The Integrin $\alpha_1\beta_2$ Hybrid Domain Serves as a Link for the Propagation of Activation Signal from Its Stalk Regions to the I-like Domain*

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Integrin activation involves global conformational changes as demonstrated by various functional and structural analyses. The integrin $\beta$ hybrid domain is proposed to be involved in the propagation of this activation signal. Our previous study showed that the integrin $\beta_2$-specific monoclonal antibody 7E4 abrogates monoclonal antibody KIM185-activated but not Mg$^{2+}$/EGTA-activated leukocyte function-associated antigen-1 (LFA-1; $\alpha_2\beta_2$)-mediated adhesion to ICAM-1. Here we investigated the allosteric inhibitory property of 7E4. By using human/mouse chimeras and substitution mutations, the epitope of 7E4 was mapped to Val407, located in the mid-region of the $\beta_2$ hybrid domain. Two sets of constitutively active LFA-1 variants were used to examine the effect of 7E4 on LFA-1/ICAM-1 binding. 7E4 attenuated the binding of variants that have modifications to regions membrane proximal with respect to the $\beta_2$ hybrid domain. In contrast, the inhibitory effect was minimal on variants with alterations in the $\alpha_2$- and $\beta_2$-I-like domains preceding the hybrid domain. Furthermore, 7E4 abrogated LFA-1/ICAM-1 adhesion of phorbol 12-myristate 13-acetate-treated MOLT-4 cells. Our data demonstrate that interaction between the hybrid and I-like domain is critical for the regulation of LFA-1-mediated adhesion.

Integrins are key proteins involved in cell-cell and cell-matrix interactions, mediating essential biological processes such as embryogenesis, the immune response, and inflammation (1, 2). They are heterodimeric type I membrane glycoproteins formed by noncovalent association of an $\alpha$ and a $\beta$ subunit. In human, 9 of the 18 $\alpha$ subunits have an I (Inserted)-domain found between blades 2 and 3 of a seven-bladed $\beta$-propeller structure at its N-terminal end. For this particular subset of integrins, the $\alpha$ subunits are involved in ligand binding via their MIDAS$^1$ motifs (3–6). The $\beta$ subunit is linearly organized into an N-terminal PSI-domain (for Plexins, Semaphorins, and Integrins) (7), a spacer region, an I-domain like structure (also known as the I-like domain or $\beta$ A-domain), a mid-region, a cysteine-rich region containing four tandem IEGF (Integrin Epidermal Growth Factor)-domains, and a terminal domain followed by the transmembrane and cytoplasmic segment (Fig. 1A). In the integrins without an I-domain in their $\alpha$ subunits, the $\beta$ I-like domain participates directly in ligand binding (8). However, in the integrins with an I-domain in their $\alpha$ subunits, the $\beta$ I-like domain may, in addition, serve a regulatory role in the integrin-mediated adhesion (9).

Previously, we constructed integrin $\beta_2/\beta_7$ chimeras in which the N-terminal region (NTR, the combined PSI domain and spacer segment), I-like domain, and the mid-region of the $\beta_2$ subunit were replaced with those of the $\beta_7$ subunit (10). The epitopes of five $\beta_2$-specific mAbs were mapped by using these chimeras and were found to require both NTR and mid-region for their expression. Coupled with the observation that removal of the I-like domain did not affect the folding of the NTR-midregion complex, we concluded that the NTR and mid-region interact extensively. This conclusion was in agreement with the crystal structure of $\alpha_2\beta_2$, in which the I-like domain of the $\beta_2$ subunit is connected via its N and C termini to a hybrid domain formed by the spacer and mid-region, assuming an I-set immunoglobulin fold (11). The function of the hybrid domain has been suggested to involve the shape shifting of the I-like domain in $\alpha_2\beta_2$ (12, 13). Various mAbs (activating, inhibitory, and reporter) against integrins have provided much information on the regulation of integrin function (1, 14, 15). Therefore, in our previous study, the properties of the five anti-$\beta_2$ mAbs to the hybrid domain were investigated. Of note, 7E4 abrogated mAb KIM185-activated LFA-1-mediated adhesion to ICAM-1 but not Mg$^{2+}$/EGTA-activated LFA-1/ICAM-1 binding. Although the end effect of both activating agents on LFA-1 is similar, their modes of action differ. Whereas the epitope and site of action of KIM185 lie in the IEGF-4 and ICAM-1, the effect of Mg$^{2+}$/EGTA lies predominantly on the MIDAS and ADMIDAS of the $\beta$ subunit (1). Taken together, we reasoned that the properties exhibited by 7E4 provide a unique opportunity to address how the activation signal is propagated via the hybrid domain of LFA-1.

