Epitope Mapping of Monoclonal Antibody to Integrin αLβ2 Hybrid Domain Suggests Different Requirement of Affinity States for Intercellular Adhesion Molecules (ICAM)-1 and ICAM-3 Binding

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Running Title: Hybrid domain movement and integrin affinity states.

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Integrin undergoes different activation states by changing its quaternary conformation. The integrin β hybrid domain acts as a lever for the transmission of activation signal. The displacement of the hybrid domain can serve to report different integrin activation states. The mAb MEM148 is a reporter antibody that recognizes Mg/EGTA-activated but not resting integrin αLβ2. Herein, we mapped its epitope to the critical residue Pro374 located on the inner face of the β2 hybrid domain. Integrin αLβ2 binds to its ligands ICAM-1 and ICAM-3 with different affinities. Integrin is proposed to have at least three affinity states and the position of the hybrid domain differs in each. We made use of the property of mAb MEM148 to analyze and correlate these affinity states in regard to αLβ2/ICAM binding. Our study showed that Mg/EGTA activated αLβ2 can adopt a different conformation from that activated by activating mAbs KIM185 or MEM48. Unlike ICAM-1 binding which required only one activating agent, αLβ2/ICAM-3 binding required both Mg/EGTA and an activating mAb. This suggests that αLβ2 with intermediate affinity is sufficient to bind ICAM-1 but not ICAM-3, which requires a high affinity state. Further, we showed that the conformation adopted by αLβ2 in the presence of Mg/EGTA depicting an intermediate activation state, could be reverted to its resting conformation.

Integrins represent a large family of type I heterodimeric (α and β subunits) membrane proteins capable of bidirectional signal transduction serving cell growth, differentiation, and apoptosis (1). The β-I-like domain is flanked by the hybrid and PSI (for Plexins, Semaphorins, and Integrins) domains (Fig. 1A) (2,3). The hybrid domain has been shown to be important in the propagation of activation signal from one end of the β subunit to the other (2,4-6). Recently, superimposed structural coordinates of liganded-open αVβ3 headpiece with that of unliganded-closed αVβ1 revealed an approximately 10 Å shift and a concomitant rotation of the hybrid domain relative to the last helix of the β-I-like domain upon ligand-binding (7). mAbs that attenuate this swing-out motion of the integrin αβI hybrid domain prevent effective allosteric activation of the β-I-like domain (5,6).

Collective observations from electron microscopy images and crystal structures of integrin αIIbβ3, αVβ3, and α5β1 suggest that integrin may undergo at least three activation states depicted by different quaternary conformations (3,7-13). A bent integrin with the hybrid domain in close proximity of the I-EGF (Integrin-Epidermal Growth Factor) 3, and 4 domains represents the resting state. The extended integrin with the hybrid domain distally separated from I-EGF 3, and 4 but orientated towards the α subunit β-propeller depicts a low affinity state, whereas the extended integrin with a swing-out hybrid domain away from the α subunit β-propeller represents a high affinity state.

The conceptualization of different integrin affinity states also derives from earlier functional studies. Observations were made on integrins having different ligand binding properties under
different cellular or extracellular conditions. Resting platelet integrin α_mβ_3 binds immobilized fibrinogen, but binding to soluble fibrinogen, fibronectin or von Willebrand factor requires platelet activation by agonist (14-16). Divalent cation Mn^{2+} activates integrin α_β_1 to bind VCAM-1 whilst adhesion to fibronectin-derived CS-1 peptide requires additional activating mAb (17). Real time analysis of integrin α_β_1 binding to fluorescent conjugated ligand mimetic via chemokine receptor activation on leukocytes also suggests integrin acquiring multiple affinity states (18). For the integrin α_β_2, ligand ICAM-1 exhibits higher affinity for purified α_β_2 from T cells than ICAM-3 (19). Prior exposure of α_β_2 to ICAM-1 increased its binding to ICAM-3 (20). Soluble ICAM (sICAM)-3 binds to α_β_2 with 9-fold lower affinity than sICAM-1 (21). We also reported the requirement of two α_β_2 activating mAbs KIM185 and KIM127 for its adhesion to ICAM-3 as compared to ICAM-1 (22). Crystal structures of engineered intermediate affinity α_1 I-domain (L161C/F299C) and high affinity α_1 I-domain (K287C/K294C) in complex with ICAM-1 showed differences in their interactions (23). Together, it is apparent that distinct α_β_2 affinity states could serve binding to different ICAM ligands. Structural data of intact α_β_2 interacting with ICAM are lacking. Hence, structural data derived from the studies of β_1 and β_2 integrin are useful as a hypothetical activation model to correlate α_β_2 functional states to its conformation.

