Functional characterization of the beta2 integrins

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Functional Characterization of the β2 Integrins

A Thesis Submitted for the Degree of Doctor of Philosophy

at Nanyang Technological University

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at

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Nanyang Technological University

Abstract

αLβ2 (CD11a/CD18, LFA-1), αMβ2 (CD11b/CD18, Mac-1) and, αXβ2 (CD11c/CD18, p150, 95) are members of β2 integrins involved in leukocyte function during immune and inflammatory responses. The first part of this study, the epitope of a β2-specific antibody MEM148 was fine mapped to the hybrid domain and it is dependent on the movement of the hybrid domain upon activation. Furthermore, we made use of the activation-reporter property of MEM148 to analyze the different αLβ2 affinity states required for ICAM-1 and ICAM-3 binding. 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.

The second part of this study also compared the activation mechanism of I-domain of αLβ2 and αMβ2. We found that mutation of the isoleucine residue in the proposed "socket for isoleucine" in full-length αL I-domain does not lead to an active αLβ2, although mutation of the equivalent residue in αM I-domain does convey constitutive activity to αMβ2.

Finally, we showed that the src family kinase Hck co-capped selectively with αMβ2 but not αLβ2 or αXβ2. This was disrupted when the αM cytoplasmic tail was substituted with that of αL or αX. Co-capping was recovered by αL or αX cytoplasmic tail truncation or forced separation of the α and β cytoplasmic tails via salt-bridge disruption.

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References

Publications from this study

1. Tang RH, Tng E, Law SK, Tan SM. (2005)
Epitope mapping of monoclonal antibody to integrin alphaL beta2 hybrid domain suggests different requirements of affinity states for intercellular adhesion molecules (ICAM)-1 and ICAM-3 binding. ***J Biol Chem***. 2005 280(32):29208-29216.

2. Walters SE, **Tang RH,** Cheng M, Tan SM, Law SK. (2005)
Differential activation of LFA-1 and Mac-1 ligand binding domains.***Biochem Biophys Res Commun***. 2005 337(1):142-148.

3. Tang RH, Law SK, Tan SM. (2006)
Selective recruitment of Src family kinase Hck by leukocyte integrin αMβ2 but not αLβ2 or αXβ2. ***FEBS Lett.*** In press.

Publication from other study

1. Geng X, **Tang RH,** Law SK, Tan SM. (2005)
Integrin CD11a cytoplasmic tail interacts with the CD45 membrane-proximal protein tyrosine phosphatase domain 1. ***Immunology***. 115(3):347-357.

Abbreviations

ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
DMSO	dimethyl sulphoxide
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra acetic acid
EGTA	ethylene-glycol-bis(β-aminoethylether)-tetra acetic acid
FACS	fluorescent activated cell sorter
HEPES	N-[2-hydroxyethy]piperazine-N'-[2-ethanesulfonic acid]
PIPES	piperazine-N', N'-bis-[2-ethanesulfonic acid]
kbp	kilobase pairs
kDa	kilodaltons
Ig	immunoglobulin
IP	immunoprecipitation
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MW	molecular weight
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
PMSF	phenylmethylsulphonylfluoride
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl)-aminoethane
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

The single-letter and triplet codes for amino acid residues are used. Restriction enzymes are referred to by their three-letter names derived from that of the source microorganism. Other abbreviations are defined in the text where first encountered.

Chapter One: Introduction

1.1 Overview

Cell adhesion and migration contribute to biological processes such as differentiation, embryonic development, and wound healing as well as to the progression of diseases and pathological conditions such as cancer and inflammatory responses that can result from either acute or chronic exposure to environmental toxicants, such as cancer and inflammatory responses (Akiyama, 1996).

Most leukocytes are normally nonadherent, and circulate in the bloodstream as round and non-polarized cells (Blois et al., 2005). However, leukocytes also need to interact with other types of cells in various ways. The interaction of the leukocyte with blood vessel endothelium and subsequent recruitment of leukocytes into tissue is a multi-step process (Springer, 1994). Leukocytes attach loosely to endothelium by the interaction of selectins with carbohydrate ligands on the endothelium. When this interaction occurs, leukocytes can be activated by, for example, immobilized chemokine on the surface of endothelium and will trigger firm adhesion via the integrins (Campbell et al., 1998). This is followed by the polarization of the leukocyte with membrane protrusion (lamellipodium) forming a leading edge guiding the cell body containing the nucleus, and an elongated uropod at the rear end of the cell (Sanchez-Madrid and del Pozo, 1999). Leukocytes migrate on the endothelium and invade between neighboring endothelial cells. The migration process includes (1) pseudopod extension and attachment to the surface, (2) cell polarization, (3)

contraction of the cell body, (4) release of rear attachment sites, and (5) uropod retraction (Springer, 1994). Effective leukocyte transendothelial migration requires interactions between extracellular matrix proteins such as fibronectin, laminin and collagens, with specific adhesion receptors including integrins.

Integrins are a large family of heterodimeric (α/β subunits) cell adhesion molecules that mediates intracellular signal transduction (Hynes, 2002). They are crucial for maintaining cell physiology and are therefore required in numerous biological processes. In the immune system, integrins support leukocyte adhesion on endothelium and are involved in leukocyte tissue homing (Laudanna et al., 2002). Unlike selectins, another group of cell adhesion molecule that slows down the rolling of leukocytes on endothelium, the integrins mediate firm adhesion of these cells, allowing them to undergo transendothelium migration. In addition, integrins also act as co-stimulatory molecules for T cell receptors in the immunological synapse (Dustin, 2001), mediate interactions with bacteria (Strijp et al., 1993) and phagocytosis (Cain et al., 1987).

Each integrin is a heterodimer composed of an α and a β subunit via non covalent association (Hynes, 2002). In humans, 18 α subunits and 8 β subunits associate to form 24 different receptors identified to date (Figure 1.1), and the rules which regulate heterodimer formation is not clear. Tissue expression patterns of integrins vary. For example, β1 integrins are expressed on almost all cell types (Zutter and Santoro, 1990), whereas β2 integrins expressions are restricted to leukocytes (Springer, 1990). Individual integrins can often bind more than one ligand and many integrins also share common

ligands (Plow et al., 2000). For example, integrin αMβ2 recognizes ligands ICAM-1, iC3b, fibrinogen and factor X while fibronectin is a ligand of 12 different integrins (Table 1.1). In addition, a particular integrin may exhibit varying ligand specificities in different cell types, as observed in α2β1 which is specific for both collagen and laminin on most cell types, but is specific for collagen and not laminin on platelets (Hynes, 1992). The major extracellular ligands of integrins are summarized (Plow et al., 2000), (Table 1.1).

1.2 Integrin structure

The integrins are type I membrane glycoproteins formed by non-covalent association between an α and a β subunit (Tan and Law, 2006). The integrins consist of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail (Figure 1.2). The β subunits contain many extracellular disulphide bonds and are N-glycosylated, mainly containing high mannose and high molecular weight complex type sugars (Asada et al., 1991). The extracellular region of the integrin α subunit is linearly organized to contain from the N-terminus a β-propeller, thigh, calf-1 and calf-2 domains (Takagi et al., 2002). In some α subunits, an I-domain is inserted in the β-propeller. The β subunits contain from the N-terminus a PSI domain, hybrid domain, I-like domain, four I-EGF repeats and a tail domain. Detailed discussion of these domains will follow in other parts of this chapter.

A major advance in the understanding of integrin structure and function came from the resolution of the crystal structure of the extracellular domain of integrin αVβ3 (Xiong et

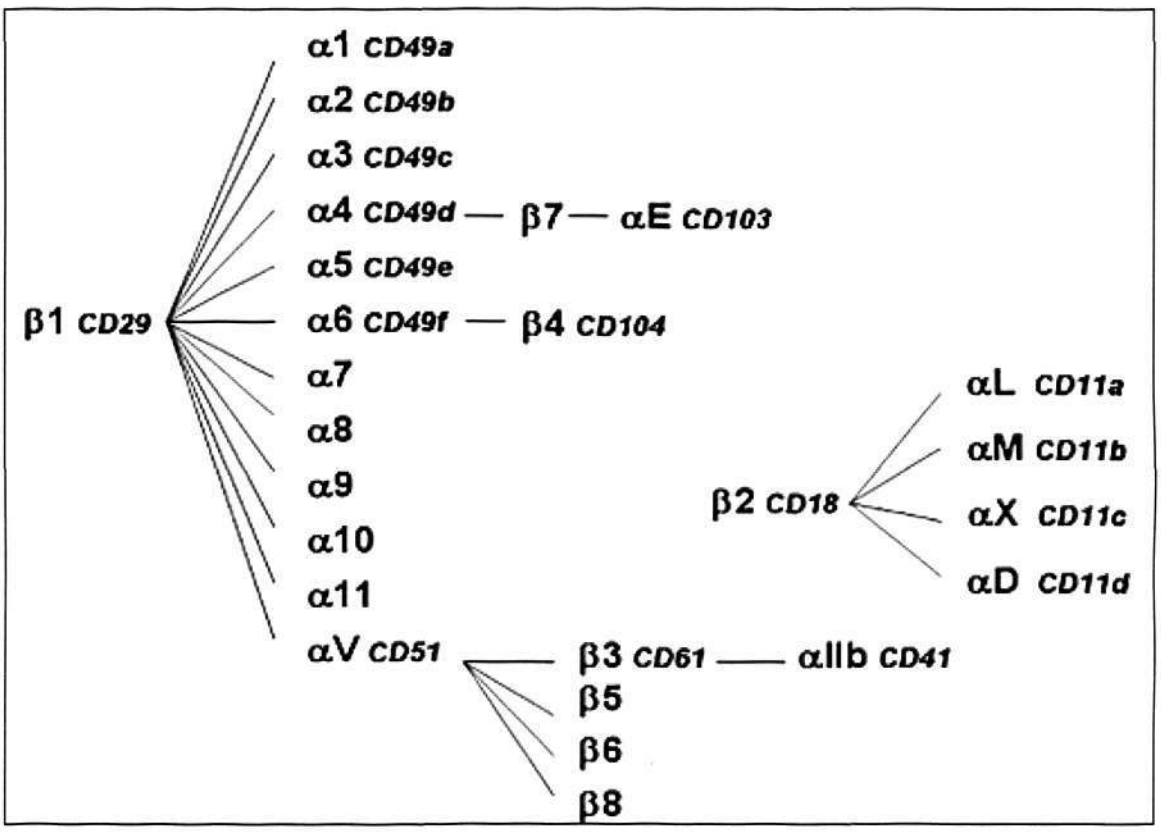


FIG 1.1. The integrin superfamily. Integrins consist of α and β subunits in different but specific combinations. In humans, 24 different heterodimers are reported.

Adenovirus penton base protein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
Bone sialoprotein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
Borrelia burgdorferi	$\alpha_{IIb}\beta_3$
Candida albicans	$\alpha_M\beta_2$
Collagens	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{11}\beta_1$, $\alpha_{1b}\beta_3$
Denatured collagen	$\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Cytotactin/tenascin-C	$\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$
Decorsin	$\alpha_{IIb}\beta_3$
Disintegrins	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
E cadherin	$\alpha_E\beta_7$
Echovirus 1	$\alpha_2\beta_1$
Epiligrin	$\alpha_3\beta_1$
Factor X	$\alpha_M\beta_2$
Fibronectin	$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$
Fibrinogen	$\alpha_5\beta_1$, $\alpha_M\beta_2$, $\alpha_v\beta_3$, $\alpha_x\beta_2$, $\alpha_{IIb}\beta_3$
HIV Tat protein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
iC3b	$\alpha_M\beta_2$, $\alpha_x\beta_2$
ICAM-1	$\alpha_L\beta_2$, $\alpha_M\beta_2$
ICAM-2,3,4,5	$\alpha_L\beta_2$
Invasin	$\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$
Laminin	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$
MAdCAM-1	$\alpha_4\beta_7$
Matrix metalloproteinase-2	$\alpha_v\beta_3$
Neutrophil inhibitory factor	$\alpha_M\beta_2$
Osteopontin	$\alpha_v\beta_3$
Plasminogen	$\alpha_{IIb}\beta_3$
Prothrombin	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Sperm fertilin	$\alpha_6\beta_1$
Thrombospondin	$\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
VCAM-1	$\alpha_4\beta_1$, $\alpha_4\beta_7$
Vitronectin	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$
von Willebrand factor	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$

Table 1.1. Integrin extracellular ligands. (Adapted from Plow et al., 2000).

al., 2001; Xiong et al., 2002). Previous electron microscopy images of integrins revealed a molecule having a large globular "head" with two "stalks" resembling a two-pin plug (Nermut et al., 1988). Indeed, the αVβ3 crystal structure (Figure 1.3) revealed two "stalks" supporting a globular "head", which is formed by the α subunit β-propeller domain and the β subunit I-like domain. Perhaps most importantly, the crystal structure of αVβ3 revealed a surprising quaternary structure. The "stalks" of both subunits were severely bent bringing the "head region", which includes the β-propeller and thigh domain in the α subunit, and the I-like domain and hybrid domain of the β subunit into close proximity of the presumed cell membrane (Figure 1.2). This quaternary structure does not conform to previous observations of the integrin structures in solution (Du et al., 1993; Hantgan et al., 1999). One possible reason of the sharp bending of the integrin is that the molecule possesses intrinsic flexibility at the genu that allows for bending in solution. The crystal packing forces may favor the bent structure over others and extensive contacts in the crystal may stabilize this form (Xiong et al., 2003a). Another possibility is that the bent form is the dominant form in solution. Recent EM images of integrins support the latter possibility (Takagi et al., 2002b). However, the crystal structure of integrin-ligand complex is not resolved, which is a limitation to the characterization of integrin activation states.

1.2.1 *The α subunit*

The α subunits are 25-65% homologous and vary in size between 120 and 180 kDa. It is linearly organized from the N to the C terminus into seven β-propeller repeats (in I-

domain-containing integrins, I-domain is inserted between repeat II and III), thigh domain, calf-1 and calf-2. This is followed by a transmembrane domain and a cytoplasmic tail (Takagi et al., 2002) (Figure 1.2) .

1.2.1.1 α subunit head region

The N-terminal region of the integrin α-subunit comprises seven repeats of about 60 amino acids each. These were predicted to form a seven bladed β-propeller domain (Figure 1.4) (Springer, 1997). By analogy with the Gα/Gβ complex in G proteins, it was also predicted that this α subunit β-propeller may associate with the highly conserved I-like domain in the β subunit. These were confirmed by the αVβ3 crystal structure (Xiong et al., 2001).

Each blade of the propeller is a single β-sheet composed of four anti-parallel β sheets. The inner strand (strand 1) of each blade lines the channel at the center of the propeller, with strands 2 and 3 of the same repeat radiating outward, and strand 4 of the next repeat forming the outer edge the of the blade. The β-propeller is made circular by juxtaposition of strand 3 of blade 7 and strand 4 of blade 1. The central channel is lined predominantly with amide and carbonyl oxygen groups from the seven inner strands, with only a few side chains projecting into the cavity (Xiong et al., 2001).

The loops connecting the blades in the β-propeller were originally thought to be EF-hand-like but are now reported to form β-hairpin loops (Xiong et al., 2001; Springer et al., 2000). Four solvent-exposed Ca^{2+} binding sites are found in these hairpin loops between

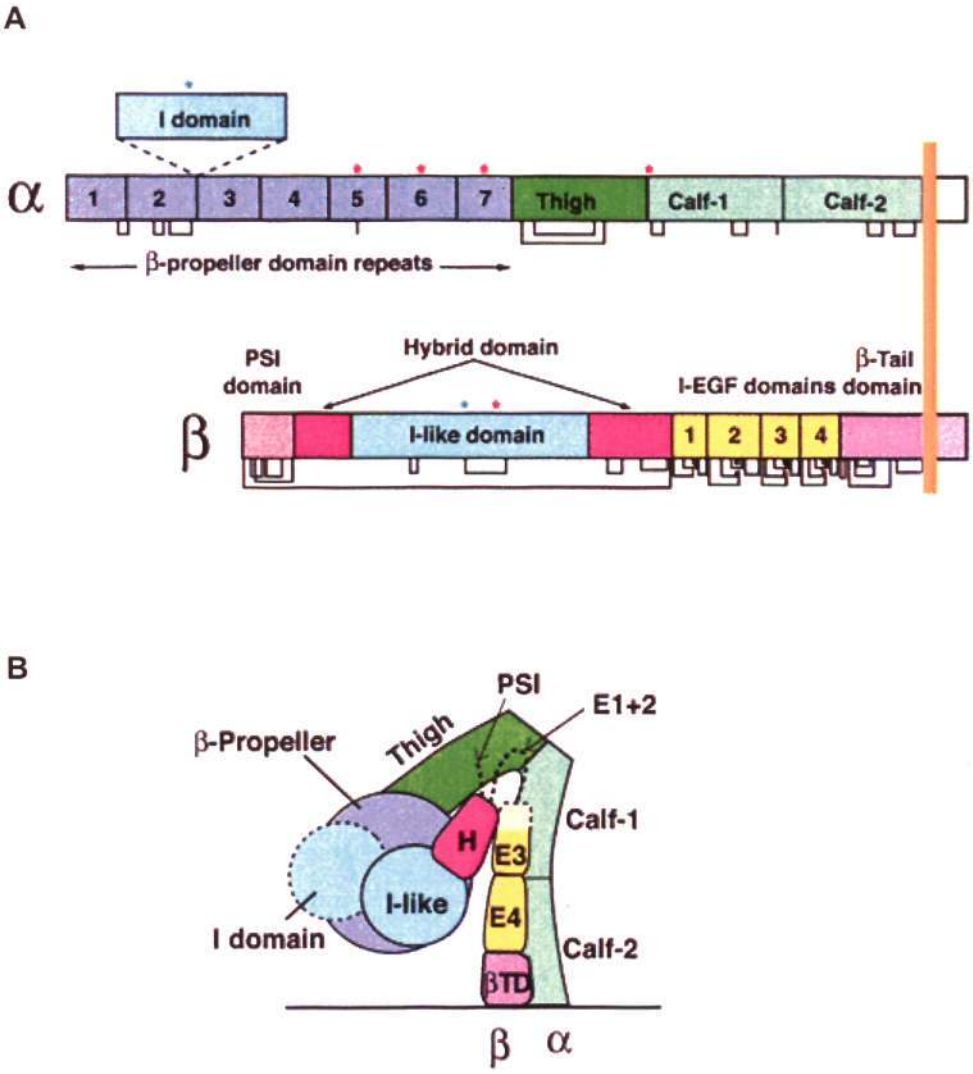


FIG 1.2. Integrin domain organization. (A) Organization of domains within the primary structure of integrin. Depending on the α subunit, it may contain an I-domain insertion as denoted by the dotted line. Asterisks show Mg^{2+} (blue) and Ca^{2+}(red) binding sites. Lines below the stick diagrams show disulfide bonds. (B) Arrangement of domains of a bent integrin αVβ3, with an I domain added. Each domain is color coded as in (A). (Adapted from Takagi and Springer , 2002)