To this end, we extended our investigation by fine mapping the epitope of 7E4 using human/mouse chimeras and amino acid substitution analyses. We were able to locate the key residue for the 7E4 mAb. In addition, the allosteric inhibitory property of 7E4 on LFA-1/ICAM-1 binding was examined by using $\alpha_1$ and $\beta_2$ variants, which confer constitutive activation of the resultant heterodimer.

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EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The following antibodies were from various sources: MHHM24 (anti-αL) (17) was obtained from A. J. McMichael (John Radcliffe Hospital, Oxford, UK); KIM185 (anti-β2) (18) was from M. K. Robinson (Celltech, Slough, UK); MEM48 (anti-β2) (19) was from V. H. Farndale (Molecular Genetics, Prague, Czech Republic); KB43 (anti-αX) (20) was from K. Pulford (LRF Diagnostic Unit, Oxford, UK); 7E4 was from Beckman Instruments. ICAM-1/Fc was prepared as described previously (21). Mouse cDNA library (macrophage LPS stimulated) was a gift from K. F. Nolan (Sir William Dunn School of Pathology, Oxford, UK).

cDNA Expression Constructs—The αL and β2 cDNA in the expression vectors pcDNA3 (Invitrogen) were described previously (22). β2 human mouse chimeras were constructed using standard molecular biology techniques. The mouse β2 fragments Mo(Met1–Asp77), Mo(Met1–Glu298), and Mo(Glu298–Asn584) were generated by PCR using a mouse cDNA library as template. An EcoRV site is found in the mouse CD18 cDNA inclusive of the codons for the invariant residues Asp-Ile at positions 77–78 (see Fig. 1B). An EcoRV site was introduced into the equivalent position in the human CD18 cDNA. Mo(Met1–Glu298) was inserted into the NheI and EcoRV sites of CD18-pcDNA3.1/zeo(+) that had their corresponding human β2 fragments removed. Mo(Met1–Glu298) was inserted into the NheI and EcoRV sites and Mo(Glu298–Asn584) into the EcoRV and SacII sites of CD18-pcDNA3.1/zeo(+) (23) that had their corresponding human β2 fragments removed. They were named pβ2HuMoA, pβ2HuMoB, and pβ2HuMoC, respectively. pβ2HuMoA was constructed by inserting Mo(Glu298–Asn584) into pβ2HuMoC that had the corresponding fragment removed. Amino acid substitutions on the mouse or human β2 constructs were made using QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The constructs bearing CD18 (24), N3518 (25), 539C3 (20), β2C23*, a β2 truncation mutant in which the codon of Cys483 (the 23rd of the 56 extracellular cysteines of the β2 subunit) was substituted to a stop codon (26), and α,L-C, with an engineered disulfide bond at K312C and K319C, were described previously. All constructs were verified by sequencing (DNA Sequencing Facility, Department of Biochemistry, University of Oxford, Oxford, UK). The initiation methionine is assigned number 1 in the protein sequence.

Cell Culture and Transfection—COS7 cells (ATCC, Manassas, VA) were cultured in complete media containing the following: RPMI 1640 with l-glutamine (Invitrogen), 10% (v/v) heat-inactivated fetal calf serum (Sigma), and 100 μg/ml kanamycin. Transfections were performed using the DEAE-dextran method as described previously (10, 20).

293T cells (ATCC, Manassas, VA) were cultured in complete media containing the following: Dulbecco’s modified Eagle’s medium with l-glutamine (JRH Biosciences Inc., Australia), 10% (v/v) heat-inactivated fetal calf serum, and 100 IU/ml penicillin and 100 μg/ml streptomycin. Constructs (3.5 μg) were transfected into 293T cells using the calcium chloride method as described previously (23) were described previously. All constructs were verified by sequencing (DNA Sequencing Facility, Department of Biochemistry, University of Oxford, Oxford, UK). The initiation methionine is assigned number 1 in the protein sequence.

