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Biochemical and Biophysical Research Communications 337 (2005) 142–148
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Differential activation of LFA-1 and Mac-1 ligand binding domains

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Received 23 August 2005
Available online 15 September 2005

Abstract

Nine integrin α subunits contain an 'inserted' or I-domain, known to involve in ligand binding. Mutation of an invariant isoleucine residue in the I-domains of αL and αM has previously been reported to activate LFA-1 and Mac-1, respectively. In this article, we report notable differences in the regulation of adhesion of these two integrins. We find that mutation of the isoleucine residue in the proposed "socket for isoleucine" in full-length αL does not lead to an active LFA-1, although mutation of the equivalent residue in αM does convey constitutive activity to Mac-1. In addition, we observe the isolated I-domain of αL to be constitutively active. This challenges reports that state the αL I-domain exists in an inactive, closed conformation, and requires the presence of activating agents for ligand binding. These results shed further light on the many questions surrounding regulation of integrin activation.

Keywords: Integrin; Activation; I-domain

Integrins are a family of transmembrane proteins that mediate cell–cell and cell–matrix adhesion. Each integrin is a heterodimer composed of an α and a β subunit in non-covalent association [1]. αLβ2, αMβ2, αXβ2 and αDβ2 constitute the β2 subfamily of integrins that are exclusively expressed on leukocytes [2,3].

The α subunits contain a β-propeller structure formed by seven repeating elements [4]. Nine of the 18 α subunits, including the four α subunits that combine with β2, contain an I-domain inserted between the second and third blades of the β propeller. This I-domain has been shown to be important in ligand binding [5]. Crystal structures of recombinant I-domains of αL [6], αM [7,8], and αX [9] have shown that these domains assume a dinucleotide binding or Rossmann fold, with a central hydrophobic β sheet surrounded by six or seven amphipathic α helices. A metal ion-dependent adhesion site (MIDAS) is located at the upper face of the I-domain [6–10].

Two conformations for integrin I-domains were identified when the integrin αM I-domain was found to crystallize in two different conformations [8]. These are generally referred to as the open, and closed, conformers, with the open conformer having the capacity to bind ligands. Mutations that stabilize the open or closed conformers have been used to measure how transition between the two conformations is regulated. Many single-residue substitutions were introduced into the I-domain of the αL subunit [10]. The cDNA of αL variants were co-transfected into COS cells with the cDNA of β2, and the adhesion properties of the transfectants to ICAM-1 were studied. One such substitution was the isoleucine at position 331 (the initiation methionine residue being defined as the first residue). When the isoleucine was replaced with an alanine, the transfectants expressing this variant was found to be more adhesive to ICAM-1 than the wild-type [10].

This isoleucine is invariant among the integrin α subunits (Fig. 1A). Using the I-domain of αM as a model, it was found that the substitution of the isoleucine with a glycine would increase iC3b binding affinity both in the isolated I-domain and in the intact Mac-1 integrin [11]. The hydrophobic side chain finger of the isoleucine was described as fastened in the closed conformation by a conserved hydrophobic socket [11]. This "socket for

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doi:10.1016/j.bbrc.2005.08.269