In this article, we described the reporter mAb MEM148, which recognizes the free integrin β_2 subunit or Mg^{2+}/EGTA-activated α_β_2 but not resting α_β_2 (2,24). We mapped the epitope of MEM148 to the hybrid domain and the location of the epitope, when modeled with the bent structure of α_β_1, faces the α subunit, and could therefore be masked. Activation as a result of integrin extension could lead to the exposure of the epitope. In addition, we make use of the activation-reporter property of MEM148 to analyze the different α_β_2 affinity states required for ICAM-1 and ICAM-3 binding.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies** - The following mAbs were gifts from different sources: MHM24 (anti-α_L) and MHM25 (anti-β_2, heterodimer dependent) were obtained from Prof. A. J. McMichael (John Radcliffe hospital, Oxford, U.K); KIM185 (anti-β_2, activating mAb) was from Dr. M. Robinson (CellTech, Slough, UK); MEM48 (anti-β_2, activating mAb) and MEM148 (anti-β_2) were from Prof. V. Horejsi (Prague, Czech Republic). ICAM-1/Fc and ICAM-3/Fc were prepared as described previously (25).

**cDNA Expression Constructs** - The α_L and β_2 cDNAs in the expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands) were described previously (26,27). Previously, we had numbered the N-terminal Met of β_2 as 1 (4) and relevant references therein. For the purpose of clarity and ease of reference with other β_2 and different β integrins functional and structural studies, herein β_2 Met ^I- is re-numbered as Met ^β_2^I-(28). Construction of β_2 Hu/Mo A in which Met ^β_2^I-(22)-Asn ^562 of human integrin β_2 was replaced with the corresponding region from mouse β_2 was described previously (4). Amino acid substitutions on the mouse or human β_2 constructs were made using QuikChange™ Site-Directed Mutagenesis Kit (Strategene). All constructs were verified by sequencing (DNA Sequencing Facility, Department of Biochemistry, University of Oxford, Oxford, U.K)(Research Biolabs sequencing service, Singapore).

**Cell Culture and Transfection** - COS-7 (monkey kidney fibroblast) and MOLT-4 (human T lymphoblast) (ATCC, Manassas, VA) were cultured in complete media RPMI 1640 containing L-glutamine (JRH Biosiences Inc., Australia), 10% (v/v) heat-inactivated foetal bovine serum (HI-FBS) (Sigma, St Louis, MO), and 100 IU/mL penicillin and 100 μg/mL streptomycin. For epitope mapping studies, COS-7 cells were transfected with wild-type β_2 or variant cDNAs by the DEAE-Dextran method (25).

**Flow Cytometry Analysis** - Flow cytometry analysis of integrin cell surface expression was performed as described previously (29). Briefly, cells were incubated with 20 μg/mL of primary mAb in RPMI 1640 for 1 h at 4°C. Thereafter, cells were washed and incubated with FITC-conjugated sheep anti-mouse F(ab’)2 secondary Ab (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 analyzed on a FACs Calibur (Becton Dickinson, Mountain View, CA). Data were analysed using CellQuest software (Becton Dickinson). Expression index (EI) was calculated by % cells gated positive (%GP) x geo-mean fluorescence intensity (GM).
Results

Mapping of mAb MEM148 Epitope - The epitope of mAb MEM148 resides in the integrin β2 hybrid domain and is not expressed in resting integrin αLβ2 but exposed upon Mg/EGTA treatment (2). Because it may serve as a useful reporter mAb for subsequent analyses of integrin affinity states, we further characterize its epitope using a panel of integrin β2 human/mouse “knock-out” mutants generated by site-directed mutagenesis (4). These human integrin β2 mutants have their residues replaced by corresponding mouse β2 residues at positions where they differ in the mid-region (Fig. 1B). COS-7 cells, which can express β2 integrin in
the absence of the α subunit (2), were transfected with the β2 human/mouse “knock-out” mutant cDNAs followed by immunofluorescence staining with mAb MEM148 and flow cytometry analyses. Expression of MEM148 epitope on transfectants was determined (Fig. 2A). The mAb MEM48, which maps to residues Leu334, Phe336, and His543 of human integrin β2 I-EGF 3 (32) was included as receptor expression control. Approximately 3-fold reduction in MEM148 epitope expression was observed for transfectants expressing β2Hu(H370S/R371I) or β2Hu(N372G/Q373K) as compared to β2Hu(wt). The β2Hu(P374S) variant showed significant (>90%) abrogation of MEM148 epitope expression.

We next employ an integrin β2 human/mouse chimera (β2Hu/MoA) (Fig. 1A) in which Met22–Asn562 of human integrin β2 is replaced by the corresponding region from mouse integrin β2 (4) to generate two “knock-in” mutants. The β2Hu/MoA(S374P) has the Ser374 of the mouse mid-region substituted by the corresponding human Pro residue. The β2Hu/MoA(A368V-S374P) has the segment Ala368–Ser374 of the mouse mid-region replaced by the corresponding human segment Val368–Pro374. In this case, mAb KIM185, which maps to integrin β2 I-EGF 4 and β-tail domain (32), was included as receptor expression control because the epitope of MEM48 is absent in β2Hu/MoA. The expression of MEM148 epitope on β2Hu/MoA(S374P) was low as compared to β2Hu(wt) (Fig. 2B). However, transfectant bearing β2Hu/MoA(A368V-S374P) restored fully the epitope of MEM148. Thus, we may conclude that although human integrin β2 Pro374 is a critical residue, other residues His370, Arg371, Asn372, Gln373 are also required for the effective presentation of MEM148 epitope.