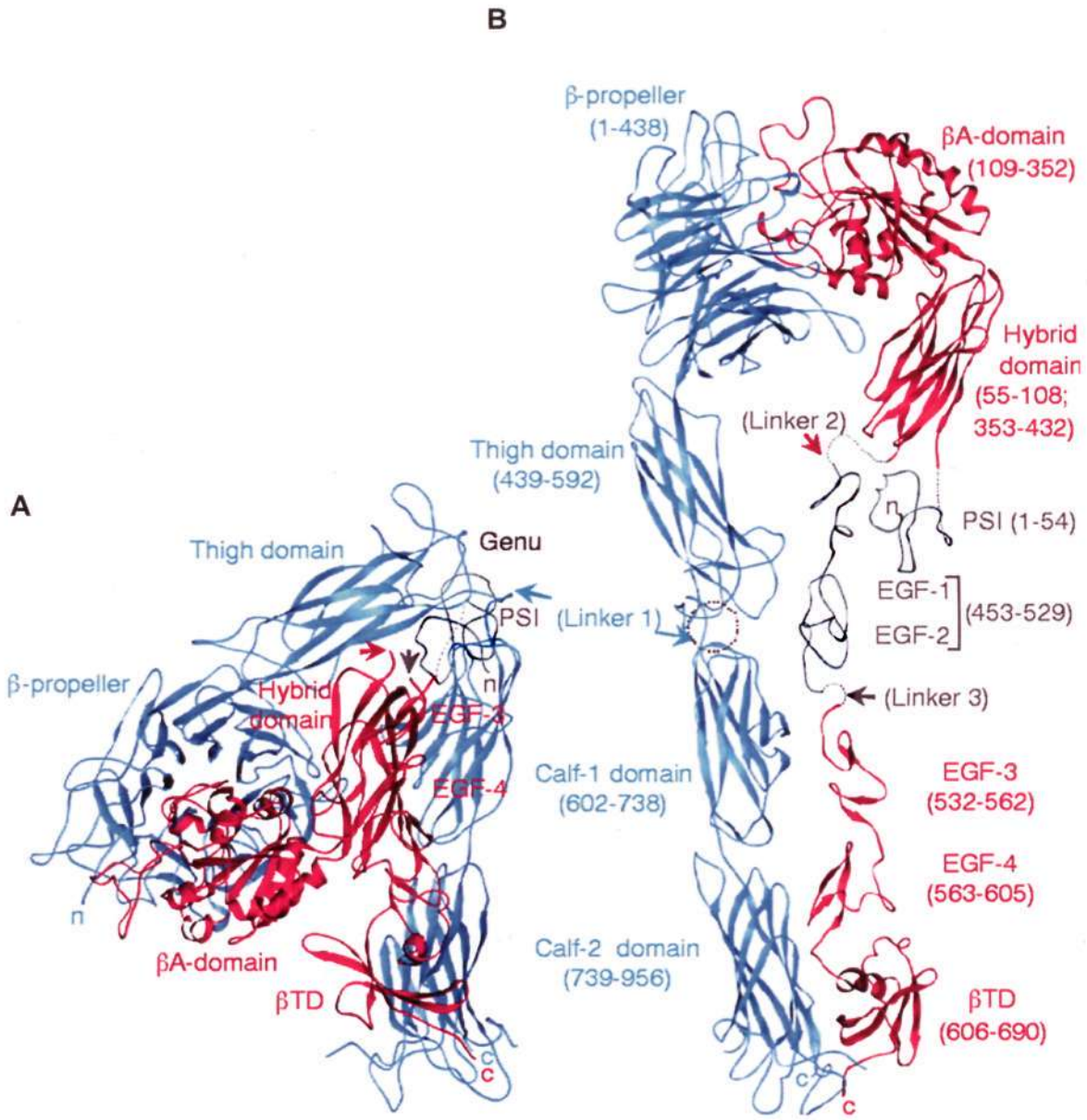


FIG 1.3. Structure of the extracellular segment of αVβ3. (A) Ribbon drawing of crystallized αVβ3 [shown in blue (αV) and red (β3)]. (B) Model of a straightened extracellular segment of αVβ3. (Adapted from Xiong et al., 2001.)

strands 1 and 2 for blade 4-7 at the bottom of the propeller from the crystal structure of αVβ3. The calcium binding residues have the consensus sequences D-h-D/N-x-D/N-G-h-x-D, where 'h' is hydrophobic and 'x' can be any residue.

Nine of the α subunits (α1, α2, α10, α11, αE, αL, αM, αX and αD) contain an 'inserted' or I-domain of approximately 200 amino acids, that is homologous to the von Willebrand factor A-domain (Larson et al., 1989; Colombatti et al., 1991). In these integrins, the I-domain is the major ligand-binding site. The β-propeller domain can also cooperate in the binding of ligands, as in αM (Yuan et al., 1992), or has no direct role, as in αL (Lu et al., 2001b). In integrins that do not have the I-domain, the β-propeller domain appears to directly participate in ligand binding (Humphries, 2000; Xiong et al., 2001). The I-domain is inserted between blades 2 and 3 on the upper surface of the β-propeller. The N and C termini of the I-domain are in close proximity; hence, the addition of an I-domain in the β-propeller results in little change to the overall structure of I-domain containing integrins (Tuckwell, 1999). There is no full structure available of an integrin dimer containing an I-domain but the structure of recombinant I-domains were determined (Emsley et al., 1997; Lee et al., 1995b; Qu and Leahy, 1995). The I-domain adopts the dinucleotide-binding or Rossmann fold, with a central hydrophobic β-sheet surrounded by α-helices (Emsley et al., 1997; Lee et al., 1995b; Mould et al., 2003b; Nolte et al., 1999). The I-domain is central to ligand recognition and binding for the I-domain-containing integrins. Integrin αL subunit lacking an I-domain have been analyzed. The resulting I-domain-less αLβ2 was unable to bind ICAM-1 or ICAM-3 (Leitinger and Hogg, 2000; Yalamanchili et al., 2000).

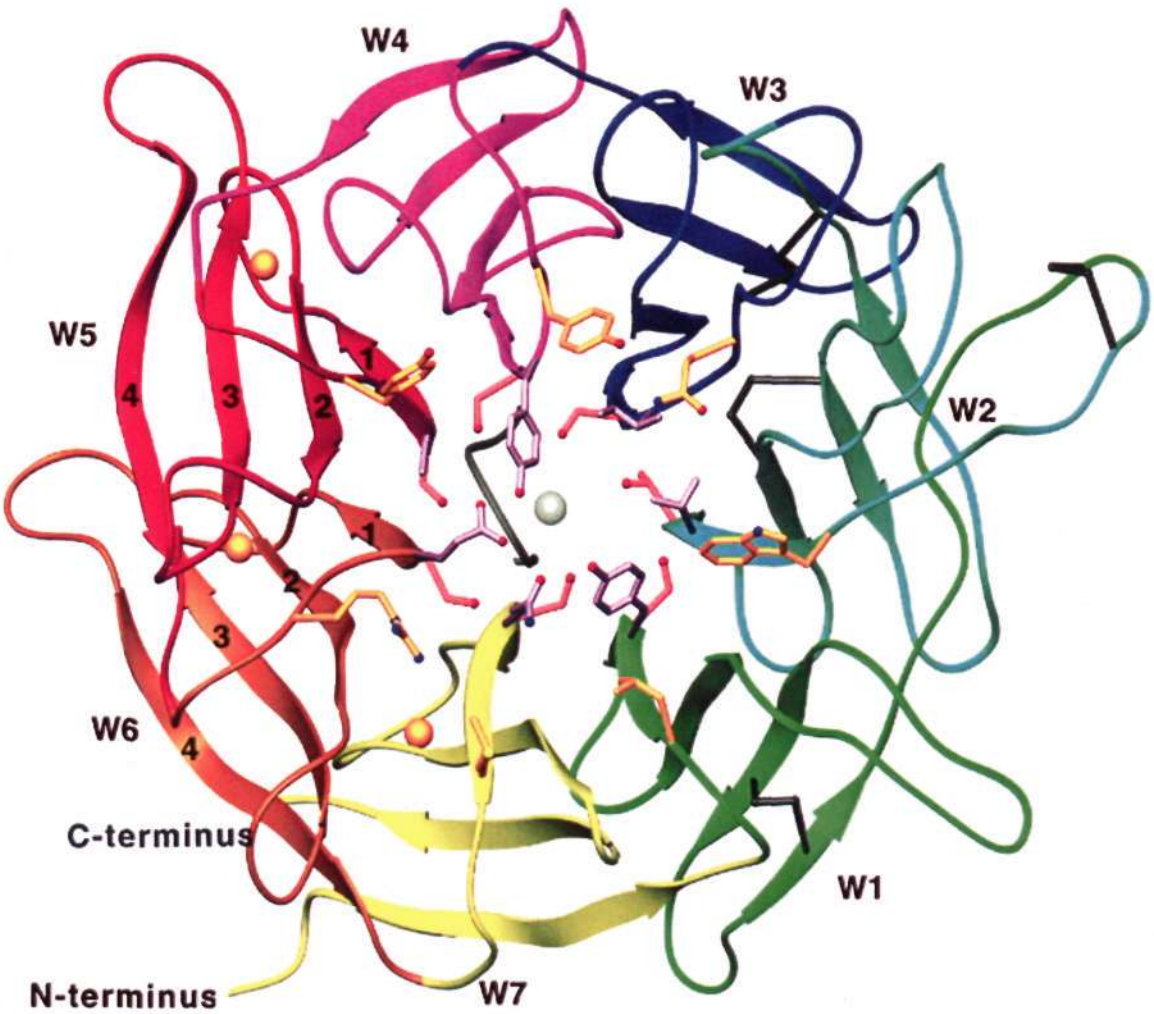


FIG 1.4. α subunit β-propeller. Ribbon diagram of the model for the integrin α4 subunit β-propeller domain, viewed from the top. Each blade of the β-propeller blade is shown in a different color and labeled W1-W7. The strands of each blade are labeled 1-4, from the center. A hypothetical polypeptide finger in the central cavity is grey. Cysteines in disulphides are black. Ca^{2+} ions and a hypothetical Mg^{2+} ion are gold and silver spheres, respectively. (Adapted from Springer, 1997)

Binding of the I-domain is dependent on the divalent cations. A metal ion-dependent adhesion site (MIDAS) is located on the upper face of the I-domain. Its key role in ligand binding is demonstrated by the observation that mutations of the MIDAS are found to abrogate ligand binding by the I-domain of the αLβ2 and αMβ2 (Kamata et al., 1995; Michishita et al., 1993). In the MIDAS, a metal ion is coordinated by three loops from the I-domain, and a glutamate of aspartate acid from the ligand completes an octahedral coordination sphere around the metal. A characteristic 'DXSXS' motif (Asp^{140}-Gly-Ser-Gly-Ser in αM I-domain), located at the β1 strand and the loop between β1 and α1 was found to contribute to three of these sites. Ser^{142}, Ser^{144} and Thr^{209} (at the C-terminal end of the α3-α4 loop) all coordinate directly. Asp^{140} interacted via a water molecule, as did Asp^{242} (at the start of the β4- α5 loop). Mn^{2+} and Mg^{2+} have been shown to enhance ligand binding, whereas high concentration of Ca^{2+} results in inactivity of the integrin (Shimaoka et al., 2002). The αM I-domain has been found to crystallize into two different conformations in the presence of different metal ions (Lee et al., 1995a/b), also referred to as the "Mg^{2+} bound" and "Mn^{2+} bound" (Figure 1.5). In the "Mg^{2+} bound" form, two serines and a threonine make electrostatic bonds to the Mg^{2+} via their side chains; two water molecules also interact with Mg^{2+} and two aspartates make indirect contacts via water molecules. The lack of direct electrostatic bonds between aspartates and Mg^{2+} enhanced the electrophilicity of Mg^{2+} (Lee et al., 1995a). In addition, from the crystal structure of the "Mg^{2+} bound" I-domain, the side chain of a glutamate residue from a neighboring I-domain completes the coordination sphere, which is reminiscence of a liganded I-domain. In the "Mn^{2+} bound" form, the thereby direct electrostatic bond to the Thr^{209} is disrupted whilst a direct electrostatic bond to Asp^{242} is formed, reducing

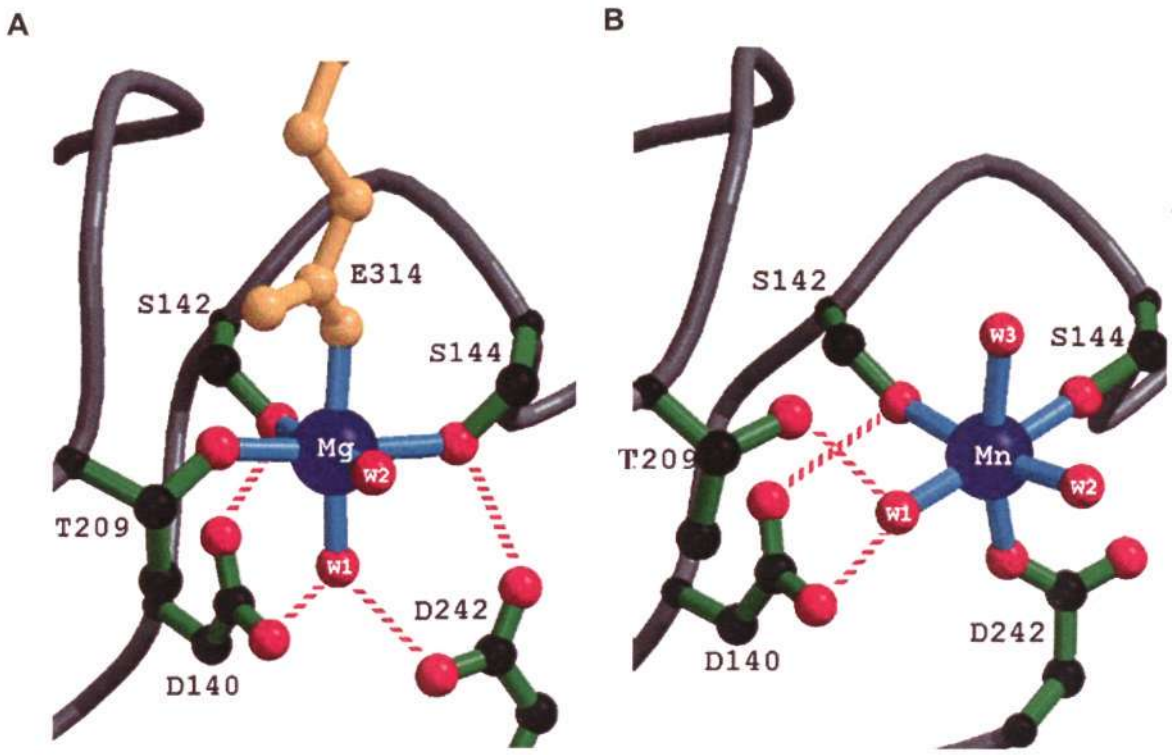


FIG 1.5. Structural comparisons of the αM $Mg^{2+}$and Mn^{2+} I-domains. (A) The MIDAS motif in the Mg^{2+} form. (B) The MIDAS motif in the Mn^{2+} form. The color code is oxygen atom (red), carbon (black), backbone trace (grey) and a glutamate residue from a neighboring molecule (gold). Water molecules are labeled w1–w3. Selected hydrogen bonds are shown as dashed red lines. (Adapted from Lee et al., 1995).

electrophilicity of the metal ion. Water molecules complete the coordination, and there is no equivalent of the exogenous glutamate in this crystal structure. Therefore, the "Mn^{2+} bound" I-domain is proposed to represent the unliganded I-domain (Lee et al., 1995a). Comparison of the two crystal forms reveals a change in metal ion co- ordination which is linked to a large 10Å shift of the C-terminal helix and the burial of two phenylalanine residues into the hydrophobic core of the Mn^{2+} form (Lee et al., 1995a).

1.2.1.2 α subunit leg

C-terminal to the β-propeller domain is the α subunit leg. This region comprises a large portion of the α-subunit extracellular domain of about 500 amino acids. There are three large β-sandwich domains in this region, designated the thigh, calf-1 and calf-2 domains (Xiong et al., 2001). Between the thigh and calf-1 is the "genu", which is capped by a divalent cation. Recent study showed that the genu/calf-1 interface is maintained integrin activation, and that extension occurs by a rearrangement at the thigh/genu interface (Xie, et al., 2004).

1.2.2 *The β subunit*

The integrin β subunit is linearly organized from the N to the C terminus into a PSI domain; a spacer segment (which comes together with the mid region to form a hybrid domain); an I-like domain; a mid region; a cysteine rich region (which shows homology to Epidermal Growth Factor fold) and a membrane proximal tail domain (βTD). This is

followed by a transmembrane domain and a cytoplasmic tail (Takagi et al., 2002) (Figure 1.2).

1.2.2.1 The PSI domain

The N-terminal cysteine-rich region of residues 1–50 shares sequence homology with membrane proteins including plexins and semaphorins. Therefore, it is known as the PSI domain for plexins, semaphorins, and integrins (Bork et al., 1999). The small hydrophobic core of the domain is formed by the highly conserved side chain of Trp^{25}. It contains four cysteine pairs connected in a 1-4, 2-8, 3-6, 5-7 pattern with the final 8^{th} cysteine being located C-terminal to the hybrid domain (Table 1.2) (Xiong et al., 2004). These cysteine rich regions cooperate to restrain the integrin in the inactive conformation (Zang et al., 2001).

1.2.2.2 The I-like domain

Integrin β subunits contain a highly evolutionarily conserved domain of about 240 residues. This domain has low sequence homology to the I-domain but shares similar secondary structure, for it contains a putative metal-binding DXSXS sequence motif similar to that of the MIDAS in the I-domain (Lee et al., 1995b; Ponting et al., 2000). Therefore, it has been termed the I-like domain. This domain is a hotspot for point mutations that result loss of integrin expression or dysfunctioned integrins (Bilsland et al., 1994). The I-like domain participates directly to bind ligand in integrins that lack I-

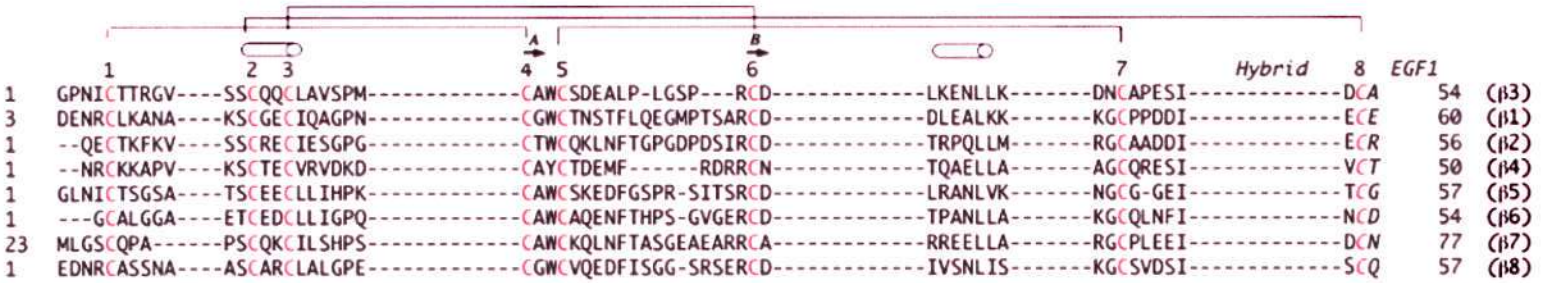


Table 1.2. Sequence alignment and cysteine pairing of the PSI domain from all eight human integrin β-subunits.
(Adapted from Xiong et al., 2004)

domains; in I-domain containing integrins, the I-like domain indirectly regulates ligand binding. From the crystal structure of αVβ3, there is a large interface between the β-propeller domain and the I-like domain (Xiong et al., 2001).