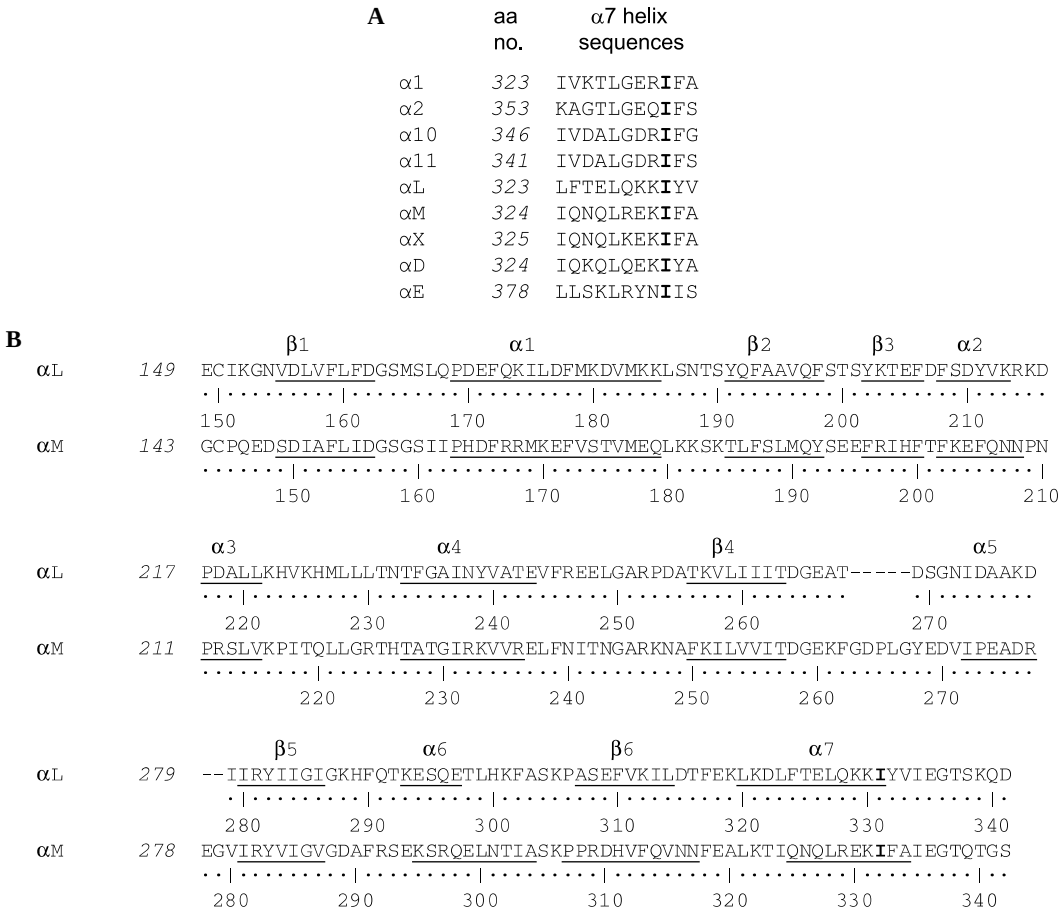

Fig. 1. Sequence alignment of integrin α subunit I-domains. (A) Alignment of α7 helices of all known human integrin α I-domains. Amino acid numbers (aa no.) of the first residues in each α7 helix are indicated in italics (in all cases, the initiating methionine is numbered 1). The invariant isoleucine in the α7 helices (Ile^{331} in the αL subunit and Ile^{332} in the αM subunit) are shown in bold. (B) Alignment of the I-domains of integrin αL and αM subunits. The Ile^{331} in αL and Ile^{332} in αM are shown in bold. Amino acids showing secondary structure (α-helices and β-sheets) are underlined. ClustalW [32] was used to align the sequences.

isoleucine," or SILEN, was postulated to be a key component in allosteric regulation, controlling affinity and shape shifting in the I-domain of Mac-1. By inference, the SILEN was suggested as the universal mechanism that regulates integrin conformation and function. This postulate seemed to have gained further support when similar findings were reported for the I-domain of the αX integrin subunit [9].

We reported previously that systematic truncation of integrin β2 leads to differential activation of LFA-1 and Mac-1 ligand binding capacity [12]. These results suggest differences in the regulation of these I-domains in the intact receptors. Indeed studies have been conducted to address how conformational changes in other regions of the integrin are propagated to result in the activation of the I-domain. Several models of integrin activation have been proposed, for example, pull-spring [13] and dead-bolt [14]. However, revelation of integrin activation, involving the I-domain, is limited by the lack of structural data for an intact integrin having an I-domain.

If integrin I-domains share a universal activation mechanism, we reason that other regions of the αL, αM or the β2 subunits must account for the differences observed in LFA-1 and Mac-1 ligand binding [12]. Alternatively, the integrin I-domains may have regulatory mechanisms which are different. To distinguish between these two possibilities we have constructed SILEN isoleucine mutants in full-length αL and αM, and the isolated I-domain of αL and have analyzed the ligand binding activity of these mutants.