**Model of αβ2 Illustrating MEM148 Epitope** - Fine mapping of MEM148 epitope allows us to pinpoint its location in the quaternary structure of integrin. A model of integrin αβ2 was generated by MODELLER using αβ3 structural coordinates as template (3) (Fig. 3A). Structure of an intact I-domain containing integrin is not solved; hence, the αI-I-domain was excluded from the model. For clarity, the αL Calf-2, β2 PS1, I-EGFs, and β-tail domains were not included. The critical residue Pro374 resides on the surface of the hybrid domain facing the αL subunit in contrast to the epitope of mAb 7E4 (Val385) (4), which is located away from the αL subunit (Fig. 3B). This could explain why mAb MEM148 fails to bind resting αLβ2, presumably it assumes a severely bent conformation similar to the resting αLβ3 (3), because its epitope is shielded in this conformation (Fig. 3C). Along the same line of reasoning, the binding of mAb MEM148 to activated αLβ2 adopting an extended conformation would therefore be favorable in conjunction with previous observations (2,24).

**The Transition of αLβ2 From One Affinity State to Another is Reversible** - The mAb MEM148 does not bind to αLβ2 on MOLT-4 cells unless the cells were treated with Mg/EGTA (2). Divalent cations have major influence on the αI-domain and β I-like domain and it is widely accepted that the activated integrin should adopt an opened and/or extended conformation. However, defining the precise mechanism for the transition of integrin from one affinity state to the next in the presence of activating divalent cations remains challenging. Recently, several quaternary integrin conformations were proposed to depict such transitions (7). Of note, the reversion from one conformation to another may be physiologically relevant to maintain a dynamic integrin population responding to different cellular activation milieu.

To this end, we determine whether Mg/EGTA-treated αLβ2 can revert back to its resting state using MEM148 as reporter mAb. MOLT-4 cells were incubated in Mg/EGTA-containing RPMI and either MEM148 or KIM127 at 37°C. The epitope of KIM127 resides in β2 I-EGF 2, and like MEM148, its epitope expression depends on integrin activation (32,33). Significant staining was detected for both mAbs only in the presence of Mg/EGTA (Fig. 4A). The integrin αL-specific mAb MHM24 was included as a control. Next, cells in the presence of Mg/EGTA were subsequently treated with EDTA to deplete existing Mg2+ before incubation with respective mAbs. Epitope expressions of MEM148 and KIM127 were significantly reduced in these samples whereas control mAb MHM24 staining was not affected.

We next tested whether Mg/EGTA-treated cells after washing in media could still express the epitopes of MEM148 and KIM127 (Fig. 4B). Mg/EGTA-treated cells were subjected to three washes in RPMI without additives followed by staining for either of the two mAbs. Low level of MEM148 or KIM127 epitope expression was detected as compared to Mg/EGTA-treated cells without washing. Cell binding assay to ICAM-1 was investigated to determine whether Mg/EGTA-treated cells followed by washing could still adhere to ICAM-1 (Fig. 4C). In the presence of
Mg/EGTA, cells adhered significantly to ICAM-1. However, adhesion was minimal when cells were treated with Mg/EGTA followed by two or three washes in RPMI wash buffer before allowing to adhere to ICAM-1. Binding specificity was shown by including α specific function-blocking mAb MHM24. Together, our data suggest that reversion of Mg/EGTA-activated αβ2 to its resting conformation is possible when the activating cation is depleted.

We further our analyses by changing the order of MOLT-4 treatment with different agents to test whether MEM148 remain bound to Mg/EGTA-treated cells even in the presence of EDTA. Cells were first treated with Mg/EGTA in the presence of MEM148 with subsequent washing in media. MEM148 staining was detected in both cases. This suggests that the αβ2/MEM148 complex is stable even when divalent cations were depleted or by extensive washing in media.

Phorbol 12-myristate 13-acetate (PMA) is known to promote α4β2 ligand-binding (4,34). PMA also induced the expression of MEM148 epitope on myeloid cells and this was contributed by a proteolytically-cleaved fragment of β2 unassociated with the αL, αM, or αX subunit rather than the respective heterodimers (35). To test whether PMA induced MEM148 epitope expression on MOLT-4, cells were surface-labeled with biotin followed by treatment with Mg/EGTA or PMA in the presence of MEM148 or other mAbs (Fig. 4E). Cells were lysed and immunoprecipitation performed followed by ECL detection. As compared to flow cytometry analyses, cell surface-labeling followed by immunoprecipitation can identify whether there are free forms of β2, which could be detected by MEM148, on the cell surface of MOLT-4 as a result of Mg/EGTA or PMA treatment. MEM148 only immunoprecipitated αβ2 when cells were treated with Mg/EGTA but not with PMA. No corresponding protein bands were detected using irrelevant mAb but αβ2 bands were detected under all conditions using the β2-specific mAb MHM23. Truncated β2 (65-70 kDa) (35) was not detected in MEM148 immunoprecipitation sample of PMA-treated MOLT-4 cells. It is possible that the truncated β2 is expressed mainly and is abundantly up-regulated on the surface of activated myeloid cells instead of lymphocytes (35).