Unlike the I-domain, the I-like domain has two other metal ion coordination sites. One of these sites, which is adjacent to the MIDAS, is termed as ADMIDAS and was previously predicted to be an inhibitory Ca^{2+} binding site (Cierniewska-Cieslak et al., 2002). The metal ion is coordinated by the carbonyl oxygen of the second serine in the 'DXSXS' sequence, the side chains of two aspartate residues, from the α1 helix, and the carbonyl oxygen of a methionine at the top of the α7 helix (Xiong et al., 2001). ADMIDAS was proposed to have a role in stabilizing the active conformation of the integrin (Mould et al., 2003a).

In addition to ADMIDAS, a ligand-induced metal-binding-site (LIMBS) is located in the I-like domain, that is only 6Å away from the MIDAS. In the studying of the interaction of a cyclic RGD ligand with extracellular αVβ3, a metal ion was found at this site in the β3 subunit. All the residues coordinating LIMBS are conserved in I-like domains (Xiong et al., 2002).

1.2.2.3 Hybrid domain

The hybrid domain is a β-sandwich domain that is folded from amino acid sequence segments on either side of the I-like domain (Takagi and Springer, 2002) (Figure 1.2A).

It has been reported that several antibodies mapped to this domain can only stain the integrin when the integrin was activated, which suggsets that the hybrind domain may undergo outward movement during integrin activation (Mould et al., 2003b; Tang et al., 2005). The outward movement of hybrid domain is shown to be coupled to the downward shift of C-terminal α7 helix of the I-like domain and appears to participate in the conformational changes involved in both activation and ligand binding (Mould et al., 2003b). In addition, the hybrid domain is shown to be important for the activation signal transduction from the membrane proximal region to head piece of integrin (Tng, et al., 2004).

1.2.2.4 I-EGF repeats

Integrin β subunits contain four I-EGF repeats in a long stalk that connects the headpiece to the membrane. These four I-EGF repeats contain high content of cysteines, so it is also called cysteine rich region. The boundaries and pairing of cysteines in these repeats have been determined (Tan et al., 2001). This region provides the crucial link between signals impinging on transmembrane and cytoplasmic domains and the conformational changes that occur in the ligand-binding headpiece. Antibodies to this region can either directly activate ligand binding or act as probes that bind only to activated integrins (Humphries, 2000; Bazzoni and Helmer, 1998; Takagi et al., 1997; Lu et al., 2001a).

1.2.2.5 β tail domain

The four I-EGF repeats are followed by a C-terminal disulphide-bond β-sheet domain

termed the β-tail domain (βTD). In the crystal structure, weak hydrophobic contacts exist between I-EGF4 and the βTD, indicating that these two domains may be flexibly linked (Arnaout, 2002). In β3 integrins, βTD is not essential for cell surface expression of integrin dimmer, and it contributes to restrain β3 integrins in a resting, low ligand-affinity state (Butta et al., 2003).

1.2.3 *Transmembrane domains*

Recent reports have suggested that the transmembrane domains of integrin subunits mediate clustering of integrins after ligand binding (Li et al., 2003). The separation of the integrin α and β TM domains is important in regulating the conformation of the extracellular domain (Figure 1.6). Therefore, it appears that TM domains do more than merely serve to connect the intra- and extracellular domains. Rather, they appear to contribute to signaling in both directions across the membrane. Several models have been proposed to explain TM signaling of integrins. These are based on different types of movements of the TM domains, such as rotation, tilting, piston movement as well as oligomerization between two or more α or β subunits (Adair and Yeager, 2002; Armulik et al., 1999; Gottschalk and Kessler, 2002; O'Toole et al., 1994; Williams et al., 1994; Li et al., 2001; Li et al., 2003; Li et al., 2005; Luo et al., 2004, Luo et al., 2005). By sequence homology, the C-terminal end of the TM domain seems likely to be general for all integrins subunits (Li et al., 2002), but the N-termini appear to vary between subunits. N-terminal borders have been defined for α2, α5, α10 and β1 and β8 (Stefansson et al., 2004).

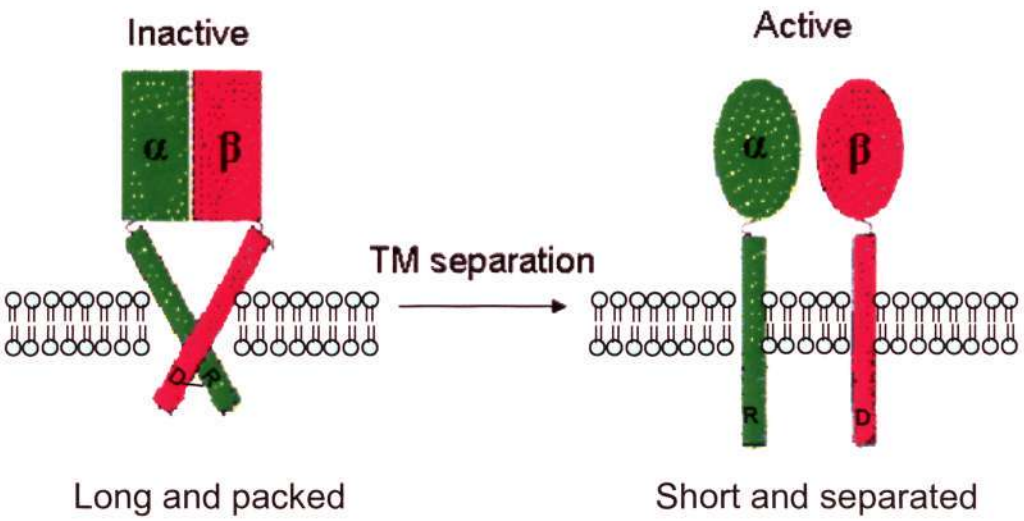


FIG 1.6. Model for integrin TM separation. Left panel: the low-affinity integrin is maintained through inter-subunit interactions at the TM and membrane-proximal domains. The TM helices specifically pack together and are in a long and tilted geometry. Right panel: the TM helical packing is broken thus giving rise to a high affinity for ligand in the extracellular domain.

1.2.4 *Cytoplasmic domains*

The cytoplasmic domains of adhesion molecules are essential for a variety of cellular functions (cell-cell and cell-extracellular matrix interactions, cell motility, receptor-ligand interactions, and receptor internalization) by establishing connection with the cytoskeleton. Association between adhesion molecules and cytoskeleton usually involve a complex of intermediary linker proteins. For example, L-selectin, the lymphocyte homing receptor (Gallatin et al., 1986), is connected to the cytoskeleton through α-actinin (Pavalko et al., 1995). α-actinin is also the linker protein for Intracellular Adhesion Molecule (ICAM) -1 and -2 (Heiska et al., 1996; Carpen et al., 1992). Cadherin, another adhesion molecule which mediates Ca^{2+}-dependent cell-cell adhesion (Takeichi 1995), is linked to cytoskeleton by catenins (Ozawa et al., 1989; Ozawa et al., 1990). Instead, integrins are linked to actin filaments via talin, filamin, and α-actinin (Horwitz et al., 1986; Otey et al., 1990; Pavalko et al., 1993 and Sharma et al., 1995).

The cytoplasmic domains of integrin α and β subunits are short, generally being 20-40 and 45-60 amino acids in length respectively. They form mechanical links to the actin-based microfilament system (Hynes, 2002). An exception to this is the long cytoplasmic tail of integrin β4, which is specialized to connect to the keratin cytoskeleton and contains fibronectin type-III domains. The cytoplasmic domains of integrins play a pivotal role in these cellular functions. Through interaction with the cytoskeleton, signaling molecules, and other cellular proteins, integrin cytoplasmic domains transduce signals from both the outside and inside of the cell (Liu et al., 2000).

All integrin α subunit cytoplasmic tails contain a conserved 'GFFKR' sequence at the membrane proximal region. This sequence has been shown to be important for maintaining the integrin in its resting state, clustering, and subunit association. Deletion of the α subunit cytoplasmic 'GFFKR' in the platelet integrin αIIbβ3, results in a constitutively active integrin whereas cytoplasmic tail deletion that retain the 'GFFKR' sequence does not activate the integrin αIIbβ3 (Lu and Springer, 1997; O'Toole et al., 1991; O'Toole et al,. 1994; Ylanne et al., 1993). Removal of this sequence from the integrin αL subunit has been found to promote αLβ2 clustering and make it constitutively active (van Kooyk et al., 1999). Mutation of either of the phenylalanines in the 'GFFKR' sequence in α6 prevents its association with β1 (De melker et al., 1997).

Most β subunit cytoplasmic tails contain the sequence 'LLvixhDRRE' (the less conserved residues are in lower cases, x is not conserved). Deletion of this sequence has been found to result in constitutively active integrins, whereas deletions that are more C-terminal block activation (Huges et al., 1995; Lu et al., 2001c). Thus β subunit cytoplasmic domains are believed to play a role in controlling the activation states of integrins and to have a regulatory role in integrin ligand binding (Huges et al., 1995). Mutagenesis studies on αIIbβ3 suggest that the Asp^{723} residue from this sequence in β3 forms a salt bridge with the residue Arg^{955} found in the GFFKR sequence of the αIIb subunit cytoplasmic tail. This stabilizes the association of the membrane proximal regions and maintains αIIbβ3 in a low affinity state (Huges et al., 1996). Furthermore, forced association of the cytoplasmic regions of αLβ2 or αMβ2 blocks activation, while dissociation of the cytoplasmic regions activates integrins (Lu et al., 2001c; Kim et al.,

2003). Indeed, lipid-modified peptides corresponding to the membrane-proximal regions of the αIIb or α2 tails activate αIIbβ3 or α2β1 when introduced into platelets, possibly by disrupting the interaction between integrin α and β tails (Stephens et al., 1998; Wang et al., 2003). More recently, fluorescence energy transfer (FRET) analysis using αLβ2 provided *in vivo* evidence for α and β subunit cytoplasmic domain associations (Kim et al., 2003).

In integrin β subunit cytoplasmic tails, there are two 'NPx(Y/F)' motifs, which are tight turn-forming sequences found in receptors (Chen et al., 1990). The N-terminal NPLY sequence was found to be required for phagocytosis of fibrinogen-coated particles by ectopically expressed αIIbβ3 integrin in fibroblastoid cells, while in β2 integrins the N-terminal NPxF plays an important role in coated pit internalization (Ylanne et al,. 1995; Rabb et al., 1993). Furthermore, tyrosine to alanine mutation in the first 'NPx(Y/F)' motif strongly inhibits integrin activation and induces structural changes at the motif itself, and within the membrane-proximal region (Ulmer et al., 2001). Mutations in the 'NPxY' also perturb the binding of numerous cytoskeletal and signaling proteins to integrin β tails (Liu et al., 2000) and αIIbβ3 activation by the talin requires an intact 'NPxY' sequence (Calderwood et al., 2002).The membrane-proximal regions of the α and β tails thus play crucial roles in integrin activation, probably by interacting with each other to restrain the integrin in a resting status. The more membrane distal regions of the β tails regulate activation through interactions with signaling proteins that might disrupt the membrane-proximal association, whereas membrane-distal α sequences regulate β tail conformation and association with activator proteins in a cell-type-specific manner (Calderwood, 2004).

1.3 Bidirectional signaling

Cells need to sense and respond to their environment. They are able to send bidirectional signals across its plasma membrane through cell surface molecules. Integrin is an important cell surface molecule in bidirectional signal transduction. Integrin extracellular domains bind a wide variety of ligands, and their intracellular cytoplasmic domains anchor to cytoskeletal proteins. In this way the exterior and interior of a cell are physically linked, allowing for bidirectional transmission of mechanical and biochemical signals across the cell membrane, leading to a cooperative regulation of cell functions, including adhesion, migration, growth, and differentiation (Qin et al., 2004).

1.3.1 *Inside-out signaling*

Integrin inside-out signaling is the process where ligation of different cell surface receptors initiates intracellular signaling events that result in increased integrin binding to ligands. Chemokines have been verified to be able to act as the physiological activators of integrin inside-out signaling on circulating leukocytes (Butcher 1991). Indeed, chemokines such as SDF-1α was reported to activate integrin αLβ2 on T lymphocytes, thus allowing the cells to adhere to the endothelium in both static and shear flow analysis (Sharmri et al., 2005; Cambell et al., 1998).

'Inside-out' activation signals are propagated from the integrin cytoplasmic tails across the plasma membrane to allosterically induce ligand-competency of the ectodomain. As the trigger point of inside-out signaling, the integrin cytoplasmic face has been intensively investigated (reviewed in Woodside et al., 2001). These studies have revealed

that (1) while intact integrin can remain latent both in unstimulated cells and in a purified state, deletion of the cytoplasmic and transmembrane region activates the receptor (Peterson et al., 1998); (2) point mutations in the membrane-proximal regions of the cytoplasmic tails or deletion of either can result in constitutive activation of the receptor (O'Toole et al., 1994; Hughes et al., 1995, 1996); (3) replacement of the cytoplasmic-transmembrane regions by heterodimeric coiled-coil peptides or an artificial linkage of the tails inactivates the receptor, and breakage of the coiled-coil or clasp activates the receptor (Lu et al., 2001a; Takagi et al., 2001b). Shortly thereafter, the model (Figure 1.7) gained direct and strong experimental support from a structural analysis in which the membrane-proximal helices of the two subunits were found to clasp in a weak "handshake" that could be disrupted by talin or constitutively activating mutations (Vinogradova et al., 2002); and (4) among the various intracellular proteins that bind to integrin cytoplasmic tails (Table 1.3), over expression of cytoskeletal protein talin, which binds to the β cytoplasmic tail, can result in integrin activation (Eigenthaler et al., 1997; Calderwood et al., 1999). These data suggest that a direct interaction between the α /β cytoplasmic tails might maintain the receptor in a latent state. Since the membrane-proximal regions of integrin α and β cytoplasmic tails are highly conserved, the generalization of this signaling mechanism to all integrins is to be anticipated.

1.3.2 Outside-in signaling

Upon the inside-out activation, integrins bind to specific extracellular matrix proteins. However, for the integrins to bind tightly to the extracellular matrix to mediate cell adhesion and migration, the integrin cytoplasmic domains must be anchored to the

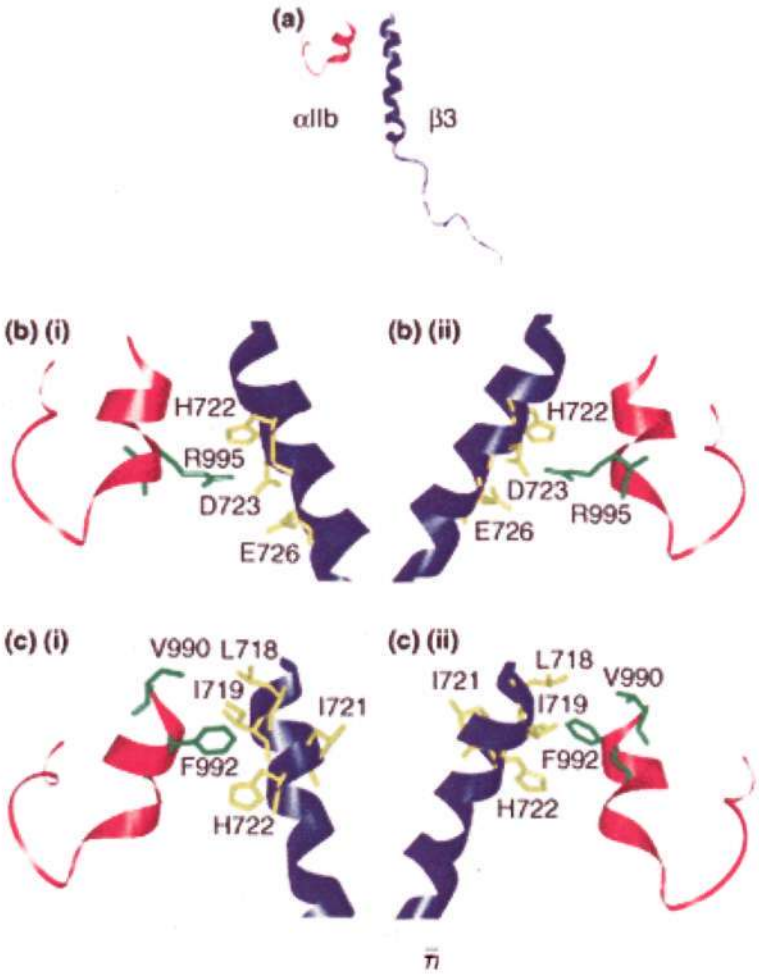


FIG 1.7. The structure and interactions of the integrin αIIbβ3 cytoplasmic tail complex. (A) Backbone ribbon diagram of the αIIb (red) and β3 (blue) tail complex. The N-terminal helical structures terminate at P998 and K738 for the αIIb and β3 subunits, respectively. In the tail of αIIb, the acidic C-terminal portion folds back and interacts with the positively charged N-terminal region through a turn. By contrast, the C-terminal of β3 is disordered. (B) Electrostatic and (C) hydrophobic interactions in the αIIbβ3 interface. Views (i) and (ii) illustrate 180° rotated views of the complex. The electrostatic interface consists of αIIb(R995)–β3(H722), αIIb(R995)–β3(D723) and αIIb(R995)–β3(E726). The hydrophobic interface consists of αIIb(V990)–β3(L718), αIIb(V990)–β3(I719), αIIb(F992)–β3(I721) and αIIb(F992)–β3(H722). (Adapted from Travis and Humphries, 2003).

Protein	Integrin chain	Binding site	Reference
calreticulin	α-tails	KXGFFKR	Coppolino et al, 1997
filamin	β1, β2,β7	724-747	Sharma et al, 1995 Pavalko et al, 1998
α-actinin ,	β1, β2	736-746(β2) 762-774(β1)	Pavalko and LaRoche 1993 Sampath et al, 1998
Rack -1	β1,β2,β3	724-743(β2)	Lilienthal and Chang, 1998
14-3-3	β1	776-790	Han et al, 2001
talin	β1A,β1D,β2,β3,β7	membrane distal, several areas	Knezevic et al, 1996 Sampath et al, 1998, Pfaff et al, 1998, Calderwood et al, 2001
ILK	β1,β2,β3	N.D.	Hannigan et al, 1996
JAB-1	β2	N.D.	Bianchi et al, 2000
FAK	β1,β2,β3	membrane proximal β subunit	Schaller et al, 1995
paxillin	β1,β3,α4,α9	membrane proximal, α subunit 983-991	Schaller et al, 1995, Tanaka et al, 1996 Liu et al, 1999, Young et al, 2001
Phospholipase Cψ	β1	conserved membrane proximal region, α-chain GFFKR	Vossmeyer et al, 2002
myosin	β3	TyrP β3	Jenkins et al, 1998
Shc	β3	TyrP β3	Cowan et al, 2000
DRAL/FHL2	α3A,α3B,α7A,β	β:C-tennina1 NXXY α:12 aa next to membrane proximal	Wixler et al, 2000
cytohesin 1 and 3	β2	723-725	Kolanus et al, 1996, Geier et al, 2000
β3 endonexin	β3	membrane distal	Shattil et al, 1995
ICAP-I	β1	785-799	Chang et al, 1997 Zhang and Hemler, 1999, Degani et al,2002
PI 3-kinase	β1	pTyr-peptide	Johansson et al, 1994
CIB	αIIb	N.D.	Naik et al, 1997, Tsuboi, 2002
IRS-1	αVβ3	N.D.	Vuori and Ruoslahti,1994
melusin	β1A,β1D	membrane proximal	Brancaccio et al, 1999
MIBP	β1A,β1B,β1D	membrane proximal	Li et al, 1999
c-src	β3	C-terminal RGT	Arias-Salgado et al, 2005
Fyn	β3	N.D.	Arias-Salgado et al,2003
Hck	β1,β2,β3	N.D.	Arias-Salgado et al,2003
Lyn	β1,β2,β3	N.D.	Arias-Salgado et al,2003
c-Yes	β1,β2,β3	N.D.	Arias-Salgado et al,2003

Table 1.3. Proteins interacting with integrin cytoplasmic tails.

cytoskeleton (Giancotti and Ruoslahti, 1999). This is achieved by "outside-in" signaling, i.e., when an integrin binds to the extracellular ligand, it clusters with other bound integrins, resulting in the formation of highly organized intracellular complexes known as focal adhesions that are connected to the cytoskeleton. The focal adhesions incorporate a variety of molecules, including the cytoplasmic domains of the clustered integrins, cytoskeletal proteins, and an extensive array of signaling molecules. The high local concentrations of these molecules facilitate cascades of downstream intracellular responses via protein–protein interactions, which are linked to the cytoskeleton as well as to complex intracellular signaling networks.