Materials and methods

Reagents and antibodies. The monoclonal antibodies (mAbs) MHM24 (anti-αL) [15] and LPM19c (anti-αM) [16] were obtained as previously described [12,17]. IB4 (heterodimer specific) was reported previously [18]. KIM185 [19] and MEM48 [20] (anti-β2, activating mAbs) were obtained from Dr. M.K. Robinson (Celltech, Slough, UK) and Dr. V. Horejsi, Institute of Molecular Genetics, Prague, Czech Republic, respectively. Purified IgGs of MHM24 and LPM19c were prepared from hybridoma supernatants using Hi-Trap protein-G columns (Pharmacia Biotech, Uppsala, Sweden). ICAM-1/Fc was prepared as described previously [21].

cDNA expression constructs. The αL, αM, and β2 cDNA clones were reported previously [12,22]. The $Ile^{331} \rightarrow Gly$ mutation (I331G) in αL, and the $Ile^{332} \rightarrow Gly$ mutation (I332G) in αM were made using QuikChange Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's

protocols. To construct the isolated, cell surface-expressed αL I-domain, *Xma*I and *Sac*II sites were introduced immediately adjacent to the respective 5′ and 3′ ends of cDNA encoding residues Glu^{149} to Asp^{341} containing the αL I-domain (Fig. 1B). The *Xma*I–*Sac*II fragment was subcloned in frame 5′ to the c-myc tag and the platelet-derived growth factor receptor (PDGFR) transmembrane domain and the first five residues of the PDGFR cytoplasmic domain in the vector pDisplay (Invitrogen). All constructs were verified by sequencing (DNA Sequencing Facility, University of Oxford, UK; or Research Biolabs PTE LTD, Singapore).

Cell culture and transfection. 293T cells (human embryonic kidney fibroblast cell line, ATCC) were cultured in DMEM supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Constructs were transiently transfected into 293T cells using the calcium phosphate method [23,24]. Briefly, 3.5 μg of αL or αM cDNA (and 3.5 μg β2 cDNA, for transfections requiring both) was used to transfect one 35 mm dish of approximately 70% confluent cells. The following day, cells were detached with 0.5 mM EDTA in PBS for use in subsequent functional analysis.

K562 cells (human erythroleukaemic cell line, ATCC) were maintained in RPMI1640 supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Stable transfection was achieved using XtremeGene transfection reagent (Roche), according to the manufacturer's instructions. Briefly, 10 μg DNA was used to transfect 2.5×10^5 cells. After transfection K562 cells were grown in media containing 20% FBS and those containing the plasmid were maintained in G418 at 2 mg/ml.

Cell adhesion assay. Microtitre wells were coated with ICAM-1/Fc or BSA as described [12]. 293T transfectants were resuspended in wash buffer (RPMI-1640 with 5% FBS and 10 mM Hepes, pH 7.5) to 1×10^6 cells/ml. Fluorescent dye, 2′,7′-bis-(2-carboxyethyl)-5(and-6)carboxy fluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR), at 1 μg/μl in DMSO, was added to a final concentration of 1 μg/ml, and labelling was carried out at 37 °C for 20 min. The cells were washed once and dispensed into each well of the ligand-coated plates (2×10^4 cells/well). Cells were incubated with various combinations of activating and blocking reagents: mAb KIM185 (2.5 μg/ml), mAb MEM48 (10 μg/ml), and Mg/EGTA (5 mM $MgCl_2$ and 1.5 mM EGTA), mAb MHM24 (10 μg/ml), and mAb LPM19c (10 μg/ml). The plates were incubated at 37 °C for 30 min. The total number of cells in each well was quantified by their fluorescence using a fluorescence multi-well plate reader (CytoFluor4000; Applied Biosystems). Plates were washed three times with wash buffer and the fluorescence of bound cells was determined.