It is also interesting to note that PMA did not induce detectable expression of MEM148 epitope on αβ2 although it is reported to promote αβ2 ligand-binding (4,34). It is possible that PMA promotes an active αβ2 conformation that is different from that triggered by Mg/EGTA for example. Alternatively, PMA increases the lateral mobility of αβ2 on plasma membrane (36) allowing receptor clustering which effects on ligand-binding.

Binding of αβ2 to Immobilized ICAM-1 and -3 Requires Different Affinity States - To understand the molecular basis of αβ2 activation with respect to ICAM-1 and ICAM-3 binding, we performed immobilized ligand binding assay using MOLT-4 cells. Cells were allowed to adhere to ICAMs in the presence of Mg/EGTA, mAb or a combination of both (Fig. 5). MOLT-4 binding to ICAM-1 was minimal in the absence of activating agents. In the presence of Mg/EGTA or any of the activating mAbs KIM185 or MEM48, binding to ICAM-1 was minimal in the absence of activating agents. In contrast, in the presence of MEM148 alone, binding to ICAM-1 was minimal. Under the condition of Mg/EGTA and any of the three mAbs under study, the binding of MOLT-4 to ICAM-1 was further enhanced. ICAM-1 binding was mediated by αβ2 on MOLT-4 cells because binding was effectively abrogated in the presence of function-blocking α specific mAb MHM24. It is therefore possible that activation of αβ2 by either Mg/EGTA or one of the activating mAbs may convert αβ2 into an intermediate affinity state. Promotion of this population of αβ2 into a higher affinity state can still be achieved by an additional activating agent. Similar profiles were observed in αβ2 COS-7 and 293T transfectants (data not shown).

ICAM-3 binding was carried out under similar conditions (Fig. 5B). Binding to ICAM-3 was minimal in the presence of Mg/EGTA or any of the mAbs. When Mg/EGTA and one activating mAb were included, binding was significantly augmented. This was in concordance with our previous findings that mAbs KIM185 and KIM127 were required for αβ2 COS-7 transfectants binding to ICAM-3 (22). The addition of two different mAbs recognizing the same receptor may result in receptor clustering. In this study, enhanced adhesion to ICAM-3 cannot be due to receptor clustering because only one activating mAb was employed while the other activating agent was Mg/EGTA. Abrogation of binding by MHM24 demonstrated interaction specificity between αβ2 and ICAM-3. Noteworthy, the combination of Mg/EGTA with
mAb MEM148 only had a marginal effect on αLβ2/ICAM-3 binding as compared to other mAbs. This could not be due to insufficient ICAM-3 ligand coated on the well (1 ng/μL) because by increasing ICAM-3 concentration 3-fold had only marginal effect on cell binding. The  quaternary conformation adopted by Mg/EGTA-activated αLβ2 may be similar to one of the intermediate affinity states proposed for β3 integrins.

**Binding of αLβ2 to soluble ICAM-1 and -3** – If resting αLβ2 assumes a severely bent conformation like αLβ3, the accessibility of ligands to the α I-domain and β I-like domain on the cell surface is unfavorable because they are oriented towards the plasma membrane. We further our analyses using sICAMs binding assays (Fig. 6) (30,31). This assay allows ICAMs to be “free” in solution rather than being immobilized on a solid-phase which may provide better accessibility to the head of the bent integrin on the cell surface. MOLT-4 cells bind to sICAM-1 when treated with Mg/EGTA, KIM185 or MEM48 but not MEM148. Binding was increased in the presence of Mg/EGTA with KIM185 or MEM48. In the presence of Mg/EGTA and MEM148, binding was augmented as compared to Mg/EGTA alone, which is similar to that observed under immobilized ICAM-1-binding assay (Fig. 5A).

sICAM-3 binding to MOLT-4 cells was detected only when cells were treated with Mg/EGTA together with KIM185 or MEM48. Similar to immobilized ICAM-3-binding assay, Mg/EGTA with MEM48 could promote MOLT-4/sICAM-3 binding albeit at a lower level as compared to Mg/EGTA with KIM185 or MEM48. Bindings were αLβ2-specific because they were effectively abrogated by MHHM24. It was noted that there was difference between immobilized and soluble ICAMs assays. The percentage cell binding of Mg/EGTA- and MEM48-treated MOLT-4 on ICAM-1, for example, was similar with that on ICAM-3 (Fig. 5A and B). However, binding to sICAM-3 was much lower than sICAM-1 with Mg/EGTA and MEM48 (Fig. 6A and B). Such difference was consistently detected in repeat experiments performed. Woska et al. also reported weaker sICAM-3 binding to purified αLβ2 as compared to sICAM-1 (21). At present, the reason for these observations is unclear.