Integrin clustering activates several signaling pathways in cells that alter gene expression, influencing cell survival, differentiation, migration and morphology (Giancotti and Ruoslahti, 1999). Signaling pathways activated by βl and β3 integrins include activation of focal adhesion kinase (FAK), a tyrosine kinase that localizes to focal adhesions. FAK is regulated by integrins and mediates cell spreading and morphological changes. FAK contains many binding sites for SH2 and SH3-domain containing proteins, and may function as an scaffolding molecule for signaling. Integrins also activate MAP kinase pathways through the recruitment of the Grb/Sos-complex, which activates Ras, and they have been implicated in both upstream and downstream of small GTP-binding proteins like Rho, Rac and Cdc42 (Ridley, 2001). Integrins also work in concert with other cell surface receptors, like growth factor receptors, to influence the behavior of the cell (Giancotti and Ruoslahti, 1999; Eliceiri, 2001).

It is noteworthy to mention that the Src family tyrosine kinases (SFKs) serve important

functions in integrin 'outside-in' signaling and it is well established that c-Src can interact with the focal adhesion kinase (FAK) within integrin-mediated focal adhesion complexes (Miranti and Brugge, 2002). The prototype member of SFKs was first identified as the transforming protein (v-Src) of the oncogenic retrovirus, Rous sarcoma virus (Brugge and Ericson 1977). A major breakthrough of the SFKs came from the finding that SFKs possess protein tyrosine kinase activity (Collett and Erikson 1978). Studies of SFKs led to the realization that these enzymes regulate many cellular events in addition to cell proliferation, e.g. cytoskeletal alterations, differentiation, survival, adhesion, and migration. This broad spectrum of activities is a consequence of the ability of SFKs to couple with many diverse classes of cellular receptors (e.g. immune recognition receptors, adhesion receptors, receptor protein tyrosine kinases, G protein-coupled receptors, etc). SFKs are involved in adhesion events because they are required to promote signaling cascades that culminate in cytoskeletal re-modeling that is necessary for cell spreading after the initial integrin-mediated attachment of the cell to its substratum. An elegant study on fibroblasts derived from the triple knockout of SFKs c-Src, c-Fyn, and c-Yes mouse embryos showed defective integrin-mediated cell spreading and downstream signaling (Klinghoffer et al., 1999). Other than colocalization studies, the physical proximity of SFKs with integrins can also be demonstrated by methods such as bioluminescence resonance energy transfer (BRET) and biomolecular fluorescence complementation (BiFC) in the study of platelet integrin αIIbβ3 with c-Src (de Virgilio *et al.,* 2004). It was shown that c-Src can interact directly and specifically with the integrin β3 cytoplasmic tail (Arias-Salgado et al., 2003). More importantly, these data suggest that the SFKs may associate constitutively with the integrins thus providing a new paradigm

of how SFK may be regulated by the integrin (Arias-Salgado et al., 2005). Integrin clustering, as a result of binding to multimeric ligands, for example, can promote SFK activation by initiating autophosphorylation of SFKs tethered to the integrin cytoplasmic tails (Shattil, 2005). The precise events and mechanisms for such activation require further careful analyses because SFK activation involves not only phosphorylation of the activation loop Tyr but also dephosphorylation of its C-terminal inhibitory Tyr, which are regulated by C-terminal Src kinase (Csk) and protein tyrosine phosphatases such as PTP-1B and CD45 in different cell types (Arias-Salgado et al., 2005; Huntington and Tarlinton, 2004; Obergfell et al., 2002). In addition to cell adhesion, the integrin-SFK complex is also critical for immunological events. For example, the β2 integrins and the SFKs (c-Hck and c-Fgr) together with the tyrosine kinase Syk and FAK related kinase Pyk2, are shown to participate in immune cells phagocytosis and respiratory burst (Fuortes et al., 1999; Le Cabec et al., 2002; Lowell et al., 1996; Mocsai et al., 2002). Taken together, the connectivity between integrins and the SFKs can potentially serve as an important platform for the development of pharmacological agents that can selectively attenuate integrin 'outside-in' signaling for treatment of conditions such as thrombosis, psoriasis, and dermatitis.

1.4 Regulation of integrins

There are two major mechanisms that can regulate the strength of integrin mediated adhesions. First, the affinity of individual integrins for their ligands can be increased by conformational changes of the integrin. Second, increased avidity can result from clustering of integrins (Stewart et al., 1996). This increase of avidity is thought to occur

regardless of affinity states, providing strong adhesion at sites of cell-cell contact. Evidence suggests that changes in both affinity and avidity are important in integrin-ligand binding (Hogg et al., 2002). It is noteworthy that changes in integrin affinity are not necessarily accompanied by changes in avidity, and vice versa. Affinity and avidity regulation may coexist or occur sequentially, and they may be driven by independent pathways (Constantin et al. 2000, Giagulli et al. 2004).

The adhesiveness of cells to substratum mediated by integrin can also be regulated by integrin surface expression level, in case they differentiate or they are differentially removed by enzymes. A direct evidence is that αLβ2 integrin shed from human leukocytes during the cutaneous inflammatory reponse to the blistering agent cantharidin (Evans et al., 2006). Loss of adhesiveness upon αLβ2 shedding may play a role in leukocyte detachment after transendothelial migration.

1.4.1 *Avidity regulation*

The strength of integrin-mediated adhesion can be controlled by several factors: (1) changes in the number of receptors expressed; (2) their local distribution can be altered (clustering) and (3) changes in cell shape which alter the contact area (cell spreading) (Stewart et al., 1996).

Several integrins are believed to be stored in intracellular pools and can be expressed on the cell surface rapidly following stimulation (Gogstad et al., 1981; Wencel-Drake et al., 1986; Bainton et al., 1987). Therefore, the avidity of a cell for a particular ligand can thus be increased rapidly and the selective mobilization of specific pools of integrins can be

used to generate differential responses to stimuli (Sengelov et al., 1993). Clustering of receptors on the cell surface would no doubt increase overall cell-adhesive efficiency, particularly when the ligands are di- or multivalent and have a similar clustered distribution on the opposing cell or substrate (Shimaoka et al, 2002). Even when receptors are in a low affinity state, they can be rearranged into clusters of focal adhesion loci, in order to increase avidity for ligand (Stewart et al., 1996). Cell spreading could also influence adhesion by increasing the potential contact area or by reducing shear applied to the cells (Springer, 1994). For example, during the process of extravasation, leukocytes are known to spread at the sites of inflammation onto the vascular endothelium.

1.4.2 *Affinity regulation*

A variety of techniques have been used to detect conformational changes in integrins. For example, treatment of αIIbβ3 with ligand peptides increased its hydrolysis by thrombin and decreased its sedimentation coefficient (Parise et al., 1987), and platelet activation caused a change in the special separation or orientation of the extracellular domains of the two subunits as measured by fluorescence resonance energy transfer (Sims et al., 1991).

Changes in the conformation of the intergin head region as a result of ligand binding are believed to be propagated through the integrin, providing the basis for bidirectional signaling. These changes are propagated down the 'legs', the transmembrane, leading to a separation of the α and β cytoplasmic tails (Liddington, 2002). The separation of the cytoplasmic domains is shown to be a key factor involved in integrin signaling (Kim et

al., 2003; Takagi et al., 2001b; Takagi et al., 2002b), although the mechanism by which conformational signals are communicated in integrins remains unknown. For I-domain-containing integrins, changes in conformation have been focused around the I-domain. It was reported that the integrin α subunit I-domain binds to ligand and causes a 10Å downward shift of the C-terminal helix, which is attached to an extended loop containing a conserved glutamate (Lee et al., 1995a). It is proposed that this could bind to the MIDAS site in the I-like domain and act upon it as a ligand relay (Alonso et al., 2002). Alternatively, it is hypothesized that the C-terminal helix acted like a "bell rope" to pull open the I-domain (Shimaoka et al., 2002a). This proposal has recently been modified and it is now proposed that the I-domain α7 helix and its linker are better modeled as a "pull spring" than a "bell rope" (Yang et al., 2004). Results of second-site reversion mutations, whereby one residue is mutated resulting in inactivity of the protein, and another residue in close proximity is mutated resulting in the restoration of activity, suggest that the conserved glutamate residue functions as an intrinsic ligand for the I-like domain, and that when integrins are activated, the I-like domain MIDAS binding to this glutamate residue pulls the spring, and thereby activates the I-domain (Yang et al., 2004).

For non I-domain-containing integrins, it has been suggested that a small ligand binds at the interface between the propeller and the I-like domain. The C-terminal helix is proposed to move down, causing the I-like domain to rotate away from the propeller domain opening up the top of the propeller to engage larger ligands such as fibronectin. This is consistent with observations that RGD motif in fibronectin engages the I-like domain while the synergy site in the adjacent Fn3 domain engages the propeller (Mould

et al., 1997).

Since the crystallized αVβ3 integrin was able to bind peptide ligand mimetics, it was suggested that the structure represented the 'active' state of the integrin. Work to determine the nature of the structural arrangement between I-EGF2 and I-EGF3 was conducted using NMR (Beglova et al., 2002). This module pair was then superimposed onto the crystal structure of αVβ3 and found to pack against the α subunit calf-1. A number of activating antibodies map to this region, and one of these was found to be buried inside the thigh (Xie et al., 2004). It was therefore proposed that activation of the integrins involved an opening of the legs. It was also suggested that the bent integrin represented the inactive conformation in vivo and that the integrin stands up through a 'switch-blade' or 'flick-knife' opening of the integrin that is associated with its activation (Takagi et al., 2002b). Binding of antibodies to these epitopes would favour the extended integrin conformation, producing a mechanism for antibody-induced integrin activation (Shimaoka et al., 2002b).

In contrast, a 'deadbolt' model has also been proposed (Xiong et al., 2003b). In this model, an elongated loop of the βTD locks the I-like domain in an inactive state in the native structure by preventing tertiary changes in its C-terminal α7 helix and the preceding loop, which is required for activation. Inside-out activation transmitted through the integrin cytoplasmic tails may lead through movements of the TM helices to unlock the adjacent βTD 'deadbolt'. The 'deadbolt' would slide away from the I-like domain, rendering it ligand binding ability (Xiong et al., 2003b).

Finally, the separation of the α and β TM domains was found important for integrin activation (Luo et al., 2004). It is reported that the cytoskeletal protein talin binds to β cytoplasmic tail and displaces the α tail from its interaction with the β tail, which in turn leads to an unclasping and a membrane-associated structural change of the cytoplasmic face (Vinogradova et al., 2004). This unclasping initiates the opening of the integrin C-terminal stalks, including the TM domains, which is necessary for the switchblade extension of the integrin for high affinity ligand binding (Takagi et al., 2002b). Clustering of integrins via TM domain oligomerization (Li et al., 2003) may compensate for the high energy required for lateral separation of the TM domains (Qin et al., 2004).

1.5 Integrin activation states

Integrin undergoes different activation states by changing its quaternary conformation. The different requirement of integrin affinity is determined by various physiological conditions. The different quaternary rearrangements in the integrin ectodomain are proposed in illustration (Figure 1.8).

For the I-domain lacking integrins αVβ3 and αIIbβ3, three different conformational states have been demonstrated by electron microscopy and crystal structures: (i) a closed conformation (resting state), not able to bind its natural ligand; (ii) an extended conformation with closed headpiece (low affinity state); and (iii) an extended conformation with open headpiece (high affinity state) (Takagi et al., 2002; Xiong et al., 2002; Xiao et al., 2004; Adair and Yeager 2002).

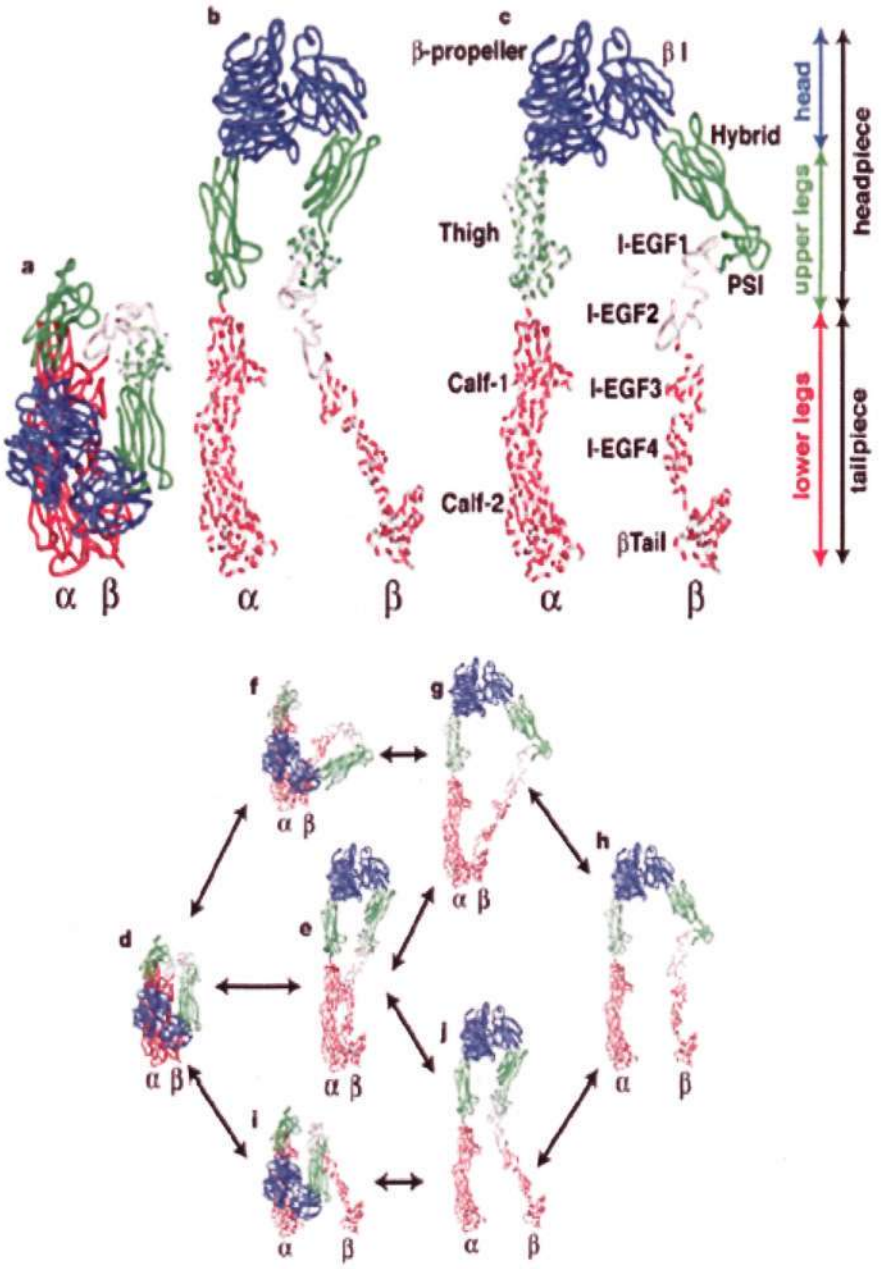


FIG 1.8. Quaternary rearrangements in the integrin ectodomain. a–c, Three conformational states visualized in electron microscopy and in crystal structures. d–j, proposed intermediate integrins between known conformational states. The upper pathways may be stimulated by ligand binding outside the cell, and the lower pathways by signals within the cell that separate the α and β subunit transmembrane domains. In a-j, solid color represents the domains which are known directly from crystal structures; dashed with grey reresents the domains which are placed from crystal structures into electron microscopy image averages; and solid grey represents for I-EGF1 and I-EGF2, which are modeled on I-EGF3 and I-EGF4. (Adapted from Xiao et al., 2004)

For the I-domain containing integrins such as αLβ2 and αMβ2, the existence of these conformational states has been demonstrated as well (Jin et al., 2004). On the cell surface, I-domains may exist in the three conformations termed closed, intermediate, and open, as seen in crystal structures. Both the open and intermediate states are active in binding to ICAM-1 in adhesion assays, whereas only the open state is of sufficient affinity to bind soluble ICAM-1 (Shimaoka et al., 2003).The intermediate state of the αL I domain is reported to be well folded, with the packing of the β6-α7 loop and upper portion of α7. These findings suggest that the intermediate conformation of the β6-α7 loop represents a discrete conformational state that could exist in intact integrins, and that the intermediate conformation could represent the second active state of αLβ2 that is active in cell adhesion but does not bind ligand with high affinity (Shimaoka et al., 2003).

1.6 Integrin related inheritable diseases

Several inheritable diseases have been attributed to defects in specific integrins. Leukocyte adhesion deficiency type I (LAD I) is caused by defects in integrin β2 subunit. Defects in both αIIb and β3 have been associated with Glanzmann's Thrombasthenia (GT). Defects of the β4 subunit have also been described in association with Junctional Epidermolysis Bullosa (JEB) which is due to a defect in hemidesmosome formation (Table 1.4).

1.6.1 *Leukocyte adhesion deficiency*

Leukocyte adhesion deficiency (LAD) was first described around 25 years ago in patients with delayed separation of the umbilical cord, neutrophilia, neutrophils defects, and

Disorder	Nature of integrin deffect	Symptom
Leukocyte adhesion deficiency (LAD) type I	β2-integrin subunit gene defect(Arnaout, 1990).	Recurrent bacterial and fungal infections, gingivitis, impaired wound healing and pus formation, and chronic leukocytosis
Glanzmann's thrombasthe nia (GT)	αIIbβ3 gene defect in either subunit (Bray, 1994).	Impaired platelet aggregation resulting in uncontrolled bleeding.
Junctional epidermolysis bullosa	Altered expression of α6β4: A gene defect in the β4 subunit is implicated in this defect (Vidal et al., 1995).	Blistering caused by separation of the dermis from epidermis within the lamina lucida of the dermal-epidermal basement membrane.

Table 1.4. Examples of human genetic diseases caused by integrin defects.

systematic bacterial infections (Crowley et al., 1980; Hayward et al., 1979). Key functions of leukocytes are impaired, notably the migration of neutrophils to the sites of extravascular inflammation (Anderson, 2001). Flow cytometry and protein analysis indicated the absence of leukocyte integrins on the surface of cells; and molecular analysis demonstrated heterogenenous molecular defects in the leukocyte β2 subunit (Anderson and Springer, 1987; Kishimoto et al., 1987a).