Flow cytometric analysis. Cells were incubated with 20 μg/ml primary mAb in RPMI 1640 for 1 h at 4 °C. The cells were then washed once and incubated with FITC-conjugated sheep anti-mouse $F(ab')^2$ secondary antibody (1:400 dilution; Sigma) for 45 min at 4 °C. Stained cells were washed once and fixed in 1% (v/v) formaldehyde in PBS. Cells were analysed on a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA).

Results

Mutation of I332G in the full-length integrin αM subunit shows constitutive ligand binding activity

293T cells were transfected with full-length wild-type αM or αM(I332G) in combination with wild-type β2. Cell surface expression of the wild-type and mutant Mac-1 was determined by flow cytometry to be comparable (Fig. 2A). The transfectants were assessed for their adhesion to denatured BSA [25] (Fig. 2B). The mutant I332G in the αM subunit yielded Mac-1 that was active.

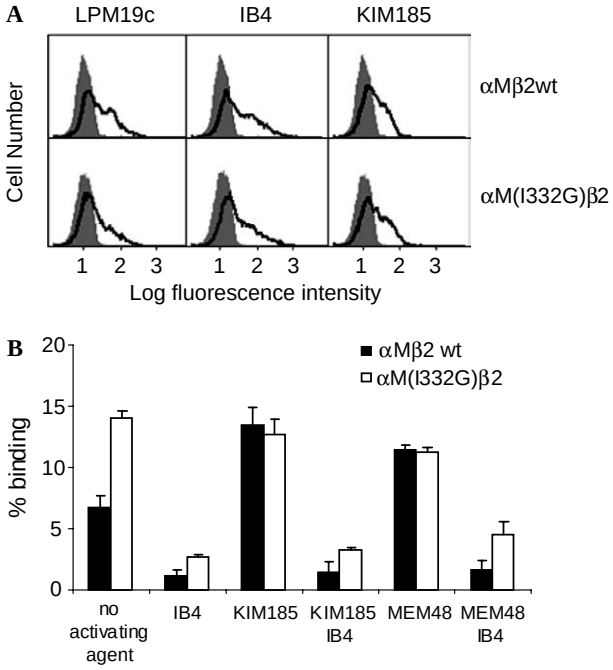

Fig. 2. αM I332G variant is confirmed to be constitutively active in binding to ligand. (A) Cell surface expression of wild-type (wt) αMβ2 and αM(I332G)β2 as determined by flow cytometry. Background histograms (shaded) were obtained using the mAb MHM24. The mAbs LPM19c, IB4 and KIM185 were used to detect expression of the αM subunit, heterodimer, and the β2 subunit respectively (solid lines). (B) Full-length αMβ2 wt and αM(I332G)β2 mutant binding to BSA. Binding of αM(I332G)β2 was 2-fold higher than that of wild-type αMβ2 in the absence of any activating antibodies. Binding of the wild-type αMβ2 was increased in the presence of mAbs KIM185 or MEM48 but αM(I332G)β2 could not be activated further. Binding to ligand was specific since it was abrogated in the presence of function-blocking heterodimer-specific mAb, IB4.

Ligand binding could not be further activated in the presence of either of the activating antibodies KIM185 or MEM48.

Mutation of I331G in the full-length integrin αL subunit has minimal effect on LFA-1 binding to ligand

We repeated the experiment with the mutation in LFA-1. 293T cells were transfected with full-length wild-type αL or αL(I331G) in combination with wild-type β2. Cell surface expression of wild-type αLβ2 was comparable to the αL(I331G)β2 as determined by flow cytometry (Fig. 3A). The transfectants were also assessed for their adhesion to ICAM-1 [26] (Fig. 3B). Both showed minimal basal activity in the absence of any activating agents, and both were activated to a similar degree when adhesion was carried out in the presence of the activating antibody KIM185, or Mg/EGTA, or both. Binding to ICAM-1 was specific since it was abrogated in the presence of function-blocking αL-specific mAb, MHM24. Similar results were obtained with COS-7 transfectants (data not shown).