**DISCUSSION**

The headpiece of the integrin αLβ2 may be analogous to the integrin αIβ1 consisting of the α I-domain (in I-domain containing integrins), β-propeller, thigh domain, β I-like domain, hybrid, PSI, and I-EGF 1 (7). The β2 I-like domain of integrin αLβ2 allosterically regulates ligand binding of the α I-domain through binding of the β2 metal ion-dependent adhesion site (MIDAS) to Glu310 found in the last helix of the αI-domain; this was shown by replacing αI Glu310 with Cys and either β2 MIDAS Ala310 or Tyr315 to Cys allowing disulphide connection between the two domains (31). Downward displacement of this helix by β2 I-like domain with a “pull-string” motion triggers I-domain conversion from a closed to an open conformation ready for ligand binding (31). The hybrid domain is linked directly to the β I-like domain (3). Our previous study on αLβ2 hybrid domain maps the epitope of mAb 7E4 to Val385 and demonstrates the importance of the hybrid domain for activation signal transfer from β2 membrane proximal region to the αI I-domain (4). Preventing the movement of integrin αLβ1 hybrid domain away from its β1 I-like domain by mAb SG/19 maintains αLβ1 in low affinity for fibronectin, as determined by surface plasmon resonance (6).

Herein, we report the binding determinant of mAb MEM148 consisting of the critical residue Pro374 and residues His370-Glu373 of the integrin β2 hybrid domain. Distinct from the exposed epitope Val385 of mAb 7E4, these residues are hidden in the αLβ2 model using the bent αLβ1 as template (Fig. 3). This shares similarity to mAbs 15/7 and HUTS-4 epitopes located on integrin β1 hybrid domain that are partly masked by integrin αLβ-propeller (5). Accessibility of mAb MEM148 to its epitope(s) would therefore be sterically unfavorable when αLβ2 adopts a bent and closed headpiece conformation. It accounts for the lack of mAb MEM148 reactivity to unactivated αLβ2 observed previously (2,24). However, when exposed to Mg/EGTA, αLβ2 can undergo a conformational change possibly via unbending thereby exposing the epitope of MEM148.

Crystal structures of the integrins αIβ3 and αIβ3 with ligand mimetics, electron microscopy images of soluble integrin αIβ2 with
cyclo-RGDfV peptide in the presence of Mn$^{2+}$ or Ca$^{2+}$, and that of integrin $\alpha_L\beta_2$ with physiological ligand fibronectin provide insights into the possible conformations that can be adopted by the integrin during its transition from resting to a high affinity state (3,7,10-12). Based on these observations, it was proposed that integrin activation involves at least three affinity states (7). Each conformer can be distinguished not only by the degree of bending but also the conformation of its headpiece as determined by the relative orientation of the hybrid with respect to the $\beta$ I-like domain. Previously, it was reported that $\alpha_L\beta_2$ binding to ICAM-1 and -3 exhibit different affinities (19-22). In this study, we found that mAb MEM148 in combination with activating agents Mg/EGTA further enhanced the affinity of $\alpha_L\beta_2$/ICAM-1 binding as compared to Mg/EGTA alone. The observation could be attributed to the disruption of the extensive contacts between the $\alpha_L\beta_2$ headpiece-tailpiece interface when in the presence of Mg$^{2+}$ and with Ca$^{2+}$ depleted. This would facilitate the unbending of $\alpha_L\beta_2$ hence allowing MEM148 to access its epitope masked previously.

From our $\alpha_L\beta_2$/ICAM-3 binding data, two activating agents are required to promote binding. Collectively, this implies that the conformation adopted by $\alpha_L\beta_2$ in the presence of Mg/EGTA, mAb KIM185, or MEM48 represents affinity state(s) capable of ICAM-1 but not ICAM-3 binding. The $\alpha_L\beta_2$ conformer in the presence of Mg/EGTA could be different from those activated by mAb KIM185 or MEM48 because if they are identical, the combination of both agents would not further enhanced $\alpha_L\beta_2$/ICAM-1 and promote $\alpha_L\beta_2$/ICAM-3 binding as compared to using one agent. Indeed, our previous study showed that the mAb 7E4 binds to $\alpha_L\beta_2$ hybrid domain and prevents $\alpha_L\beta_2$ activation by mAb KIM185 but not Mg/EGTA (4). The Mg/EGTA-activated $\alpha_L\beta_2$ conformer has its hybrid domain re-oriented as evidenced by its reactivity with MEM148 described herein. However, such re-positioning as a result of Mg/EGTA activation still did not favor $\alpha_L\beta_2$ binding of ICAM-3. We also found that the combination of Mg/EGTA with mAb MEM148 did not promote effective $\alpha_L\beta_2$/ICAM-3 binding as opposed to Mg/EGTA with mAb KIM185 or MEM48. It differs from that of ICAM-1 binding in which Mg/EGTA and MEM148 had a cumulative effect. If mAb MEM148 further displaces the hybrid domain in Mg/EGTA-activated $\alpha_L\beta_2$ resulting in a higher affinity state comparable to Mg/EGTA/KIM185 or MEM48 condition, effective ICAM-3 binding should also be established. However, the lack of binding detected in this case would suggest that mAb MEM148 may only stabilize the Mg/EGTA-activated conformation of $\alpha_L\beta_2$ by binding to its re-positioned hybrid domain.