Cell Adhesion Assay—For analysis of LFA-1-mediated adhesion to ICAM-1, the wells of Polysorb microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μg/ml of ICAM-1/Fc in phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin was added to each well. The wells were blocked with 5% (w/v) bovine serum albumin in phosphate-buffered saline containing 0.1% (v/v) Tween 20 for 45 min at 4 °C. Stained cells were washed once and fixed in 1% (v/v) formaldehyde in phosphate-buffered saline. Cells were analyzed by a FACScan flow cytometer (BD Biosciences). Data were analyzed using the CellQuest software (BD Biosciences). Expression index was calculated by EI = %GP × MFI/100, where EI is expression index, %GP is % cells gated positive, and MFI is mean fluorescence intensity.

Flow Cytometry—Cells were incubated with primary mAb, at 10 μg/ml unless otherwise stated, in RPMI 1640 for 1 h at 4 °C. They were then washed twice and incubated with fluorescein isothiocyanate-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 phosphate-buffered saline. Cells were analyzed on a FACScan flow cytometer (BD Biosciences). Data were analyzed using the CellQuest software (BD Biosciences). Expression index was calculated by EI = %GP × MFI/100, where EI is expression index, %GP is % cells gated positive, and MFI is mean fluorescence intensity.

RESULTS

Epitope Mapping—In a previous study (10), we reported the mapping of several anti-β2 mAbs using β2/β2 hybrid chimeras. The epitope of mAb 7E4 was further studied by using human/mouse β2 chimeras (β2HuMoA). Four chimeras were generated: β2HuMoA contains the Met1 to Asn584 residues of the mouse β2 sequence; β2HuMoB contains the Met1 to Asp77 residues; β2HuMoC contains the Met1 to Glu298 residues; and β2HuMoD contains the Glu298 to Asn584 residues (Fig. 1A). These chimeras were co-transfected with αL cDNA into COS7 cells and analyzed by flow cytometry. The mAb KIM185 has its epitope located at EGF-4/TD (Tail Domain) of the β2 subunit (16); hence it was included to monitor surface expression of the β2 wild-type and chimeric subunits. The expression of the 7E4 epitope was greatly reduced on the αLβ2HuMoA transfectants (Table I) but was not significantly affected on the αLβ2HuMoB and αLβ2HuMoC transfectants. However, minimal expression...
Expression of antibody epitopes on human/mouse chimeric α₁β₂ integrin subunits

| α₁β₂ transfectants | 7E4 | KIM185 |
|--------------------|-----|--------|
| α₁β₂ Hu (wt)       | 100 | 100    |
| α₁β₂ Hu/N372S/A373T| 111 | 64     |
| α₁β₂ Hu/V390A/T391S| 92  | 100    |
| α₁β₂ Hu/I409V      | 89  | 95     |
| α₁β₂ Hu/N394G/Q395K| 124 | 95     |
| α₁β₂ Hu/P396S      | 63  | 100    |
| α₁β₂ Hu/V407N      | 9   | 105    |
| α₁β₂ Hu/I436T      | 139 | 53     |
| α₁β₂ Hu/T416M      | 81  | 92     |
| α₁β₂ Hu/T418S      | 85  | 102    |
| α₁β₂ Hu/I436T      | 145 | 183    |
| α₁β₂ Hu/L442R      | 122 | 173    |

α Epitope expression is measured using $EI = %GP \times MFI$, where $EI$ is expression index, $%GP$ is $%$ cells gated positive, and $MFI$ is mean fluorescence intensity. Values are calculated using CellQuest software and presented with respect to the expression of the same mAbs on the α₁β₂ wild type.

The mAb KIM185 maps to the C-terminal of the human β₂ subunit and is used as a positive control (16).

“Knock-out” mutants were constructed on the β₂ Hu/MoD background, in which the PSI, I-like domain, hybrid domain (made up of the spacer and mid-region), IEGF-1, -2, and -3, and part of IEGF-4 (Fig. 1A) were those of the mouse β₂ subunit. The 7E4 epitope expression was restored in the β₂ Hu/MoA(N407V) variant which had the mouse Asn changed to a human Val residue (Table III). Taken together, the above experiments suggest that the key residue for the 7E4 epitope is the valine residue at position 407 of the human β₂ subunit.