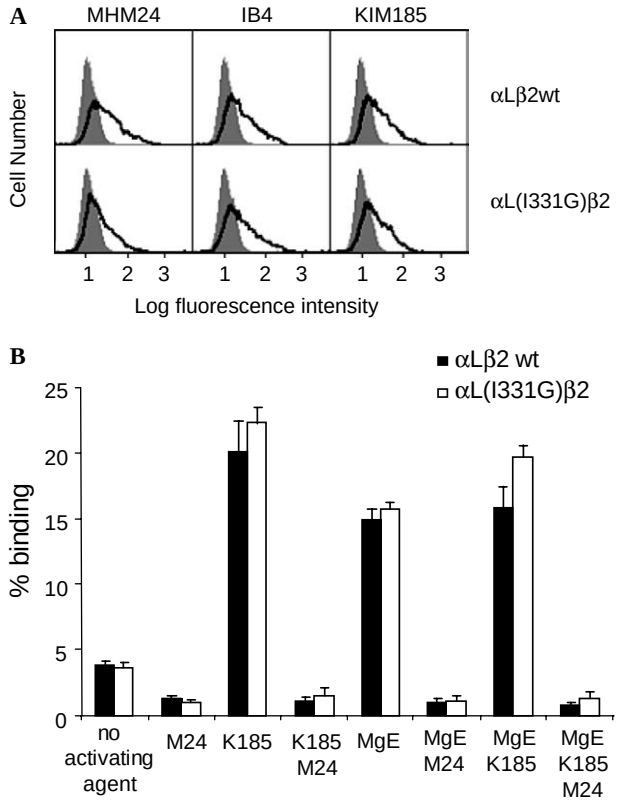

Fig. 3. αL I331G mutation shows minimal difference when binding to ligand ICAM-1 compared to wild-type αL. (A) Cell surface expression of wild-type (wt) αLβ2 and αL(I331G)β2 as determined by flow cytometry. Background histograms (shaded) were obtained using the mAb LPM19c. The mAbs MHM24, IB4 and KIM185 were used to detect expression of the αL subunit, heterodimer, and the β2 subunit, respectively (solid lines). (B) Full-length αLβ2 wt and αL(I331G)β2 mutant binding to ICAM-1. Both showed minimal basal activity in the absence of any activating agents, and both were activated to a similar level when adhesion was carried out in the presence of the activating antibody KIM185 (K185 in figure) or activating agents Mg/EGTA (MgE). Binding to ICAM-1 was specific since it was abrogated in the presence of function-blocking αL-specific mAb, MHM24 (M24 in figure).