In LAD-1, mutations are found in INTG2, the gene located at 21q22.3 that encodes the β2 integrin (Corbi et al., 1988). Most of the point mutations are found in the I-like domain, which is encoded by exons 5-9 of INTG2 (Roos and Law, 2001). These mutations result in impaired integrin heterodimer formation and expression on the cell surface. The clinical severity of the disease correlates with the level of leukocyte integrin cell surface expression. Expression levels of β2 integrins are absent or less than 1% in the severe phenotype and less than 10% in the moderate phenotype (Bunting et al., 2002).

Also 'knock-outs' of integrin genes in mice have been reported, and these models lead us to know much about the function of individual integrins *in vivo* (Bouvard et al., 2001). β2 null mice exhibit a phenotype closely resembling human LAD-I (Sharffetter-Kochanek et al., 1998; Mizgerd et al., 1997). The degree of neutrophil extravasations into different tissue depends on the model of inflammation indicates that other integrins can substitute for β2-integrins in some circumstances. Inactivation of leukocyte integrin α-chains has revealed individual functions for αLβ2 and αMβ2 respectively. Neutrophil adhesion to endothelium occurs mainly via αLβ2, and less of αMβ2 (Ding et al., 1999). Leukocytes lacking αLβ2can still respond to viral infection, but T cell proliferation and cytotoxicity

in response to alloantigen are severely impaired, implicating additional roles for αLβ2 (Schmits et al., 1996; Shier et al., 1996). αLβ2 deficiency in mice leads to inability to reject tumors (Schmits et al., 1996, Shier et al., 1996; Shier et al., 1999) and are also important for T cell re-circulation (Andrew et al., 1998; Berlin-Rufenach et al., 1999). αMβ2 deficient neutrophil adhesion to endothelium, phagocytosis, and degranulation is impaired (Coxon et al., 1996; Lu et al., 1997). In addition, mast cell number in peritoneal cavity and peritoneal wall is reduced and their function is impaired (Rosenkranz et al., 1998).

Two other forms of LAD, types II and III, have been characterized. LAD-II is a result of a defect in sialyl Lewis X epitope expression, which is a carbohydrate ligand for selectins (Phillips et al., 1995). Patients with LAD-II have decreased adhesion of leukocytes to the endothelial vessel wall and migration into tissues. This is a result of reduced contact of L-selectins on the leukocytes and E- or P-selectins on the endothelial cells with their respective sialated fucosyl ligands on the opposite cells. LAD-III was recently characterized as a defect in the ability of the integrins to undergo activation by G-protein-coupled receptors (Alon and Etzioni, 2003).

1.6.2 *Glanzmann's thrombasthenia (GT)*

In Glanzmann's thrombasthenia, the defect is found in the platelet integrin αIIbβ3, which functions as a receptor for fibrinogen, von Willebrand factor and perhaps other ligands (Plow et al., 2000). αIIbβ3 is crucial for platelet aggregation and contributes to platelet

adhesion, platelet spreading and protein trafficking (Bennett, 2005).

GT is an inherited recessive bleeding disorder clinically characterized by quantitative or qualitative deficiency in αIIbβ3 on the surface platelets. The molecular defect responsible for the disease may either be on the α or the β subunit, resulting in impaired αIIbβ3 cell surface expression (Simsek et al., 1993; Wilcox et al., 1995).

As in LAD, a number of genetic defects responsible for GT have been identified, including deletions, insertions, and point mutations. GT has also been classified into different categories based on function and expression levels: type I or severe (less than 10% expression), type II or moderate (10-20% expression), and variant (50-100% expression, but dysfunctional) (Baker et al., 1997). One example of a variant case involves the point mutation D119Y (Loftus et al., 1990). In this case, normal levels of αIIbβ3 are found on the cell surface, but functional analysis revealed an inability to bind ligand. This point mutation lies in the β3-MIDAS motif recognized as important in ligand binding (Bajt et al., 1992; Goodman and Bajt, 1996).

Molecular analysis of other GT β3 variants has previously pinpointed other functionally important regions in the ligand binding region (R214Q and R214W) (Kouns et al., 1994; Bajt, 1992) and the cytoplasmic domain (S752P) (Baker et al., 1997). As in LAD, mutations identified in GT are useful for the investigation of site(s) in integrin that is functionally critical.

1.6.3 *Junctional epidermolysis bullosa (JEB)*

Junctional epidermolysis bullosa (JEB) is a group of hereditary autosomal recessive disorders characterized by marked skin fragility and blistering in response to minor trauma (Fine et al., 1991). A subgroup of JEB patients is associated with pyloric atresia (PA-JEB). Both lethal and non-lethal cases have been reported for PA-JEB patients (Weber, 1987; Hayashi et al., 1991; Lacour et al., 1992). This disease is characterized by localized blistering of the skin and occlusion of the pylorus. A substantial reduced expression of α6β4 has been shown in several PA-JEB patients (Philips et al., 1994) whereas in one patient no β4 was detectable at all (Gil et al., 1994). In support of this finding, mutations in the β4 gene in PA-JEB have been identified that result in a reduced expression of the α6β4 integrin (Vidal et al., 1995).

1.7 The β2 integrins

The β2 integrin subfamily has four members: αLβ2 (CD11a/CD18, Leukocyte Function-associated Antigen -1 or LFA-1), αMβ2 (CD11b/CD18, Complement Receptor 3 or Mac-1), αXβ2 (CD11c/CD18, CR4 or p150, 95), and αDβ2 (CD11d/CD18). The expression of these integrins is restricted to leukocytes. The gene encoding β2 is found on chromosome 21 (21q22.3) (Solomon et al., 1988). The leukocyte integrin α genes are located in a cluster on chromosome 16 (16p11-13.1) (Noti et al., 2000; Wong et al., 1996).

β2 integrins are the major integrins expressed on leukocytes that have been implicated in leukocyte–endothelial adhesive interactions. Most of leukocyte integrins expressed on circulating leukocytes are quiescent and do not mediate adhesion. In the process of

emigrating from blood into tissues, leukocytes are exposed to a variety of stimuli, including chemokines, which rapidly activate integrin function through changes in affinity and/or avidity. (Chan et al., 2003). Unlike leukocyte integrins which are highly regulated in ligand-binding, non-leukocyte integrins often bind their ligands constitutively. For example, α6β4 integrin (mainly expressed on epithelial (Kajiji et al., 1989), endothelial (Kennel et al., 1992; Klein et al., 1993) and Schwann cells (Sonnenberg et al., 1990; Einheber, 1993)) constitutively binds with the high affinity to laminin 4 and laminin 5 (Spinardi et al., 1995); moreover, platelet integrin αIIbβ3 on unstimulated platelets adhere to immobilized fibrinogen (Savage and Ruggeri 1991).

1.7.1 *αLβ2*

αLβ2 is expressed on all leukocytes (Barclay, 1997). Anti- αLβ2 antibodies have shown that this integrin participates in cytotoxic T cell killing, antigen-induced T cell proliferation and differentiation, T cell dependent Ig production, monocyte homotypic aggregation, NK cell killing, and leukocyte transendothelial extravasation (Davignon et al., 1981; Hildreth et al., 1983; Larson and Springer, 1990; Springer et al., 1987).

The only identified ligands for αLβ2 are the Intracellular Adhesion Molecules (ICAMs) (Figure 1.9), which are members of the Immunoglobulin-superfamily (IgSF) adhesion molecules. ICAMs are single chain type I membrane glycoprotein. Five ICAMs have been identified and have been given numerical designations based on the chronological order of discovery (Hayflick et al., 1998): ICAM-1 (Marlin and Springer, 1987), ICAM-2 (Staunton et al., 1989), ICAM-3 (de Fougerolles and Springer, 1992; Fawcett et al., 1992), ICAM-4 (Landsteiner-Wiener antigen) (Bailly et al., 1995), and ICAM-5 (telencephalin)

(Tian et al., 1997).

ICAM-1 (CD54) is found expressed at low to moderate levels on the surface of endothelial and epithelial cells, leukocytes, dermal fibroblasts, melanocytes, and many carcinoma cell types (Anderson and Siahaan, 2003). Its expression, however, can be significantly increased in the presence of cytokines (TNF-α, IL-1, IFN-γ) (Hersmann et al., 1998) and reactive oxygen species (Hubbard and Rothlein, 2000). ICAM-1 has five immunoglobulin-like domains (D1-D5) and mutational studies have revealed that the crucial residues for αLβ2 binding are found in D1 (Casasnovas et al., 1998). On the cell surface, ICAM-1 exists predominantly in a dimeric form that binds more efficiently to αLβ2 than the transmembrane-lacking monomeric form (Miller et al., 1995; Reilly et al., 1995). Through interactions with αLβ2 and αMβ2, ICAM-1 plays an important role in leukocyte-leukocyte, epithelial-leukocyte, and endothelial-leukocyte contact. Mice deficient in ICAM-1 have impaired neutrophil emigration, an inability to stimulate T cell proliferation in mixed lymphocyte cultures, resistance to septic shock, and a reduced susceptibility to cerebral ischemia-reperfusion injury (Sligh et al., 1993; Soriano et al., 1996; Xu et al., 1994).

ICAM-2 (CD102) has two Ig domains and similarly to ICAM-1, the N-terminal domain is the most important in binding to αLβ2 (Casasnovas et al., 1997; de Fougerolles et al., 1991; Li et al., 1993). ICAM-2 plays an important role in epithelial-leukocyte binding associated with inflammation (Hayflick et al., 1998). It is predominantly expressed at high levels on endothelium and occurs at low levels on resting lymphocytes and monocytes. On resting endothelial cells, the basal expression level of ICAM-2 is much

higher than that of ICAM-1. Unlike ICAM-1, inflammatory cytokines do not upregulate ICAM-2 expression on endothelial cells (Diacovo et al., 1994; Nortamo et al., 1991).

ICAM-3 (CD50) is closely related to ICAM-1 and is also composed of five Ig-like domains, which shares 52% homology with those of ICAM-1 (Barclay, 1997). ICAM-3 is expressed on leukocytes and it is the only ICAM significantly expressed on neutrophils (de Fougerolles and Springer, 1992). ICAM-3 is also highly expressed on epidermal Langerhans cells where presentation of alloantigen or peptide antigen by these cells to $CD4^+$ T cells may require an interaction between ICAM-3 and αLβ2(Acevedo et al., 1993; Griffiths et al., 1995; Staquet et al., 1995; Teunissen et al., 1995). More recently, eosinophil activation has been shown to result from specific adhesion of ICAM-3 to $CD4^+$ T cells and eosinophils (Douglas et al., 2000).

ICAM-4 was originally characterized as the Landstein-Wiener antigen in 1994 and was included as an ICAM molecule in 1995 (Bailly et al., 1995). Like ICAM-2, it is a two Ig domain molecule, and structural models have been proposed for ICAM-4 based on the crystal structure of ICAM-2 (Hermand et al., 2000). Selective binding of ICAM-4 to different integrins may be important for a variety of normal erythrocytes functions and is also relevant to the pathology of thrombotic disorders and vasooclusive events in sickle cell diseases (Mankelow et al., 2004).

ICAM-5 is the only ICAM expressed in the brain, where it is restricted to the telencephalon (Yoshihara et al., 1994). It binds to αLβ2 (Mizuno et al., 1997; Tian et al., 1997). ICAM-5 plays a role in neurite outgrowth by homophilic interactions (Tian et al.,2000b), and may switch binding partner after oligomerization during development to

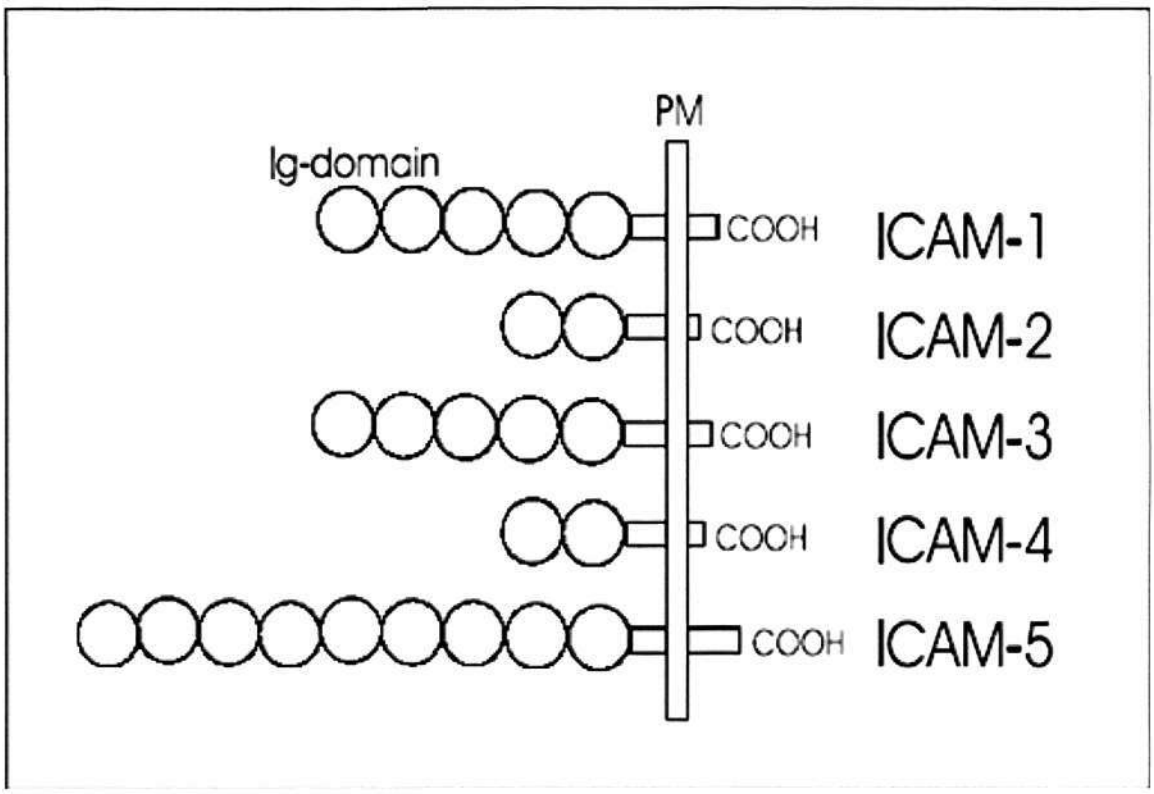


FIG 1.9. Schematic structures of the ICAMs. ICAM-1 and ICAM-3 have five Ig-domains While ICAM-2 and ICAM-4 have only two. ICAM-5 is the largest ICAM, with nine Ig-domains, and is expressed in the brain.

mediate binding to leukocytes.

1.7.2 *αMβ2*

αMβ2 heterodimers are mainly expressed on leukocytes of the myeloid monocytic lineage and natural killer lymphocytes (Larson and Springer, 1990). It has a critical role in adhesive reactions during inflammatory responses and is involved in neutrophil trafficking. It is one of the most versatile adhesion molecules and has the ability to recognize a wide range of ligands of very different biological functions (Hynes, 1992). To date, more than 30 proteins and non-protein molecules have been reported to bind αMβ2 (Yakubenko et al., 2002).

αMβ2 binds to the complement proteolysis product iC3b (Beller et al., 1982; Ueda et al., 1994). On macrophage, it has a pivotal role for clearance of bacteria and yeast by binding to iC3b-opsonized microbes and also by binding directly to microbial surface antigens such as lipopolysaccharide (Van Strijp et al., 1993; Wright and Jong, 1986), lipophosphoglycan (Talamas-Rohana et al., 1990), β-glucan (Ross et al., 1987) and *Leishmania* glycoprotein 63 (Russell and Wilhelm,1986). αMβ2 also binds ICAM-1 and ICAM-2 (Gahmberg, 1997) and in doing so may contribute to transendothelial extravasation of monocytes and granulocytes (Diamond et al., 1990; Diamond et al., 1991; Xie et al., 1995).

Binding of the zymogen factor X to αMβ2 results in the acceleration of its conversion to activated factor Xa and constitutes an alternative pathway for the initiation of the coagulation serine protease cascade (Altieri and Edgington, 1988; Altieri et al., 1988b;

Plescia and Altieri, 1996). Further, the ability of αMβ2 to bind fibrinogen (Altieri et al., 1988a; Ugarova and Yakubenko, 2001), heparin (Coombe et al., 1994; Diamond et al., 1995), and ICAM-4 (Bailly et al., 1995) may indicate that it has a role in regulating the blood clotting process and erythrocyte turnover.

In addition, αMβ2 interacts with complement factor H (Discipio et al., 1998), GPIb (Simon et al., 2000a), uPAR (Simon et al., 200b), E-selectin (Kotovuori et al., 1993), neutrophil inhibitory factor (Moyle et al., 1994) and many extracellular matrix proteins such as fibrinogen, laminin, collagens, vitronectin, thrombospondin, Cyr61, and connective tissue growth factor (Davis, 1992; Thompson and Matsushima, 1992; Miller and Castella, 1982; Monboisse et al., 1991; Walzog et al., 1995; Nathan et al., 1989; Schober et al., 2002). The versatile function of αMβ2 is further demonstrated by the finding that oligodeoxynucleotide are bound and internalized by the integrin (Benimetskaya et al., 1997).

The apparent lack of structural relationship between its ligand, together with the observation that αMβ2 mediates cell spreading on ligand-coated surfaces, led to the finding that αMβ2 binds denatured protein such as denatured BSA (Davis, 1992).

Despite the known ligand binding promiscuity of αMβ2, only one peptide motif, (D/E)(D/E)(G/L)W, was obtained when phage display libraries were used to isolate peptides that bind to the αM I-domain (Stefanidakis et al., 2003). Prior to this, it was identified that leukocyte-specific β2 integrins bind sequences containing a leucine-leucine-glycine (LLG) tripeptide motif. A novel antagonist peptide, CPCFLLGCC, was identified as the most active peptide ligand to purified αMβ2 (Koivunen et al., 2001).

It has been suggested that the ability of αMβ2 to bind to such a variety of ligands arises from the variable surface on the αM I-domain, which contains many docking residues that selectively engage different ligands (Ustinov and Plow, 2002). Further insights into the molecular basis of ligand binding promiscuity from chimeric-based studies indicate that αM (Lys^{245}-Arg^{261}) might constitute a consensus binding region (Yakubenko et al., 2002).

1.7.3 *αXβ2*

αXβ2 is expressed mainly on myeloid cells, with high levels on tissue macrophages. Its tissue distribution overlaps with that of αMβ2 and its specific function remains to be elucidated. However, it is known to contribute to neutrophil and monocyte adhesion to endothelium, chemotaxis and phagocytosis (Keizer et al., 1987; Stacker and Springer, 1991). It was initially defined as complement receptor type 4 (CR4) when it was shown to bind iC3b (Bilsland et al., 1994; Micklem and Sim, 1985) but it also binds to fibrinogen (Loike et al., 1991; Nham, 1999; Ugrarova and Yakubenko, 2001), LPS (Ingalls and Golenbock, 1995; Wright and Jong, 1986), LPG (Talamas-Rohana et al., 1990), ICAM-1 (Blackford et al., 1996) and denatured proteins (Davis, 1992).

1.7.4 *αDβ2*

αDβ2 is expressed predominantly on myelomonocytic cells, particularly on macrophage foam cells and splenic red pulp macrophages (Notu, 2002). αDβ2 is the most recently discovered member of the β2 integrin family (Van der Vieren et al., 1995) and so its function has not been fully characterized, although it may be implicated in atherosclerosis

(Van der Vieren et al., 1995). αDβ2 is known to bind ICAM-3 (Van der Vieren et al., 1995) and VCAM-1 (Vascular Cell Adhesion Molecule-1, CD106) (Van der Vieren et al., 1999).