Effect of 7E4 on the Adhesion of MOLT-4 and Transfectants Expressing Wild-type and Constitutively Active LFA-1 to ICAM-1—Previously we have shown that the mAb 7E4 blocks KIM185-activated LFA-1-mediated adhesion to ICAM-1 but not adhesion activated by Mg²⁺/EGTA (10). In this work, we determined whether 7E4 can block the adhesion of several “constitutively active” LFA-1 to ICAM-1. β₂, NV1 is a β₂/β₁ chimera in which the cysteine-rich region of the β₂ subunit was replaced that of β₁ (23). β₂ R593C is a mutant found in an LAD-1 patient that supported expression of a constitutively active LFA-1 in an in vitro transfection system (20). Since then, two other mutations have been investigated, β₂ C366S described for the Irish Setter (24) and β₂ N351S for another LAD-1 patient (25), both were found to support the expression of constitutively active LFA-1. ² 7E4 was found to abolish the adhesion of 293T transfectants expressing the LFA-1 variants.

² S. K. A. Law, unpublished observations.
Expression of the 7E4 epitope on 293T transfectants with variant αL and β2 subunits.

| Epitope expression | IB4 | 7E4(20) | 7E4(10) | 7E4(3) | KB43 |
|-------------------|-----|--------|--------|--------|------|
| αL wt | β2 wt | 55.4 | 39.5 | 37.2 | 44.5 | 2.0 |
| αL wt | β2C36S | 11.4 | 7.3 | 6.5 | 7.6 | 3.3 |
| αL wt | β2N351S | 44.3 | 34.5 | 34.7 | 30.0 | 2.9 |
| αL wt | β2R593C | 34.2 | 21.9 | 25.4 | 16.9 | 2.0 |
| αL wt | β2NV1 | 19.1 | 20.8 | 20.8 | 22.0 | 2.1 |
| αL wt | β2C23* | 32.2 | 21.6 | 23.9 | 29.3 | 1.9 |
| αL,C-C wt | β2 wt | 33.3 | 24.1 | 26.6 | 30.0 | 2.0 |

a Expression indices are shown.

b The absolute level of αL,β2-LFA-1 expression, as indicated by the heterodimer-specific mAb IB4, is quite variable among the different transfectants. The expression levels of LFA-1 with the β2C36S and β2NV1 are consistently lower than the wild type and other LFA-1 variants. Staining of IB4 was done at 10 μg/ml.

c Cell staining of 7E4 were carried out in three different concentrations of mAb at 20, 10, and 3 μg/ml, respectively.

d Staining of KB43, an irrelevant mAb, was carried out at 10 μg/ml.

**DISCUSSION**

Based on electron micrographs of the representative integrins αIIbβ3 and α9β1, the general structure of the integrin molecule had been taken as a two-pronged plug having a glob-
ular head and two legs anchoring them into the membrane (33–35). From numerous functional studies and other studies, it was also generally accepted that conformational change is an integral component in regulating integrin adhesiveness (36). The conformational change had not been defined, but much experimental data pointed to the model that a resting integrin is highly constrained and a release of these constraints results in activation. The x-ray solution of α1β2 showed a structure in which the two legs were bent, folding the integrin heterodimer into a compact conformation (11). Extensive analyses of the electron micrograph images of the recombinant extracellular fragment of the α1β2 integrin in different activation states were performed (33). It was found that the resting integrin in Ca\(^{2+}/Mg^{2+}\) corresponds to the compact bent conformer, whereas the active integrin induced by Mn\(^{2+}\) or the cyclic RGD ligand corresponds to the extended conformer. These observations were in line with the “switchblade” model of integrin activation proposed based on the NMR analysis of the structure of the IEGF-2 and -3 of the β2 integrin subunit (37).

Previously, we have reported the effect of the mAb 7E4 on LFA-1-mediated adhesion of MOLT-4 to immobilized ICAM-1 (10). If LFA-1 was activated by the mAb KIM185, 7E4 can block the adhesion. However, if Mg\(^{2+}/EGTA\) was used to activate LFA-1, 7E4 has no effect. The switchblade model suggests that resting LFA-1 is presumably bent, and activation involves the transition of the molecule to the extended conformation. Support for this model for both KIM185 and Mg\(^{2+}/EGTA\)-mediated activation of LFA-1 can be found with the study of the mAb KIM127. The epitope of the mAb KIM127 has been mapped to the IEGF-2 and IEGF-3 of the integrin β2 subunit (16, 38). Superposition of the NMR structure of these two domains on the α1β2 template (11) showed that the residues forming the epitope are buried (37). It has been shown that the expression of the KIM127 epitope is associated with the activation of LFA-1, which would require the binding of the mAb KIM185 (39) or the incubation of the cells in Mg\(^{2+}/EGTA\). Taken together, both KIM185 and Mg\(^{2+}/EGTA\) activate LFA-1 by exposing the KIM127 epitope, which may be interpreted as the conversion of the bent conformer into the extended conformer. KIM185-activated adhesion must start with the binding of the antibody to LFA-1 at its epitope site in the IEGF-4 and βT domain of the integrin α2 subunit (16). This binding induces a conformational change rendering LFA-1 the capacity to bind ICAM-1 at its ligand-binding site that is located at the I-domain of the α2 subunit. Binding of 7E4 to the hybrid domain may thus be viewed as a blockade in communication between the activation site, i.e. KIM185-binding site, and the ligand-binding site. Mg\(^{2+}/EGTA\), on the other hand, is likely to induce the conformational change of LFA-1 at the MIDAS and ADMI-DAS sites of the I-like domain of the β2 subunit (1). Because the divergent -binding sites and the ligand-binding sites are both distal to the hybrid domain, 7E4 does not affect ICAM-1 binding induced by Mg\(^{2+}/EGTA\).