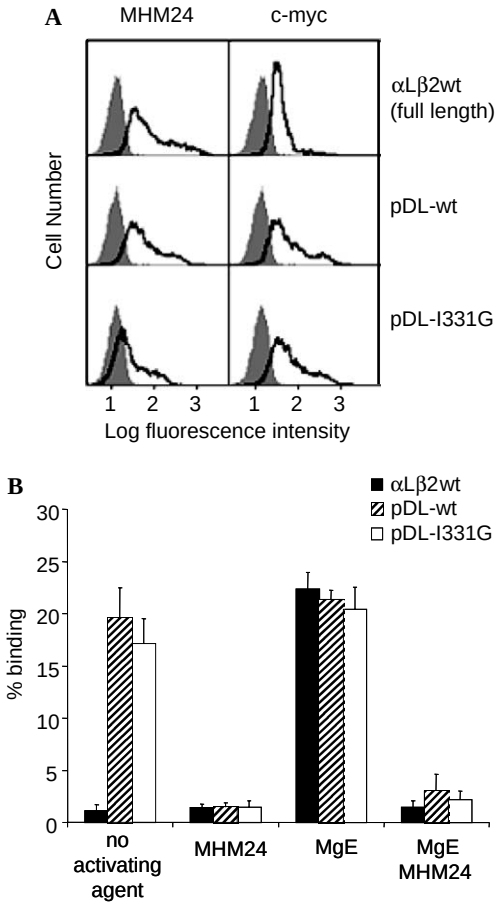

Fig. 4. Isolated wild-type αL I-domain and mutant αL I331G show constitutive ligand-binding activity in 293T cells. (A) Cell surface expression was comparable as determined by FACS using the anti-c-myc antibody for the pDisplay constructs and MHM24 for the αL I-domain. (B) Isolated αL I-domain binding to ligand ICAM-1. pDisplay αL I-domain wild-type (pDL-wt) was found to be constitutively active and could bind ICAM-1 even in the absence of activating agents Mg/EGTA (MgE). A similar binding profile was observed for pDL-I331G. Intact full-length αLβ2 could bind to ICAM-1 only in the presence of KIM185 or Mg/EGTA. Addition of MgE to the isolated I-domain constructs did not result in any further activation of the receptors, confirming their constitutive ligand-binding activity.

Isolated αL I-domain wild-type and mutant I331G show constitutive ligand binding activity

These differences in the activity of Mac-1 and LFA-1 isoleucine mutants prompted us to investigate further the activation of the isolated αL I-domain. αL I-domain cDNA coding for residues Glu^{149} to Asp^{341} (Fig. 1B) was engineered in fusion with a C-terminus coding for the c-myc tag and PDGF transmembrane and cytoplasmic tail in the pDisplay vector (see Materials and methods). In addition, αL I-domain having the I331G substitution was generated. These constructs will be referred to as pDL-wt and pDL-I331G. Expression and analyses in 293T cells were performed as before.

Cell surface expression was comparable as determined by flow cytometry using the anti-c-myc antibody for the pDisplay constructs and MHM24 which recognizes the αL I-domain (Fig. 4A). Interestingly, transfectants expressing pDL-wt were found to be constitutively active and could bind ICAM-1 even in the absence of activating agents Mg/EGTA (Fig. 4B). Similar binding profiles were observed for pDL-I331G. In contrast, intact full-length αLβ2 could bind to ICAM-1 only in the presence Mg/EGTA. Furthermore, addition of Mg/EGTA to the isolated I-domain constructs did not result in any further activation of the receptors, confirming their constitutive ligand-binding activity.

The I-domain boundaries were based on the crystal structure of the αL I-domain. It was noted that these isolated I-domain constructs contained an extra cysteine residue (Cys^{149}) which may have interfered with the proper folding of the I-domains. To this end, constructs coding for residues Gly^{153} to Asp^{341}, which lacked Cys^{149} were generated in pDisplay vector, and transfected into 293T cells. No

difference was observed, compared to the Cys^{149} containing I-domain constructs, with respect to their expression and their constitutive capacity to bind ICAM-1 (data not shown).

Binding activity at different coating level of ICAM-1

To ensure that the high ligand binding observed for the isolated I-domains was not a result of the presence of excess ligand, the binding experiment was repeated at different amounts of ICAM-1 coated. ICAM-1 was serially diluted and coated on the plate at concentrations of 1, 0.3, 0.1, 0.03, and 0.01 ng/μl (50 μl are added in each well). In the absence of Mg/EGTA activation, wild-type αLβ2 showed minimal binding under all concentrations of ICAM-1 coating (Fig. 5). The transfectants expressing either pDL-wt or pDL-I331G I-domain showed parallel increase in binding to higher coatings of ICAM-1. Activation of the cells expressing wild-type αLβ2 integrin with Mg/EGTA resulted in an adhesion profile similar to transfectants expressing the isolated I-domains.