It was hypothesized that other intermediates may exist during $\alpha_L\beta_3$ activation (10). These intermediates may be depicted by half-bent structures with separation of their $\alpha$ and $\beta$ subunits but still connected at their heads. Solution structure of intact integrin in Mg$^{2+}$ only is lacking; nonetheless, it is possible that Mg/EGTA-activated $\alpha_L\beta_2$ adopts a conformation akin to one of these intermediates. This may explain the reactivity of mAb MEM148 to Mg/EGTA-activated $\alpha_L\beta_2$. The Mg/EGTA-activated $\alpha_L\beta_2$ “intermediate” is also a dynamic structure that can be reverted back to its resting bent conformation. We found that the reactivity of mAbs MEM148 and KIM127 was diminished in EDTA-treated $\alpha_L\beta_2$, which was beforehand activated by Mg/EGTA. EDTA does not directly affect on these mAbs because stainings could be detected for single integrin $\beta_2$ expressed on COS-7 cells in the presence of 5 mM EDTA (unpublished observations). Because mAbs MEM148 (2) and KIM127 (32) report Mg/EGTA-activated $\alpha_L\beta_2$ hybrid domain and I-EGF 2 movement during $\alpha_L\beta_2$ activation respectively, our data favor a global conformational reversion to its resting state rather than a localize effect.

Structural data revealed an intermediate affinity $\alpha_L$ I-domain (Leu$^{61}$-Cys/PhPhe$^{299}$-Cys lock) capable of ICAM-1 binding in the presence of Mg$^{2+}$ (23). The transition between low, intermediate, and high affinity conformations of $\alpha_L$ I-domain revealed a rachet-like movement of its $\beta_6$-$\alpha_7$ loop (23,37). Recently, molecular dynamics simulation reveals that intermediate affinity $\alpha_L$ I-domain can be generated by applying pulling-force on the $\alpha_7$ helix (38), which is allosterically regulated by the $\beta_2$ I-like domain. We found that Mg/EGTA-treated $\alpha_L\beta_2$ could be further activated to bind ICAM-1 by addition of activating mAb KIM185 or MEM48. Mg/EGTA treatment would have converted $\alpha_L\beta_2$ into an intermediate affinity state. Addition of these mAbs would disrupt the interface between the $\alpha_L$ and $\beta_2$ leg pieces with eventual propagation of conformational change to the hybrid domain and $\beta_2$ I-like domain. This allows further displacement of the I-domain $\alpha_7$ helix transforming the $\alpha_L\beta_2$
from an intermediate to a high affinity state for ICAM-1 binding.

Why does binding to ICAM-1 and ICAM-3 require different $\alpha_\ell$/$\beta_2$ conformations? The difference may be effected by dissimilar dissociation rates of the interactions and the conformations of the binding pockets. With respect to the ICAMs, the height of ICAM-1 and -3 on the cell surface is similar, both having five C2-set IgSF domains. The binding sites in the ICAMs for $\alpha_\ell$/$\beta_2$ also shares similarity: Glu$^{34}$ and Gln$^{73}$ in IgSF domain 1 (D1) of ICAM-1 (39) and the corresponding residues Glu$^{37}$ and Gln$^{35}$ in D1 of ICAM-3 (40-42). However, Lys$^{39}$, Met$^{44}$, Tyr$^{66}$, and Asn$^{68}$ of ICAM-1 are important for binding to $\alpha_\ell$ I-domain (23) but Asn$^{23}$, Ser$^{25}$, and Phe$^{54}$ of ICAM-3 are required for effective interaction (40). In addition, ICAM-3 IgSF domain 1 is extensively glycosylated as compared to ICAM-1 IgSF domain 1 (19). These may contribute to the dissimilarity between $\alpha_\ell$/$\beta_2$/ICAM-1 and -3 binding (43). The binding sites of ICAM-1 and -3 on the $\alpha_\ell$ I-domain have also been determined. Residues surrounding the $\alpha_\ell$ I-domain MIDAS and those in proximity are found to be critical for ICAM-1 and -3 binding (23,43-45). In another study, the Ile-Lys-Gly-Asn motif, located in the amino-terminal of the $\alpha_\ell$ I-domain, was shown to be important for ICAM-3 but not ICAM-1 binding (46). This is intriguing because the MIDAS is positioned at the top of the $\alpha_\ell$ I-domain whilst its amino terminal is located at its bottom. Recent crystal data of an engineered high affinity $\alpha_\ell$ I-domain in complex with ICAM-3 D1 suggests that both ICAM-1 and ICAM-3 share a common docking mode with $\alpha_\ell$ I-domain (43). The exact nature and differences of the interactions between intact ICAMs and $\alpha_\ell$/$\beta_2$ require further investigations.