If 7E4 blocks LFA-1 mediated adhesion by preventing signals originating from regions proximal to the membrane to the ligand-binding site, we should be able to test this hypothesis by using a number of LFA-1 variants that are constitutively active. Indeed, our results presented here showed segregation of these variants. The constitutive activity of LFA-1 due to modification on the membrane side of the hybrid domain is abolished by 7E4. These variants are α1β2R593C (20), α1β2NV1 (23), α1β2C23* (26), and LFA-1 activated by KIM185 that binds to the “foot” of the β2 subunit. Most interestingly, also in this list is the LFA-1 variant with the C36S mutation (24) in the PSI domain. Because the PSI domain is folded back under the hybrid domain, the mutation C36S can therefore be considered to be located on the membrane proximal side of the hybrid domain. Furthermore, activation of LFA-1 by PMA, originating from the inside the cell, was attenuated by 7E4. The corollary is that LFA-1 with modification distal to the hybrid domain should not be affected by 7E4 because they exert their effects to the ligand-binding site beyond the hybrid domain. They include the activation by Mg\(^{2+}/EGTA\) (10), the LAD mutation N351S in the I-like domain of the β2 (25), and the engineered disulfide lock in the I-domain of the α1 subunit (27, 30).

The β I-like domain is proposed to regulate the activity of the α I-domain. It has been known that mutations in the MIDAS residues in the I-like domain of the β2 integrin subunit would not affect LFA-1 expression, but the LFA-1 variants were incapable of ligand binding, irrespective of being treated with activating mAbs or Mg\(^{2+}/EGTA\) (40–42). Subsequently, an invariant glutamate residue, located in the linker between the I-domain and the β-propeller in all I-domain containing α subunits had been identified. Mutation of this glutamate residue, Glu\(^{335}\) in the α1 subunit, rendered the resultant LFA-1 inactive (43). Taken together, these results suggest that an integral part of LFA-1 activation may lie in the binding of the β2 MIDAS motif with Glu\(^{335}\) in α1. This hypothesis received strong support from experiments in a recent publication in which the Glu\(^{335}\) of the α2 subunit, and either one of the two residues on the β2 subunit, Tyr\(^{137}\) or Ala\(^{232}\), were converted to cysteines (44). The two resultant LFA-1 variants were found to have the α2 and β2 subunits disulfide-linked and are constitutively active with respect to ICAM-1 binding. Because both Tyr\(^{137}\) and Ala\(^{232}\) are in the proximity of the MIDAS motif of the β2 I-like domain, the LFA-1 variants may be considered as being “locked” into an active conformation similar to when the β2 MIDAS binds to the invariant Glu in the α1 subunit.

The epitope of 7E4 is complex and requires the split regions of the hybrid domain for expression (10). By using single residue substitutions described in this article, the species-specific residue was identified to be Val\(^{477}\). Other residues, common to both human and mouse, must also contribute to the conformation of the epitope. Modeling on the α1β2 template showed that it is close to the C-terminal helix of the I-like domain (Fig. 5). Thus, binding of 7E4 to the hybrid domain may alter its capacity to interact with the I-like domain, preventing LFA-1 from being activated perhaps by disabling the binding of the β2 MIDAS motif to the invariant glutamate in α1. In recent studies on the α1β1 integrin (12, 13), it was suggested that the “swinging” of the hybrid domain away from the I-like domain is associated with the integrin being activated. In this article, our data do not provide support nor contradict this particular relative motion of the hybrid domain in integrin activation. However, it does concur that the hybrid domain plays a role in the regulation of LFA-1 activity.

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