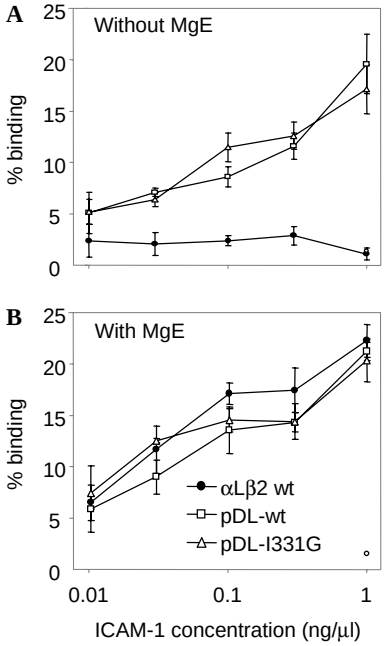

Fig. 5. αL I-domain binding to ligand ICAM-1 at different concentrations. (A) In the absence of activation by Mg/EGTA (MgE), the full-length wild-type αLβ2 showed minimal binding under all ICAM-1 concentrations. The binding for wild-type isolated I-domain and with the I331G mutation constructs decreased steadily as the ICAM-1 concentration was reduced. The binding for each construct at each ICAM-1 concentration, however, remained almost identical, showing that the similar binding at 1.0 ng/μl seen earlier was not a result of excess ligand. With addition of MgE (B), full-length αLβ2 bound ICAM-1 at similar levels to pDL-wt and pDL-I331G under all ICAM-1 concentrations. The binding of full-length wild-type αLβ2 is blocked with MHM24 (○).

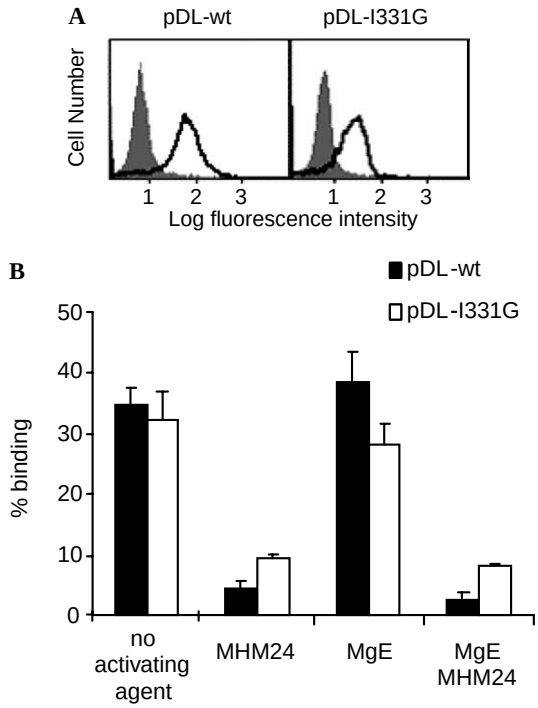

Fig. 6. Isolated wild-type αL and mutant αL I331G I-domains show constitutive ligand-binding activity in K562 cells. (A) K562 stable cell lines expressing pDL-wt and pDL-I331G were established and clones showing similar levels of expression, detected using MHM24, were selected. (B) Isolated I-domain binding to ligand ICAM-1. pDisplay αL I-domain wild-type (pDL-wt) and pDL-I331G were found to be constitutively active and could bind ICAM-1 even in the absence of activating agents Mg/EGTA (MgE). Addition of MgE to the isolated I-domain constructs did not result in any further activation, confirming their constitutive ligand-binding activity in K562 cells.

Constitutive binding of isolated αL I-domains in K562 cells

To exclude the possibility that the constitutive binding activity of pDL-wt or pDL-I331G I-domain in 293T cells was due to cell-type differences, K562 stable cell lines expressing these constructs were made. Clones showing similar levels of expression were selected (Fig. 6A), and the adhesion of these to ICAM-1 was tested. Consistent with results obtained in 293T, K562 expressing pDL-wt or pDL-I331G I-domain exhibited constitutive ICAM-1 binding in the absence of any activating agents. Adhesion was not further augmented in the presence of Mg/EGTA (Fig. 6B).

Discussion

There is much evidence to suggest that activation of integrins occurs through conformational change [27]. Early crystal structures of the I-domain of integrin αM revealed two distinct conformations—a ligand-occupied 'open' form and an unoccupied 'closed' form [7,8]. These differ primarily in the position of the C-terminal α7 helix which undergoes a 10 Å downward shift in the ligand-occupied conformation. This movement of the α7 helix has a

potential physiologic regulatory role in integrin ligand binding. Analyses of other integrin I-domains, namely those of α2 [28], and αL [29], suggested that this conformational change may be universal in the activation of the I-domains of integrins.