In conclusion, our study using $\beta_2$ hybrid domain-specific reporter mAb MEM148 suggests the requirement of different affinity states for integrin $\alpha_\ell$/$\beta_2$ binding to ICAM-1 and -3. A proposed model explaining these observations would be the displacement of the $\beta_2$ hybrid domain in Mg/EGTA-treated $\alpha_\ell$/$\beta_2$ which renders $\alpha_\ell$/$\beta_2$ capable of ICAM-1 binding. However, this movement did not generate an $\alpha_\ell$/$\beta_2$ conformer that could bind to ICAM-3 effectively, which requires additional activation signal. It will be interesting in future studies to test the proposed model of $\alpha_\ell$/$\beta_2$ activation by structural analyses of intact $\alpha_\ell$/$\beta_2$ with ICAMs under different conditions.

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FOOTNOTES

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The abbreviations used are: HI-FBS, heat-inactivated foetal bovine serum; ICAM, intercellular adhesion molecule; mAb, monoclonal antibody; MIDAS, metal ion-dependent adhesion site; IgSF, immunoglobulin superfamily.

FIGURE LEGENDS

Fig. 1 A, Linear organization of the human integrin β2 subunit. The human integrin β2 subunit is shown with domains and regions indicated. The mouse fragment in human/mouse chimera β2Hu/MoA is shaded in black. B, Sequence alignment of human and mouse integrin β2 mid-region. The amino acids that differ are indicated by solid circles. The disulphide bridges are connected by dashed lines. The segment that is suggested to be part of the PSI domain (7) is underlined with the Cys\textsuperscript{425} involved in long range disulphide bond indicated by *.

Fig. 2 Epitope expression of mAb MEM148 on β2 “knock-out” and “knock-in” mutants. A, β2 “knock-out” mutants were generated and stained for mAb MEM148 followed by flow cytometry analyses. The epitope expression of MEM148 is calculated by MEM148 EI (expression index)/MEM48 EI for each mutant. The mAb MEM48 maps to the C-terminal region of human β2 subunit and was used as a reference antibody in this case. B, β2 “knock-in” mutants were analyzed for the restoration of MEM148 epitope expression. The epitope expression of MEM148 is calculated by MEM148 EI/KIM185 EI. The mAb KIM185 maps to β1 I-EGF 4 and β-tail domain (32) and was included as reference mAb because the epitope of MEM48 is absent in β2Hu/MoA. EI was calculated by %GP (percentage cells gated positive) x GM (geo-mean fluorescence).

Fig. 3 Model of the integrin αLβ2. A, The model of αLβ2 was generated by the MODELLER using the αVβ3 structural coordinates (3) as template. For clarity, only the β-propeller, thigh, Calf-1 of the αL subunit, and the I-like domain, and hybrid domain of the β2 subunit are shown. The critical determinants Pro\textsuperscript{374} (yellow) for mAb MEM148 and Val\textsuperscript{385} (red) for mAb 7E4 (4) are indicated. B, Surface representation with different rotation of the model. Pro\textsuperscript{374} (yellow) is located in the interface between the αL and β2 headpiece and between the αLβ2 headpiece and tailpiece interface. Val\textsuperscript{385} (red) is exposed. C, Diagram illustrating the masking of Pro\textsuperscript{374} (*) in resting β2 integrins and its exposure upon activation. The I-domain is not included. C: Calf; E: I-EGF; BT: β tail domain; H: hybrid.

Fig. 4 Analyses of integrin αLβ2 reversion from Mg/EGTA-activated state to resting state. A, Flow cytometry analysis of mAb MEM148 epitope expression. MOLT-4 cells were stained with mAb KIM127 or mAb MEM148 under different conditions at 37 °C: in the absence of additives; in the presence of Mg/EGTA; or in the presence of Mg/EGTA followed by EDTA treatment. All FITC-conjugated secondary antibody staining was performed at 4 °C. %GP: percentage cells gated positive; GM: geo-mean fluorescence; EI: expression index. B, In this experiment, instead of EDTA-treatment, MOLT-4 cells treated with Mg/EGTA were subjected to three washes in RPMI media followed by staining with mAb KIM127 or MEM148. Other conditions are the same as before. C, MOLT-4 cells were allowed to adhere to ICAM-1 in the presence of activating agents Mg/EGTA as described under materials and methods. In separate samples, Mg/EGTA-treated cells were subjected to two or three washes in RPMI wash buffer before dispensing into ICAM-1-coated wells. αL specific function-blocking mAb MHM24 was included to demonstrate binding specificity. D, Experiment was performed as in (A and B) except that cells were first treated with Mg/EGTA in the presence of MEM148, washed twice in media followed by EDTA treatment or further washes before secondary antibody staining and flow cytometry analyses. E, MOLT-4 cells were surface-biotinylated. Labeled cells were stimulated with Mg/EGTA or PMA (100
ng/mL) in the presence of irrelevant mAb, MHM23 or MEM148. Cells were washed and lysed in lysis buffer followed by immunoprecipitation and ECL detection (Experimental procedures).

