrins.
Reference


Fig. 1
Fig. 2

ICAM-1

% of Binding

WT  αLMt-y  αLMg  αLMt  αLMc-y  αLMc1  αLMc2

Schematic Expression
Fig. 3

[Graph showing % of Binding for different conditions: WT, aMLt, aMLg, aMLc-y, aMLc1, aMLc2. Bars representing BSA with schematic expression below each condition.]