1.8 Aim of study

1.8.1 *Integrin activation states*

The requirement of different affinity states of αLβ2 to different ICAMs has been observed previously (Buckley et al., 1997; Al-Shamkhani and Law, 1998). However, the relationship between αLβ2 affinity states and its conformations is unclear, because structural data of full length integrin was lacking at that time. The resolution of αVβ3 crystal structure (Xiong et al., 2001) allows us to understand how αLβ2 affinity states are linked to its conformation. Hence, we hypothesize that the movement of β2 hybrid domain is involved in the αLβ2 transition between different activation states. This section of the study made use of an activation reporter mAb MEM148 to demonstrate that αLβ2 hybrid domain movement is involved in ICAM-1 and ICAM-3 binding.

1.8.2 *Differential activation of αLβ2 and αMβ2 I-domain*

Nine integrin α subunits contain an I-domain which is involved in ligand binding. Many single-residue mutations were introduced into the αL I-domain to study its activation mechanism (Huth et al., 2000). One such substitution was the isoleucine at position 331, which is invariant among the integrin α subunit I-domains. Mutations of this invariant isoleucine were reported to activate both αLβ2 and αMβ2 in terms of ligand binding (Huth et al., 2000; Xiong et al., 2000). However, it was also reported that systematic

truncation of integrin β2 leads to differential activation of αLβ2 and αMβ2 ligand binding capacity (Tan et al., 2000). These results raise the question of whether the integrin I-domains share a universal activation mechanism. Therefore, we seek to test whether αLβ2 and αMβ2 integrin I-domains share a universal activation mechanism. In this part of study, we introduced isoleucine mutations into full length αL and αM, and the isolated I-domains of αL and αM. The ligand binding activities of these mutants were analyzed.

1.8.3 *Integrin mediated signal transduction*

Integrins lack intrinsic enzymatic activities but are capable of transducing intracellular signal events that is achieved by recruitment of signaling proteins to their cytoplasmic tails. The pairing between an integrin α subunit with a β subunit is specific and several α subunits can share a common β subunit. To date, the hypothesis that the different α cytoplasmic tails can exert different effect on the binding of cytosolic proteins to the β cytoplasmic tail has not been well characterized. Therefore, we set force to detect whether cytoplasmic tails of α subunits can affect the binding of cytosolic proteins to the β2 cytoplasmic tail. In the third part of this study, we investigate the influence of different α subunit cytoplasmic tails on the recruitment of a src family kinase Hck by αMβ2.

Chapter Two: Materials and Methods

2.1 General reagents

General reagents and solvents were of analytical grade, and were obtained from Sigma Aldrich Chemical Company Ltd., BDH Chemicals Ltd., Becton-Dickenson Ltd., GIBCO-BRL Ltd., Pierce Ltd., unless otherwise stated. Solutions were sterilized where required, by autoclaving or by passing through a 0.22μm filter (Millipore).

2.1.1 *Enzymes*

All restriction endonucleases and other enzymes were obtained from New England Biolabs, Promega Ltd., Fermentus Ltd, Roche diagnostics unless otherwise stated and were used according to the manufacturers' instructions.

2.1.2 *Commercially available kits*

ECL Detection Kit	GE Health Care
Plamid Maxi Kit	QIAGEN Ltd.
QIAprep Spin MINIprep Kit	QIAGEN Ltd.
QIAquik PCR purification Kit	QIAGEN Ltd.
mRNA Extraction Kit	Roche Dianostic

2.1.3 *cDNA clones provided by others*

CD11a (integrin αL)	From Prof. LAW SK (School of Biological Sciences, NTU)
CD11b (integrin αM)	From Prof. LAW SK (School of Biological Sciences, NTU)
CD11c (integrin αX)	From Prof. LAW SK (School of Biological Sciences, NTU)
CD18 (integrin β2)	From Prof. LAW SK (School of Biological Sciences, NTU)
ICAM-1-(D1-D5)-Fc	From Dr, DL Simmons, IMM, Oxford
ICAM-3-(D1-D5)-Fc	From Dr DL, Simmons, IMM, Oxford

2.1.4 *Cells*

MOLT-4 cells (human T lymphoblast)	Purchased form ATCC
THP-1 cells (human monocyte)	Purchased form ATCC
293T cells (human embryonic kidney cell with SV40 large T antigen)	Purchased form ATCC
293 cells (human embryonic kidney cell)	Purchased form ATCC
CHO-K1 cells (Chinese hamster ovary cell)	Purchased from ATCC

2.1.5 *Monoclonal antibodies*

MHM24	anti-αL	From Prof. McMichael (John Radcliffe Hospital, Oxford, UK)
LPM19c	anti-αM	From Prof. LAW SK (School of Biological Sciences, NTU)
KB43	anti-αX	From K. Pulford (LRF Diagnostic Unit, Oxford, UK)
MHM23	anti-β2 heterodimer dependent	From Prof. McMichael (John Radcliffe Hospital, Oxford, UK)
1B4	anti-β2 heterodimer dependent	From Prof. LAW SK (School of Biological Sciences, NTU)
MEM48	anti-β2 activating mAb	From Prof. Horejsi (Prague, Czech Republic)
H52	anti-β2	From Prof. LAW SK (School of Biological Sciences, NTU)
7E4	anti-β2	From Beckman Instruments
MEM148	anti-β2	From Prof. Horejsi (Prague, Czech Republic)
KIM127	anti-β2	From Dr. Robinson (UCB, CellTech, UK)
KIM185	anti-β2 activating mAb	From Dr. Robinson (UCB, CellTech, UK)
KIM202	anti-β2	From Dr. Robinson (UCB, CellTech, UK)

2.1.6 *Ligands for cell binding analysis*

iC3b	From Dr. AW Dodds (MRC Immonochemistry Unit, Oxford)
ICAM-1-(D1-D5)-Fc	Prepared by Miss Elianna Bte Mohamed Amin (SBS, NTU)
ICAM-3-(D1-D5)-Fc	Prepared by Miss Elianna Bte Mohamed Amin (SBS, NTU)

2.1.7 *Expression Vectors*

pcDNA3.0	Invitrogen
p-Display	Invitrogen
pXJ40-HA	Described in Xiao et al., 1991 and Manser et al., 1997

2.2 Solutions, buffers, and media

2.2.1 *Laboratory stocks*

10 mg/ml BSA	Stored at -20°C
0.1 M DTT	Stored at -20°C
0.5 M EDTA, pH 8.0	Stored at RT
100 mM EGTA, pH 7.4	Stored at RT

0.1% ethidium bromide	Stored at 4°C
1 M HEPES, pH 7.4	Stored at 4°C
1 M $MgCl_2$	Stored at RT
3 M NaAc, pH 4.8	Stored at RT
5 M NaCl	Stored at RT
0.1 M Sodium Bicarbonate Buffer	Stored at RT
2x protein solubilisation buffer	0.16 M Tris pH 8.0, 8 M Urea, 1.6% (w/v) SDS,
bromophenol blue	0.08% (w/v)
10%(w/v) SDS	Stored at RT
10x TBE	0.9 M Tris pH 8.0, 0.9 M Boric acid
	25 mM EDTA
1M Tris-HCl, pH 8.0	Stored at RT

2.2.2 *Media*

All media were sterilized by autoclaving unless otherwise stated.

20mg/ml ampicillin	20 mg/ml in ddH_2O, pH 8.4,
	filtered (0.22μm) and stored at -20°C
LA broth	LB medium containing 60 μg/ml ampicillin
LA plate	LB agar plate containing 60 μg/ml ampicillin
MOLT-4 cell culture media	RPMI1640 (JRH) containing 10% (v/v)
	heat-inactivated FBS, 100 IU/ml penicillin,
	100 μg/ml streptomycin

THP-1 cell culture media	RPMI1640 (JRH) containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, 100 µg /ml streptomycin
CHO-K1 cell culture media	Ham's F12K containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin
293 and 293T cell culture media	DMEM (JRH) containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin
LB medium	1% (w/v) Bacto-tryptone (BD), 0.5% (w/v) yeast extract (BD), 1% (w/v) NaCl
LB agar	LB medium plus 1.5% (w/v) Bacto-agar (BD)

2.2.3 *Solutions*

blocking buffer (western)	PBS containing 1% (w/v) non-fat milk, 0.1% (v/v) Tween20
blotting buffer (western)	12.5 mM Tris pH 8.0, 96 mM glycine, 10% (v/v) ethanol
cell freezing media	10% DMSO in heat-inactivated FBS
100 mM iodoacetamide	prepared fresh in ddH_2O
immunoprecipitation buffer	50 mM Tris pH 7.5, 150 mM NaCl, 1% (v/v) NP-40, 0.5 mM PMSF, 2.5 mM iodoacetamide

100 mM PMSF	prepared in ethanol, stored at -20 °C
10x SDS-PAGE running buffer	0.25 M Tris, 1.9 M glycine, 1% (w/v) SDS
4x resolution gel buffer	0.25 Tris, 0.4% (w/v) SDS, pH 8.8
4x stacking gel buffer	0.5M Tris, 0.4% (w/v) SDS, pH 6.8
cytoskeleton stabilization buffer	100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA pH 7.4, 10 mM PIPES, pH 6.8
sodium bicarbonate buffer	1.36 g sodium carbonate, 7.35 g sodium bicarbonate, pH 9.2

2.3 Methods

2.3.1 *General Methods for DNA Manipulation*

2.3.1.1 Quantitation of DNA

The concentration of DNA was determined by its absorbance at wavelength 260nm based on the calculation; 50 mg/ml double-stranded DNA gives an OD_{260} of 1. The OD_{280} was also read and the ratio of OD_{260}/OD_{280} was calculated to estimate the purity of the DNA solutions. An OD_{260}/OD_{280} ratio between 1.6-2.0 was considered satisfactory.

2.3.1.2 Restriction endonuclease digestion

Restriction endonuclease digestions were usually carried out in a 20-100 µl reaction volume, with 2-5 U of enzyme used for up to 500 ng DNA, for 1-18 h at an appropriate temperature. Commercially available 10x buffers were used according to the manufacturers' instructions. Digests with more than one enzyme were carried out simultaneously in a suitable buffer. When the buffer requirements were incompatible, restriction digestions were performed sequentially with individual buffer for each enzyme.

2.3.1.3 DNA separation by agarose gel electrophoresis

A 50 ml 0.8% (w/v) agarose gel was routinely used for analysis of 0.1-8 kb DNA fragments. Agarose was melted in 1x TBE, cooled to 55°C, before adding ethidium bromide to a final concentration of 1 mg/ml followed by casting in a mini-gel apparatus. The gel was left to set at RT for at least 30 min. Electrophoresis was carried out in

horizontal apparatus with the gel submerged in 1x TBE. DNA samples were loaded with one-fifth volume glycerol loading dye. A standard DNA ladder was also run to allow estimation of the sizes of the sample DNA fragments. DNA fragments were visualized by fluorescence over a UV light (302 nm, UV transiluminator TM-20, UVP), under which DNA/EB complexes fluoresce and the image was recorded with a Mitsubishi video copy processor.

2.3.1.4 Purification of DNA fragments by agarose gel electrophoresis

When a particular fragment of DNA in a mixture of fragments was required e.g. in ligations or as a PCR template, it was routinely separated from other fragments by agarose gel electrophoresis. Gel slice containing the DNA fragments to be purified was cut from the gel using a razor blade, carefully avoiding their exposure to the UV light. DNA was extracted using a QIAquik Gel Extraction Kit.

2.3.1.5 DNA ligation

DNA vectors with complementary ends to be used for ligation were prepared by restriction enzyme digestion and where necessary treated with alkaline phosphatase and purified using agarose gel electrophoresis. Vector DNA (~ 10 ng) and insert DNA (20-40 ng) were ligated by incubation at RT for 2-3 h, using 1U of T4 DNA ligase with ligase buffer provided together with the enzyme in a 20 µl reaction volumn. A reaction with uncut vector, a reaction without insert DNA and a reaction without both insert DNA and T4 DNA ligase were included in the experiments as controls.

2.3.1.6 Preparation of *E.coli* competent cells

The *E.coli* strain DH5α was used to prepare competent cells in advance which were stored at -70°C. A fresh plate of cells was prepared by streaking out cells from frozen stocks and growing overnight at 37°C. On the second day, an individual colony was picked and grown in 10 ml LB broth culture overnight. On the third day, 5 ml of overnight culture was transferred into each of two flasks containing 500 ml LB broth. Incubate at 37°C with aeration until the culture reaches OD_{550} of 0.5. This should take approximately 2 h. The cells were transferred to centrifuge bottles and spin at 4°C for 8 min at 8000 rpm. Pellets were gently resuspended in 250 ml ice cold 0.1 M $CaCl_2$ and combined into a single bottle. Cells were resuspended again in 250 ml ice cold 0.1 M $CaCl_2$ and centrifuged at 8000 rpm for 8 min at 4°C. Finally the pellet was resuspended in 43 ml ice cold 0.1 M $CaCl_2$ in ddH_2O with 7 ml sterile glycerol. Competent cells were distributed into convenient aliquots (0.2 ml) in cold microfuge tubes. Cells were store at -70°C. A portion of the cells was saved to assay for viability, purity and competence.

2.3.1.7 Transformation of plasmid DNA

For each transformation, only 50-100 µl of competent cells are necessary. Competent cells were defrozed on ice. DNA was added to cells (the volume of DNA should not exceed 40% of the cell volume). The mixture was incubated on ice for 20-30 min followed by a heat shock in a 42°C water bath for 2 min. 1 ml LB broth was added to the tube. The tube was incubated at 37°C with constant shaking for 30 min to 1h. 50-500 µl of the mixture was streaked out onto plates containing the appropriate antibiotics.

2.3.1.8 Purification of plasmid DNA

For small scale purification of plasmid DNA, 5ml LB with appropriate antibiotics was inoculated with a single colony that contains recombinant plasmid from an agar plate and incubated at 37°C with constant shaking overnight. A QIAprep Spin Miniprep Kit (QIAGEN) was used to extract DNA according to the manufacturers' instructions.

For large scale purification of plasmid DNA, a Plasmid Maxi Kit (QIAGEN) was used according to the manufacturers' instructions. This kit routinely yielded between 200-750 μg DNA from 100 ml of overnight bacterial cultures.

2.3.1.9 Polymerase chain reaction (PCR)

DNA fragment between two oligonucleotide primers were amplified by PCR through repeated cycles of three incubation steps where the DNA template is denatured, allowed to anneal with the primers and a new strand synthesized using a thermo-stable DNA polymerase. A typical cycle included denaturation at 94°C for 1 min, annealing for 1 min at a temperature ~5°C below the lower Tm of the two primers, and finally an extension at 72°C for an appropriate time (normally 1 min per kb). Tm refers to the melting temperature for each oligonucleotide calculated according to its composition allowing 2°C for each A or T and 4°C for each C or G.

2.3.1.10 Standard PCR protocol

PCR was routinely performed in a 50 μl reaction volumn containing less than 1 μg template DNA, 1 μM of each oligonucleotide primer, 200 μM of each dNTP, and 1 U

DNA polymerase. *Taq* polymerase (Fermentas) was used for PCR colony screening. Pfu DNA polymerase (Fermentas), which possesses a 3'-5' proofreading activity resulting in a twelve fold increase in fidelity of DNA synthesis over *Taq* DNA polymerase, was used for high fidelity DNA synthesis. Each polymerase has its own reaction buffer, normally supplied by the manufacturer. The reaction mixtures were subjected to a varying number of cycles of amplification using the DNA Thermal Cycler (MJ research).

2.3.1.11 Identification of colonies that contain recombinant plasmids of interest

After transformation of competent cells, colonies of interest were identified using either PCR screening or restriction digestion, depending on the availability of suitable PCR primers for screening and the degree of background indicated by the control plates. Each colony to be tested was used to inoculate 15 µl LB. 1 µl of this inoculated LB broth was added to a 25 µl PCR reaction containing 0.5 U *Taq* polymerase. A negative control and, where possible, a positive control were included in the experiment. Colonies containing the recombinant plasmid of interest were identified by the size of their PCR products using agarose gel electrophoresis. Positives from PCR screening were confirmed using restriction digestion.

2.3.1.12 Site-directed mutagenesis

Point mutations were made using QuikChange Site-Directed Mutagenesis Kit (Stratagene), and following the manufacturer's protocols. Overlay primers with the desired mutations were used in the long PCR cycle. The restriction enzyme Dpn I was used to digest the original template. The Dpn I treated PCR product was transformed in

competent *E.coli* and plated onto LB agar plates with appropriate antibiotics. All constructs were verified by sequencing (Research Biolabs).

2.3.1.13 Reverse transcription PCR

QIAGEN One Step RT-PCR Kit was used for reverse transcription PCR of mRNA. The reverse transcription of mRNA to cDNA was carried out by Omniscript™ Reverse Transcriptase/ Sensiscript™ Reverse Transcriptase at 50°C for 30 minutes. The extraction of mRNA will be described in later part of Materials and Methods.

2.3.2 *General methods for cell culture*

All cell lines were maintained in 5% CO_2 at 37°C in a humidified tissue culture incubator using Nunc tissue culture flasks or dishes.

2.3.2.1 Cell storage in liquid nitrogen

Cells were sedimented at 400 g for 5 min, resuspended in cell freezing media at a concentration of $5x10^6$ cells/ml and dispensed into Cryo Vials (Greiner). Cells were frozen in a NALGEN™ Cryo 1°C Freezing Container (Nalgen) for 24 h at -70°C to achieve a 1°C/min cooling rate. Thereby the vials were transferred into liquid nitrogen for long term storage.

2.3.2.2 Cell recovery from liquid nitrogen

Cells were removed from the liquid nitrogen storage and quickly brought to 37°C in a water bath. Cells were washed in 10 ml warmed media to remove DMSO. Each cell

pellet was resuspended in complete media and cultured in 75 cm^2 flask.

2.3.2.3 Culture of 293 and 293T cells

293 and 293T cells were maintained in DMEM with 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 and humidified tissue culture incubator at 37°C. Cells were passaged when they were subconfluent. Cells were washed twice in PBS, incubated in 0.25% (w/v) trypsin (GIBCO) for 5 min at RT, followed by tapping of each flask to dislodge the adherent cells. Trypsin was inactivated by adding full media and cells were directly seeded into fresh culture media in new flasks at desired cell density.

2.3.2.4 Culture of CHO-K1 cells

The culture of CHO-K1 cells was similar to that of 293 and 293T cells except that CHO-K1 cells were grown in Ham's F12K with 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.3.2.5 Culture of MOLT-4 and THP-1 cells

MOLT-4 and THP-1 cells were grown in cell culture flasks in RPMI1640 with 10% (v/v) heat- inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were passaged by diluting cells with fresh media.