Fig. 5 Binding of MOLT-4 cells to immobilized ICAMs. A, MOLT-4 cells binding to ICAM-1-coated wells is described under materials and methods. Binding was specific because addition of αL specific mAb MHM24 abrogated binding under different conditions. ME: Mg/EGTA. (Student’s t-test on * and §, assuming unequal variance, p<0.01). B, MOLT-4 cells binding to ICAM-3. C, MOLT-4 cells binding to different concentrations (0, 0.3, 1, 3 ng/µL) of ICAM-3. Only MHM24 blocking of ME/MEM48 binding at highest concentration of ICAM-3 was included for clarity.

Fig. 6 Binding of MOLT-4 cells to soluble ICAMs. A, MOLT-4 cells were allowed to bind to sICAM-1 as described under materials and methods. The level of binding was determined by the intensity of fluorescence detected on the cell surface by flow cytometry. Fluorescence staining of MOLT-4 binding to sICAM in the absence of any additives was used as background for gating purpose. Binding was represented by the expression index calculated by %GP (percentage cells gated positive) x GM (geometric mean fluorescence). Binding was specific because addition of αL specific mAb MHM24 abolished binding under all conditions. ME: Mg/EGTA. B, MOLT-4 binding to sICAM-3 crosslinked with FITC-labelled secondary antibody. Data are representative of three experiments conducted.
FIG 1

A

PSI

Hybrid

spacer

mid-region

IEGF

β-tail domain

N 1 2 3 4 C

β₂Hu/MoA

N562

B

β₂ Mo 342 SSVFVLDHSTSPLDTKVTVSSGCSNSASSIGKS 374
β₂ Hu 342 SSVFVLDHSTLPLDTKVTVSSGCSNSASSIGKS 374

β₂ Mo 375 RGDCDGQ1INPVTQKVTACEIQEQQSFVIR 407
β₂ Hu 375 RGDCDGQ1INPVTQKVTACEIQEQQSFVIR 407

β₂ Mo 408 ALGTDTVQVRPOCEQCRDQ 430
β₂ Hu 408 ALGTDTVQVRPOCEQCRDQ 430
FIG 2

A

- $\beta_2$Hb[wt]
- $\beta_2$Hb(N350S/A351T)
- $\beta_2$Hb(V368A/T369S)
- $\beta_2$Hb(H370S/R371I)
- $\beta_2$Hb(N372G/Q373K)
- $\beta_2$Hb(P374S)
- $\beta_2$Hb(V385N)
- $\beta_2$Hb(I387V)
- $\beta_2$Hb(T394M)
- $\beta_2$Hb(T396S)
- $\beta_2$Hb(I414T)
- $\beta_2$Hb(L420R)

Epitope expression

B

- $\beta_2$Hb[wt]
- $\beta_2$Hb(A368V-S374P)
- $\beta_2$Hb(A373P)
- $\beta_2$Hb(A368V-S374P)

Epitope expression
**D**

|                | MHM24 | MEM148 |
|----------------|-------|--------|
| %GP: 98.9      |       |        |
| GM : 16.7      |       |        |
| EI : 16.5      |       |        |
| %GP : 1.3      |       |        |
| GM : 8.0       |       |        |
| EI : 0.1       |       |        |

**E**

![Image of gel electrophoresis](http://www.jbc.org/)

- **ME**
- **PMA**
- Irrelevant mAb
- MHM23
- MEM148

**FIG 4**
FIG 5

% Cell Binding vs. ICAM-3 (ng/µL)

- ME / MEM48
- ME / MEM48 / MHM24
- ME / KIM185
- ME / MEM148
- ME
FIG 6

A

sICAM-1

Expression Index

ME
KIM185
MEM48
MEM148
ME / KIM185
ME / MEM48
ME / MEM148

without MHM24
with MHM24

B

sICAM-3

Expression Index

ME
KIM185
MEM48
MEM148
ME / KIM185
ME / MEM48
ME / MEM148

without MHM24
with MHM24
Epitope mapping of monoclonal antibody to integrin αLβ2 hybrid domain suggests different requirement of affinity states for intercellular adhesion molecules (ICAM)-1 and ICAM-3 binding

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