Crystal structure analysis of the isolated I-domain of αM showed that the side chain of Ile^{332} packs into a hydrophobic pocket between the α7 helix and the opposing β-sheet in the closed conformation. Here Ile^{332} coordinates with Ile^{151}, Leu^{180}, Ile^{252}, and Tyr^{283}. It was proposed that packing of this residue into the isoleucine socket, or SILEN, might constrain the αM I-domain in the closed conformation [11]. This implies that activation of αMβ2 involves the displacement of the isoleucine from the socket. Substitution of Ile^{332} to a glycine (I332G variant) in the I-domain of αM was reported to result in a constitutively active αMβ2 [11]. Our results herein confirmed this observation. Further, adhesion was not augmented in the presence of activating mAbs KIM185 and MEM48. Together, these results suggest that αM Ile^{332} is a key residue in αM I-domain regulation. Docking of Ile^{332} into SILEN will therefore determine in part the activity of the αM I-domain.

The isoleucine found in αM at position 332 is invariant among all integrin I-domains and so the Ile-SILEN contribution has been proposed as a universal mechanism for regulating integrin adhesion activities. Additional support was drawn from the analyses of the αX I-domain. An αXI-I333G variant was shown to have 200-fold higher affinity for iC3b as compared to wild-type αXI [9]. In a separate study, alanine scanning mutagenesis of the αL I-domain yielded many LFA-1 variants that showed higher ligand-binding capacity than the wild-type [10]. Substitution of αL Ile^{331}, which corresponds to the invariant isoleucine in the integrin I-domains, to alanine generated an αLβ2 variant that was approximately 3-fold more adhesive to ICAM-1 as compared to wild-type.

Herein, we report that mutation of αL Ile^{331} did not render the resultant αLβ2 variant constitutively active. When expressed on 293T, it showed an ICAM-1 binding profile indistinguishable from that of wild-type αLβ2. Adhesion was only promoted in the presence of mAb KIM185 or Mg/EGTA. On examination of the data from Huth et al. [10], it appears that although the LFA-1 with the Ile^{331} mutated to an Ala was more adhesive than the wild-type, it was far from being "constitutively active" since an additional twofold enhancement could be brought about with the activating mAb, 240Q. Because we found that full-length wild-type αLβ2 and αL(I331G)β2 showed no difference in their ligand binding properties, we reason that Ile^{331} is not the critical residue contributing to allosteric regulation of LFA-1.

Of interest, our data revealed that isolated wild-type αL I domain exists in a constitutive active state. I331G substitution had no significant effect on its activity. Soluble wild-type I-domain was expressed and shown to have an affinity to ICAM-1, with a K_D of ~1.7 mM, by surface plasmon resonance [30]. In a separate study K562 transfectant expressing the isolated αL I-domain using the pDisplay vector was shown not to adhere to ICAM-1 coated surfaces [31]. In our hands, αL wild-type I-domain in pDisplay vector showed reproducible constitutive ICAM-1 adhesion using the same system on K562 cells. Same results were obtained using 293T cells. The reason for this apparent discrepancy remains to be investigated. Nevertheless, our data revealed that the proposed Ile-SILEN regulation of I-domain is applicable to specific I-domain containing integrins, e.g., αM, but may not serve as a prototypic model for the regulation of other I-domains. Such differential regulation of β2 integrins activities was reported in our previous study on the functional regulation of αLβ2 and αMβ2 via β2 subunit truncation [12].

It has been widely assumed that all I-domains remain in an inactive, closed conformation and that activation is achieved through conformational change. Our data, however, suggests that the isolated αL I-domain exists in a default active conformation and that the αL I-domain is prevented from binding ligand in the intact receptor unless activated. It is possible that the β2 subunit plays a role in constraining the αL I-domain in an inactive conformation in intact LFA-1. More structural data of an I-domain-containing integrin is required to shed light on the mechanism of integrin activation involving the I-domain.

Acknowledgments

This work is supported in part by Agency for Science, Technology and Research (A*STAR), Biomedical Research Council grant A*STAR03/1/22/18/219 and by Nanyang Technological University Grant M52080022.

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