2.3.3 *Transfection of cells*

2.3.3.1 Transfection of 293 and 293T cells by the calcium phosphate method (for 10 cm dish)

Cells were split into 10 cm dishes the night before to give 60-70% confluence at the day of transfection. On the next day, 10 ml of fresh media containing 25μM chloroquine was added to replace the old media one hour prior to the transfection. 10μg DNA was added to ddH_2O (1095 μl total) in a 15 ml sterile tube, followed by adding in 155 μl 2M $CaCl_2$. 1250 μl of 2xHBS (8.0g NaCl, 0.37g KCl, 201mg $Na_2HPO_4 \cdot 7H_2O$, 1.0g glucose, 5.0g HEPES/500ml pH 7.05 and filter sterile, store at 4°C) was added to that tube drop by drop with gentle mixing. Finally, this mixture was added directly to the cells drop by drop. This step should be done within 1-2 min after adding 2xHBS. The cells were then incubated for 7-11 h. After incubation, 10 ml of fresh media was added to replace the old media which contain chloroquine. Cells, or culture supernatant, can be harvested 48-72 h after transfection

2.3.3.2 Transfection of CHO-K1 cells by lipofectamine method (for 24-well plate)

One day before transfection, cells were plated in 500 μl of growth media without antibiotics to achieve a 70-90% confluency at the time of transfection. On the next day, 1 μl Lipofectamine™ 2000 and 0.8 μg DNA were added to two different tubes which contained 50 μl of serum free media each. After the 5-minute incubation, the diluted DNA and the diluted Lipofectamine™ 2000 were combined and mixed gently and incubated for 20 minutes at room temperature. This mixture was then added to cells. Cells were incubated at 37°C for 24-48 h. Media may be changed after 6 h.

2.3.3.3 Harvesting transfected cells (adherent)

24-48 h after transfection, the cells in each plate were washed in PBS and detached in 5 ml of 0.5 mM EDTA in PBS by incubation at RT for 10 min with gentle rocking. Cells were collected and mixed with 10 ml full media and transferred to centrifuge tubes. Cells were spun down (400g, 5 min, 4 °C) for subsequent analysis.

2.3.3.4 FACS analysis

Cells were incubated with 20 µg/ml primary mAb in RPMI1640 for 1 h at 4°C. The cells were then washed twice 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 in RPMI1640 and fixed in 1% (v/v) formaldehyde in PBS. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using CellQuest pro software (Becton Dickinson). Expression index was calculated by (% cells gated positive) x (geomean fluorescence intensity).

2.3.3.5 Surface biotinylation and preparation of whole cell lysates

Harvested cells were pelleted by centrifugation (400 g, 5 min, 4 °C), washed twice in ice cold PBS and resuspended in ice cold PBS at ~ 2.5 x 10^6 cells/ml in microfuge tubes. 200 µl cells were mixed with 200 µl 1 mg/ml sulpho-NHS-biotin (Pierce) that was freshly prepared in PBS, and incubated on ice for 30 min. Reaction was quenched by adding 5 ml 10 mM Tris-HCI pH 8.0 in PBS and washing the cells twice in the same solution. Cells were resuspended in 200 µl PBS and transferred to a 1.5 ml eppendorf tube. The labeled cells were lysed by adding 1 ml immunoprecipitation buffer with the addition of 1 mM

PMSF and incubated on ice for 20 min. Cell debris were removed by centrifugation at 12000 g at 4°C for 10 min, and the cell lysates were transferred to fresh microfuge tubes. The lysates were stored at -20°C.

2.3.3.6 Preparation of rabbit anti-mouse IgG coupled onto protein A sephrose beads

Protein A sepherose (PAS) (Sigma) (1g) was swelled by rotating in an excess of PBS overnight at 4°C. The swelled PAS beads, which occupied a bed volume of 4 ml, was sedimented by centrifugation (3000 g, 5 min, 4°C), washed twice, and finally resuspended to a 25 % (v/v) suspension in PBS. Rabbit anti-mouse IgG (RaM, Sigma) (4 mg) was added and the mixture was rotated for 1 h at 4°C. The PAS-RaM bead were washed once in PBS, once in IP buffer, and resuspended to a 25 % (v/v) suspension in IP buffer. The PAS-RaM suspension was stored at 4°C.

2.3.3.7 Immunoprecipitation of biotinylated cell lysates

0.5 ml of biotinylated cell lysates were precleared by adding 3 µg of irrelevant mAb as appropriate, and incubated at 4°C for 45 min with rotation. 40 µl PAS-RaM suspension was added and the mixture was rotated at 4°C for 30 min. The PAS-RaM was sedimented by centrifugation (10000 g, 2 min, 4 °C) and the supernatant was transferred to a fresh tube. 3 µg of appropriate mAb was added to the precleared cell lysate and incubated at 4°C for 45 min with rotation. Subsequently, 70 µl PAS-RaM suspension was added and the mixture was incubated at 4°C for 1 h with rotation. The PAS-RaM was sedimented by centrifugation (10000 g, 4°C, 2 min) and the supernatant was discarded. The PAS-RaM was washed in 500 µl IP-buffer for three times. Thereafter, 100 µl protein solubilisation

buffer containing 40 mM DTT was added to the PAS-RaM which was then vortexed, heated at 100°C for 5 min. The immunoprecipitated proteins were resolved by directly running on SDS-PAGE gels, or were stored at -20°C until used.

2.3.3.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) with slight modifications. Protein sample (15 µl for minegel system) was mixed with an equal volume of sample loading buffer (2x) under non-reducing conditions or in the presence of 40 mM DTT under reducing conditions. Electrophoresis was carried out at 200V in a Mini Electrophoresis Set (Biorad) in SDS-PAGE eletrophoresis buffer.

2.3.3.9 Western blotting

Proteins separated by SDS-PAGE were transferred onto a PVDF membrane (Immobilon-P, Millipore) by western blotting. The PVDF membrane was prepared according to the manufacturer's instructions: the membrane was equilibrated by washing in ethanol for 10 sec, then water for 10 min until miscible, and then in blotting buffer(12 mM Tris-HCI, 95 mM Glycine, 10 % (vlv) ethanol) for 5 - 10 min. The SDS-PAGE gel was equilibrated by washing in blotting buffer once. One western blot sponges, the PVDF membrane, the SDS-PAGE gel and finally the second sponge, was assembled and placed in a semi-dry western blot apparatus (Biorad), bubbles between each layer were removed carefully. Western blotting was performed at 25 V for 30 min. After protein transfer, the PVDF membrane was transferred to blocking buffer and was rotated at RT for an hour or overnight at 4°C.

2.3.3.10 ECL detection biotinylated proteins blotted onto immobilon-P membrane

Biotinylated proteins which had been transfered onto PVDF membranes by western blotting were labeled with a streptavidin-HRP (Horse radish peroxidase) conjugate (Amersham) and were then detected by enhanced chemiluminescence (ECL). Membrane was removed from blocking buffer and were washed three times in PBS-T (PBS, 0.1% (v/v) Tween-20 (Sigma)) at RT for 10 min. Membrane was then incubated with the streptavidin-HRP conjugate (1:1000 dilution in PBS-T) at RT for 60 min with gentle agitation, and was washed again for three times in PBS-T at RT for 10 min. Thereafter, the membrane was developed using an ECL Plus Detection Kit (Amersham) according to the manufacturer's recommendations. Membrane was then exposed to Kodak X-Omat film and developed using a Kodak X-OMAT ME-1 processor.

2.3.3.11 Coating microtitre plates with ICAMs for cell adhesion assay

Goat anti-human IgG (Fc specific) (Sigma) was diluted to 5 µg/ml in sodium bicarbonate buffer , pH 9.2, and 50 µl was added to each well of a microtitre plate (Polysorb, Nunc Immuno-Plate). The microtitre plates were left at 4 °C overnight. On the next day, the solution was discarded and the wells were washed twice with 150 µl PBS per well. A solution of 0.5 % (w/v) BSA in PBS was added to each well (150 µl per well) and the plates were incubated at 37°C for 30 min. The plates were washed with PBS once (150 µl per well) before the addition of 50 µl per well of ICAM-1/Fc or ICAM-3/Fc (1 µl /ml in PBS containing 0.1 % BSA). After 2-3 h incubation at RT, the plates were washed twice in RPMI/HEPES/FBS (RPMI1640 supplemented with 10 mM HEPES, pH 7.4 and 5% (v/v) heat inactivated FBS) before use.

2.3.3.12 Coating microtitre plates with iC3b for cell adhesion assay

Nunc immuno plates polysorp (Nunc) plates were coated with 7.5 µg /ml iC3b, in 50 µl of sodium bicarbonate buffer pH 9.2 for 2 h at RT. Plates were washed once in PBS (150 µl per well), and blocked with 0.2 % PVP10 (Sigma) in PBS (150 µl per well) for 30 min at 37°C. Before use, the wells were washed twice with 150 µl RPMI/HEPES/FBS.

2.3.3.13 Cell adhesion assays

Plates were prepared as described in 2.3.3.11 and 2.3.3.12. Cells were then incubated with BCECF dye (2',7'-bis-(2-carboxyethyl)-5(and -6) carboxy fluorescein, acetoxymethyl ester) (Molecular Probes), at the concentration of 1 µg/ml for 20 min at 37°C. Labeled cells were transferred to wells ($2x10^4$ cells/well) alone or with stimulation/inhibition reagents and incubated for 30 min at 37°C. Nonadherent cells were removed by washing. Cell fluorescence, which indicated the number of cells adhering to ligand-coated wells was measured using FL600 fluorescence plate reader (Bio-Tek).

2.3.3.14 Soluble ligand binding assay

Soluble ICAMs were prepared by incubating 20 µg/ml ICAM-Fc with 100 µg/ml FITC-conjugated rabbit anti-human IgG Fc antibody (Pierce) in 50 µl of RPMI 1640 media containing 5% (v/v) heat-inactivated fetal bovine serum and 10 mM HEPES (pH 7.4) for 30 min at room temperature. Thereafter, purified mouse IgG was added to a final concentration of 100 µg/ml mAb for 20 min at room temperature. This was performed to quench cross-reactivity to mouse IgG, which was included in the experiment as activating

or function-blocking mAb. Cells (2×10^5) were resuspended in this sICAM mixture with or without αLβ2-activating agents for 30 min at 37 °C. Cells were washed with RPMI media and fixed in 1% (v/v) formaldehyde in PBS followed by flow cytometry analyses. For activating studies, Mg^{2+}/EGTA (5 mM $MgCl_2$ and 1.5 mM EGTA) and/or activating mAbs (MEM48 or KIM185) (10 µg/ml) were used. For blocking studies, function-blocking αL-specific mAb MHM24 (10 µg/ml) was included in the samples.

2.3.3.15 Human serum preparation

After collection, blood was allowed to coagulate for 30-60 min at 37 °C and then placed at 4°C overnight, for contraction. On the next day, serum was removed from the clot by spinning at 10000 g for 10 min at 4 °C and was stored at -20 °C

2.3.3.16 Preparation of rhodamine red™-X succinimidyl ester labeled zymosan

Zymosan particles (Sigma) were labeled with rhodamine red™-X succinimidyl ester (Molecular probes) according to manufacturer's instructions for visualization using confocal microscopy. Briefly, around 10 mg of zymosan particle was dissolved in 1 ml of 0.1M sodium bicarbonate buffer, pH 8.3. 1 mg of rhodamine red™-X succinimidyl ester was dissolved in 100 µl DMSO (Sigma), and was slowly added into the zymosan solution while stirring. The reaction was incubated for 1 h at RT with continuous stirring. The reaction was stopped by adding 0.1 ml of freshly prepared 1.5 M hydroxylamine, pH 8.5.

2.3.3.17 Phagocytosis assay

Labeled zymosan particles were opsonized with normal human serum. Briefly, labeled

OZ was incubated in 10% (v/v) normal human serum in modified HBSS buffer containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 30 min at 37°C. CHO-K1 transfectants grown on the Lab-Tek chamber slides (Nunc) were serum starved for 30 min and fed with serum opsonized zymosan (OZ) at a ratio of 5:1 (OZ:cell) in RPMI 1640 media without serum under culture conditions for 3 h to allow OZ uptake by phagocytosis.

2.3.3.18 Immunofluorescence and confocal microscopy

PMA-treated THP-1 cells were incubated with relevant integrin mAbs αLβ2 -specific MHM24; αMβ2 -specific LPM19c; αXβ2 -specific KB43 and FITC-conjugated sheep anti-mouse IgG antibody (1:300 dilution) (Sigma) in PBS for 30 min at 37°C to allow cross linking of the integrins. Thereafter, cells were fixed in 3.7% (w/v) paraformaldehyde in PBS for 10 min at RT. Cells were permeabilized with 0.2% (v/v) Triton X-100 in modified CSK buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES, pH 6.8) at RT for 2 min. Permeabilized cells were incubated with rabbit anti-Hck antibody (1:50 dilution) (Santa Cruz) followed by TRITC-conjugated swine anti-rabbit (1:100 dilution) (Dako Cytomation).

For the co-localization analyses involving αMβ2, Hck, Lck, and CD4, the surface antigen antibodies used were mAbs LPM19c (anti αMβ2) and SK3 (anti-CD4) (BD Biosciences Pharmingen) followed by secondary FITC-conjugated sheep anti-mouse IgG antibody (1:300 dilution) aforementioned. Staining of the SFKs in permeabilized CHO-K1 transfectants was performed using the rabbit anti-His tag antibody (Santa Cruz) followed by TRITC-conjugated swine anti-rabbit antibody.

Similar procedure was employed for co-localization of Hck with different αMβ2 mutants having β2 cytoplasmic tail truncations. For the analyses of co-localization of Hck around OZ-containing phagosome in CHO-K1 transfectants, detection of Hck was achieved using the rabbit anti-His tag antibody followed by FITC-conjugated goat anti-rabbit antibody (1:80 dilution)(Sigma) for distinction from rhodamine red-labeled OZ. In all cases, slides were finally mounted in mounting medium (Dako Cytomation) prior to immunofluorescence detection on a Carl Zeiss LSM 510 META confocal microscope and software LSM 510, version 3.2 (Carl Zeiss, Germany). All images were taken with 63X oil immersion objective.

2.3.3.19 mRNA extraction

mRNA Isolation Kit (Roche) was used for mRNA extraction according to the manufacturers' instructions . Briefly, cells were washed twice with ice cold PBS and lysed by adding lysis buffer. Biotin-labeled oligo$(dT)_{20}$ probe was added to the cell lysate and mixed. Magnetic particles were separated from storage buffer by magnetic separation and washed once with lysis buffer. Thereafter, cell lysate with oligo$(dT)_{20}$ probe was combined with the magnetic particles and incubated for 5 min at 37°C. The magnetic particles were separated from the fluid by magnetic separation and washed three times with washing buffer. Finally, redistilled water was added to the magnetic particles and incubated for 2 min at 65°C. mRNA in fluid was separated from magnetic particles and transferred to a fresh RNase-free tube.

2.4 Plasmid construction details

2.4.1 *Integrin plasmids*

2.4.1.1 Wild type plamids

The β2 cDNA in J8.1E (Douglass et al., 1998) was used as a template for construction of the β2 in mammalian expression vector pcDNA3.0 (Invitrogen) (Figure 2.1). KpnI and SpeI were used to digest β2 cDNA from J8.1E and the fragment was ligated into pcDNA3.0 vector which was digested with KpnI and XbaI (SpeI and XbaI digested DNA fragments have compatible ends). αL, αM and αX cDNA plasmids were in pcDNA 3.0 vector with the restriction cutting sites KpnI and XbaI.

2.4.1.2 Isolated I-domain plasmids

The vector pDisplay (Invitrogen) (Figure 2.2) was used for isolated I-domain constructions. The restriction enzymes XmaI and SacII were used for both αL and αM I-domain. PCR products of αL and αM I-domain were digested with XmaI/SacII and were ligated into pDisplay vector which was digested with the same enzymes.

Specific primers are listed below:

1. αL I-domain
   F 5' CCCGGGAACGTAGACCTGGTATTTCTG 3'
   R 5' CCGCGGAATGACATAGATCTTCTTCTGC 3'

2. αM I-domain
   F 5' CCCGGGGATAGTGACATTGCCTTCTTG 3'
   R 5' CCGCGGCGCAAAGATCTTCTCCCGAA 3'

2.4.1.3 Integrin chimeras and mutations

The αML and αMX cytoplasmic tail chimeras were generated by exchanging the αM

cytoplasmic tail with αL or αX cytoplasmic tail respectively. Restriction enzymes SspbI and XbaI were used for digestion. Because wild type αL cDNA in pcDNA3.0 has two SspbI cut sites, the one at the position of 1652 in αL cDNA was removed by point mutation before digestion with SspbI and XbaI (Figure 2.3). Thereafter, cytoplasmic tails of αL and αX were digested with SspbI and XbaI and ligated into wild type αM plasmid which was also digested by the same enzymes. Point mutations were made by using QuikChange Site-Directed Mutagenesis Kit (Stratagene), and following the manufacturer's protocols. Wild type plasmids were used as templates. Specific primers are listed below:

1. αL I331G FL or αL I331G I-domain
   F 5' CTGCAGAAGAAGGGCTATGTCATTGTC 3'
   R 5'GACAATGACATAGCCCTTCTTCTGCAG 3'

2. αM I332G FL or αM I332G I-domain
   F 5' CTTCGGGAGAAGGGCTTTGCGATCGAAG 3'
   R 5' CTTCGATCGCAAAGCCCTTCTCCCGAAG 3'

3. β2 D709R
   F 5' CTGATCCACCTGAGCCGCCTCCGGGAGTACAGG 3'
   R 5' CCTGTACTCCCGGAGGCGGCTCAGGTGGATCAG 3'

4. β2 N741*
   F 5' CCACGACGGTCATGTAGCCCAAGTTTGCTGAG 3'
   R 5' CTCAGCAAACTTGGGCTACATGACCGTCGTGG 5'

5. β2 N727*
   F 5' AAGTCCCAGTGGAACTAGGATAATCCCCTTTTC 3'
   R 5' GAAAAGGGGATTATCCTAGTTCCACTGGGACTT 5'

6. β2 K702*
   F 5' GCTGGTCATCTGGTAGGCTCTGATCCAC 3'
   R 5' GTGGATCAGAGCCTACCAGATGACCAGC 5'

7. β2 S734*
   F 5' CCCCTTTTCAAGTGAGCCACCACGACGGTC 3'
   R 5' GACCGTCGTGGTGGCTCACTTGAAAAGGGG 3'

8. αMLP1110*
   F 5' CCGAATGGAATCCCTTGAGAAGACTCTGAGCAGCTG 3'
   R 5' CAGCTGCTCAGAGTCTTCTCAAGGGATTCCATTCGG 3'

9. αMXN1132*
   F 5' CAAATTGCCCCAGAAAACTGAACACAGACCCCCAG 3'
   R 5' GCTGGGGGTCTGTGTTCAGTTTTCTGGGGCAATTTG 3'

2.4.1.4 Other constructs

1. HA-tagged Hck59 in pXJ40
   F 5' GCACTCGAGATGGGGTGCATGAAGTCCAAG 3'
   R 5' GCAGGTACCTCATGGCTGCTGTTGGTACTGGC 3'

2. HA-tagged Lck56 in pXJ40
   F 5' GCACTCGAGATGGGCTGTGGCTGCAGCTCAC 3'
   R 5' GCAGGTACCTCAAGGCTGAGGCTGGTACTGGCC 3'

3. CD4 in pcDNA3.0
   F 5' GCAGAATTCATGAACCGGGGAGTCCCTTTTAG-3'
   R 5' GCACTCGAGTCAAATGGGGCTACATGTC-3'

4. His-tagged Hck in pcDNA3.0
   F 5' GCAGGTACCATGGGGTGCATGAAGTCCAAG-3'
   R 5' GCACTCGAGCTAATGATGATGATGATGATGTGGCTGCTGTTGGTACTG
   GC 3'

5. His-tagged Lck in pcDNA3.0
   F 5' GCAGGTACCATGGGCTGTGGCTGCAGCTCA 3'
   R 5' GCACTCGAGCTAATGATGATGATGATGATGAGGCTGAGGCTGGTACTG
   GC-3'

* Mutated bases are underlined

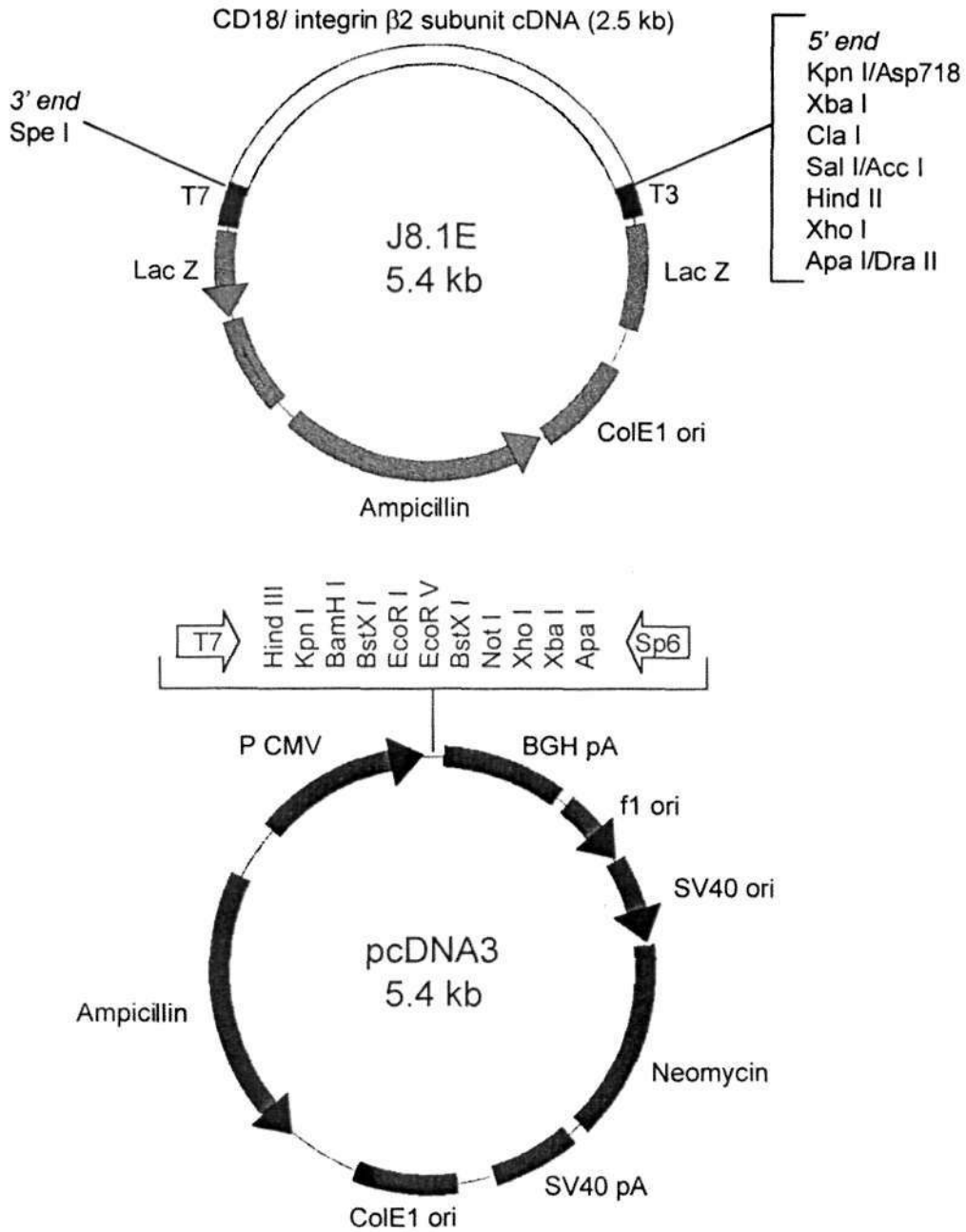


FIG 2.1. Schematic illustration of plasmid J8.1E and pcDNA 3.0.
The β2 cDNA in J8.1E (Douglass et al., 1998) was used as a template for construction of the β2 in mammalian expression vector pcDNA3.0. KpnI/SpeI was used to digest β2 cDNA from J8.1E and the fragment was ligated into pcDNA3.0 vector which was digested with KpnI and XbaI (SpeI and XbaI digested DNA fragments have compatible ends). αL, αM and αX cDNA plasmids were in pcDNA 3.0 vector with the restriction cutting sites KpnI and XbaI.

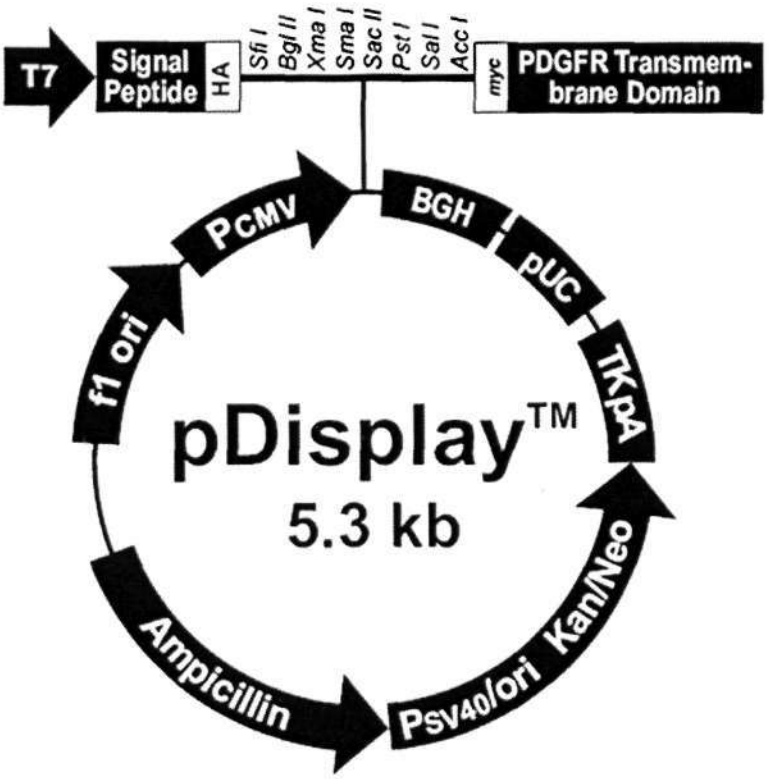


FIG 2.2 Schematic illustration of pDisplay vector.

The restriction enzymes XmaI and SacII were used for both αL and αM I- domain.

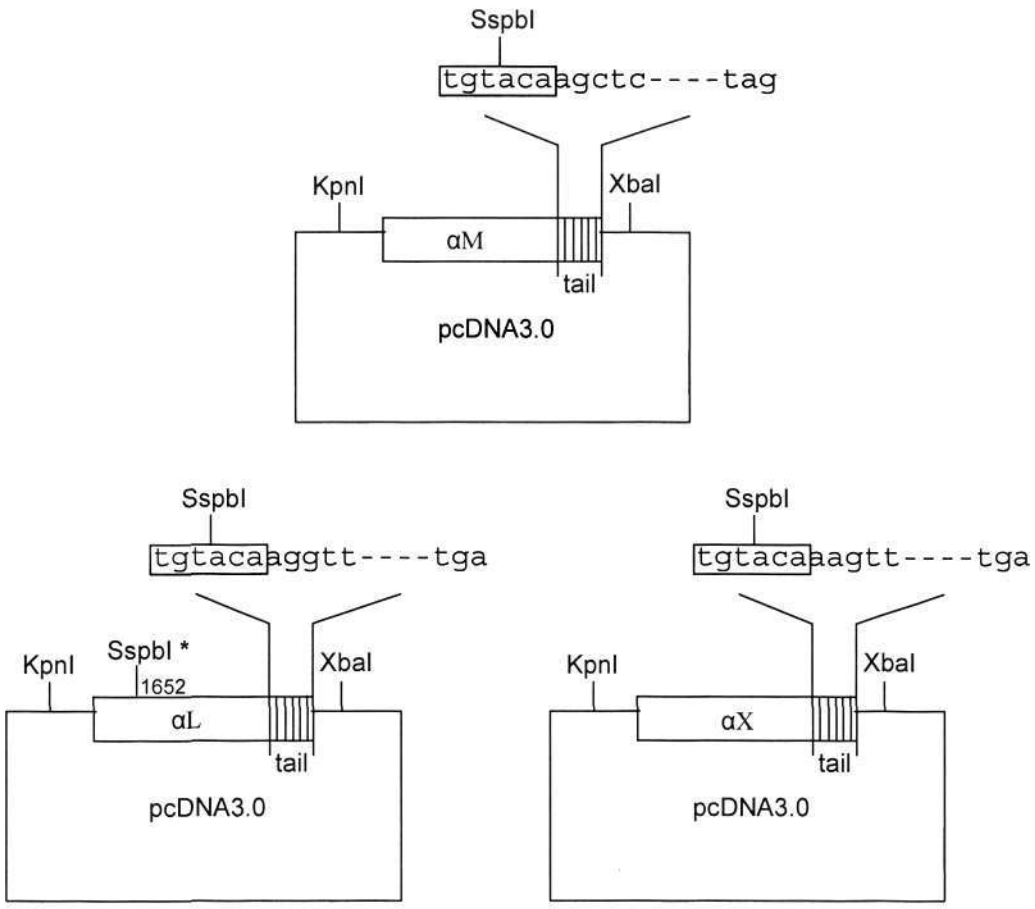


FIG 2.3 Schematic illustration of plasmid construction of αML and αMX chimera. The αML and αMX cytoplasmic tail chimeras were generated by exchanging the αM cytoplasmic tail with αL or αX cytoplasmic tail respectively. Restriction enzymes SspbI and XbaI were used for digestion. There are two SspbI sites on αL cDNA, the SspbI* site at the position 1652 was removed by point mutation before digestion.

Chapter Three: Different activation states of αLβ2 required for ICAM-1 and ICAM-3 binding.

3.1 Introduction

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 (Hynes, 2002). The β I-like domain is flanked by the hybrid and PSI (for plexins, semaphorins and integrins) domains (Figure 3.1A) (Tan et al., 2001; Xiong et al., 2001). The hybrid domain has been shown to be important in the propagation of the activation signal from one end of the β subunit to the other (Tan et al., 2001; Tng et al., 2003; Luo et al., 2004). Recently, superimposed structural coordinates of liganded-open αIIbβ3 headpiece with that of unliganded-closed αVβ3 revealed a ~10Å shift and a concomitant rotation of the hybrid domain relative to the last helix of the β I-like domain upon ligand binding (Xiao et al., 2004). mAbs that attenuate this swing-out motion of the integrin α5β1 hybrid domain prevent effective allosteric activation of the β I-like domain (Mould et al., 2003; Luo et al., 2004).

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 (Xiong et al., 2001; Xiao et al., 2004; Nermut et al., 1988; Weisel et al., 1992; Takagi et al., 2002; Takagi et al., 2003; Xiong et al., 2004; Mould and Humphries, 2004). A bent integrin with the hybrid domain in close proximity of the integrin epidermal growth factor I-EGF3 and I-EGF4

domains represents the resting state. The extended integrin with the hybrid domain distally separated from I-EGF3 and I-EGF4 but orientated toward 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 derives from earlier functional studies which will be described below. Observations were made on integrins having different ligand binding properties under different cellular or extracellular conditions. Resting platelet integrin αIIbβ3 binds immobilized fibrinogen, but binding to soluble fibrinogen, fibronectin, or von Willebrand factor requires platelet activation by agonist (Savage et al., 1991; Philips et al., 1991; Savage et al., 1992). Divalent cation Mn^{2+} activates integrin α4β1 to bind VCAM-1 (vascular cell adhesion molecule 1), whereas adhesion to fibronectin-derived CS-1 peptide requires additional activating mAb (Masumoto et al., 1993). Real time analysis of integrin α4β1 binding to fluorescent-conjugated ligand mimetic via chemokine receptor activation on leukocytes also suggests integrin acquiring multiple affinity states (Chigaev et al., 2001). For the integrin αLβ2, it has been reported that αLβ2 binds to its ligand (ICAM-1,-2,-3) differentially and such difference was probably because ICAM-1, -2, and -3 bind to distinct sites of the αLβ2 I-domain (Binnerts et al., 1994; Binnerts et al., 1999). However, based on crystal structure of an engineered high affinity αL I-domain in complex with ICAM-3 D1 (Song et al., 2005), it was suggested that both ICAM-1 and ICAM-3 share a common docking mode with αL I-domain. Ligand ICAM-1 exhibits higher affinity for purified αLβ2 from T cells than ICAM-3 (de Fougerolles et al., 1992). Prior exposure of αLβ2 to ICAM-1 increased

its binding to ICAM-3 (Buckley et al., 1997). Soluble ICAM (sICAM)-3 binds to αLβ2 with 9-fold lower affinity than sICAM-1 (Woska et al., 1998). It was also reported that two activating mAbs (KIM185 and KIM127) were needed for αLβ2 adhesion to ICAM-3 while only one activating mAb was required for αLβ2 binding to ICAM-1 (Al-Shamkhani et al., 1998). Crystal structures of engineered intermediate affinity αL I-domain (L161C/F299C) and high affinity αL I-domain (K287C/K294C) in complex with ICAM-1 showed differences in their interactions (Shimaoka et al., 2003). Together, it is apparent that distinct αLβ2 affinity states are required to bind to different ICAM ligands. Many inferences on αLβ2 conformation states are drawn from reporting antibody study, such as KIM185, KIM127 and MEM48 (Andrew et al., 1993; Lu et al., 2001a). In this part of study, the reporter mAb MEM148, which recognizes the free integrin β2 subunit or Mg^{2+}/EGTA-activated αLβ2 but not resting αLβ2 (Tan et al., 2001; Drbal et al., 2001), was characterized. The epitope of MEM148 was fine mapped to the hybrid domain and it is dependent on the movement of the hybrid domain upon activation. In addition, we make use of the activation-reporter property of MEM148 to analyze the different αLβ2 affinity states required for ICAM-1 and ICAM-3 binding.

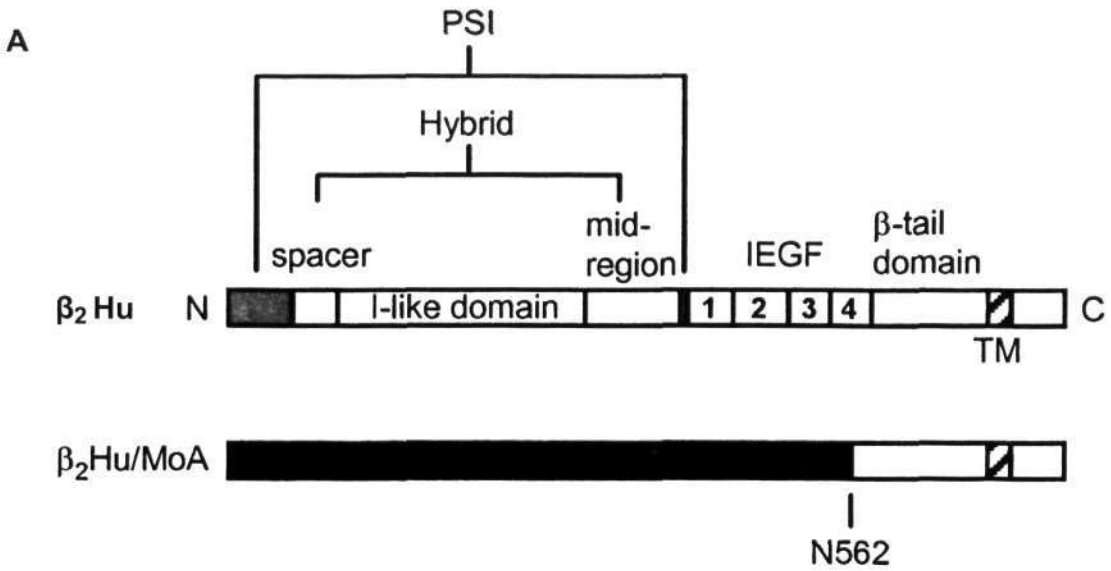
3.2 Results

3.2.1 *Mapping of mAb MEM148 epitope*

The epitope of mAb MEM148 resides in the integrin β2 hybrid domain. It is not exposed in resting integrin αLβ2 but is exposed upon Mg^{2+}/EGTA treatment (Tan et al., 2001).

Because it may serve as a useful reporter mAb for subsequent analyses of integrin affinity states, our group extended the analysis by characterizing its epitope using a panel of integrin β2 human/mouse "knock-out" mutants generated by colleague Dr. Tng (Tng et al., 2004). These human integrin β2 mutants have their residues replaced by corresponding mouse β2 residues at positions where they differ in the mid-region (Figure 3.1B). COS-7 cells, which can express β2 integrin in the absence of the α subunit (Tan et al., 2001) 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 (Figure 3.2A). The mAb MEM48, which maps to residues Leu^{534}, Phe^{536}, and His^{543} of human integrin β2 I-EGF 3 (Lu et al., 2001c), was included as the receptor expression control. An approximately 3-fold reduction in MEM148 epitope expression was observed for transfectants expressing $\beta2_{Hu}$ (H370S/R371I) or $\beta2_{Hu}$ (N372G/Q373K) as compared with $\beta2_{Hu}$ (wt). The β_{2Hu} (P374S) variant showed significant (>90%) abrogation of MEM148 epitope expression.

Next, an integrin β2 human/mouse chimera ($\beta2_{Hu/Mo}$A) (Figure 3.1A) in which Met^{-22}-Asn^{562} of human integrin β2 is replaced by the corresponding region from mouse integrin β2 (Tng et al., 2004) was used to generate two "knock-in" mutants. The $\beta2_{Hu/Mo}$ A(S374P) has the Ser^{374} of the mouse mid-region substituted by the corresponding human Pro residue. The $\beta2_{Hu/Mo}$ A(A368V-S374P) has the segment Ala^{368}-Ser^{374} of the mouse mid-region replaced by the corresponding human segment Val^{368}-Pro^{374}. In this case, mAb KIM185, which maps to integrin β2 I-EGF 4 and β-tail domain (Lu et al., 2001c), was included as the receptor expression control because the epitope of MEM48 is absent in



B

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β2 Mo 342 SSRVFLDHSTLPDTLKVTYDSFCSNGASSIGKS 374
β2 Hu 342 SSRVFLDHNALPDTLKVTYDSFCSNGVTHRNQP 374

β2 Mo 375 RGDCDGVQINNPVTFQVKVMASECIQEQSFVIR 407
β2 Hu 375 RGDCDGVQINVPITFQVKVTATECIQEQSFVIR 407

β2 Mo 408 ALGFTDTVTVQVRPQCECQCRDQ 430
β2 Hu 408 ALGFTDIVTVQVLPQCECRCRDQ 430
```

FIG 3.1. (A) Linear organization of the human integrin β2 subunit. The human integrin β2 subunit is shown with the domains and regions indicated. The mouse fragment in human/mouse chimera β2 Hu/MoA is *shaded* in *black*. *TM*, transmembrane domain. (B) Sequence alignment of human and mouse integrin β2 mid-region. The amino acids that differ are indicated by *solid circles*. The disulfide bridges are connected by *dashed lines*. The segment that is suggested to be part of the PSI domain (Xiao et al., 2004) is *underlined* with the Cys^{425} involved in the long range disulfide bond indicated by an *asterisk* (*).

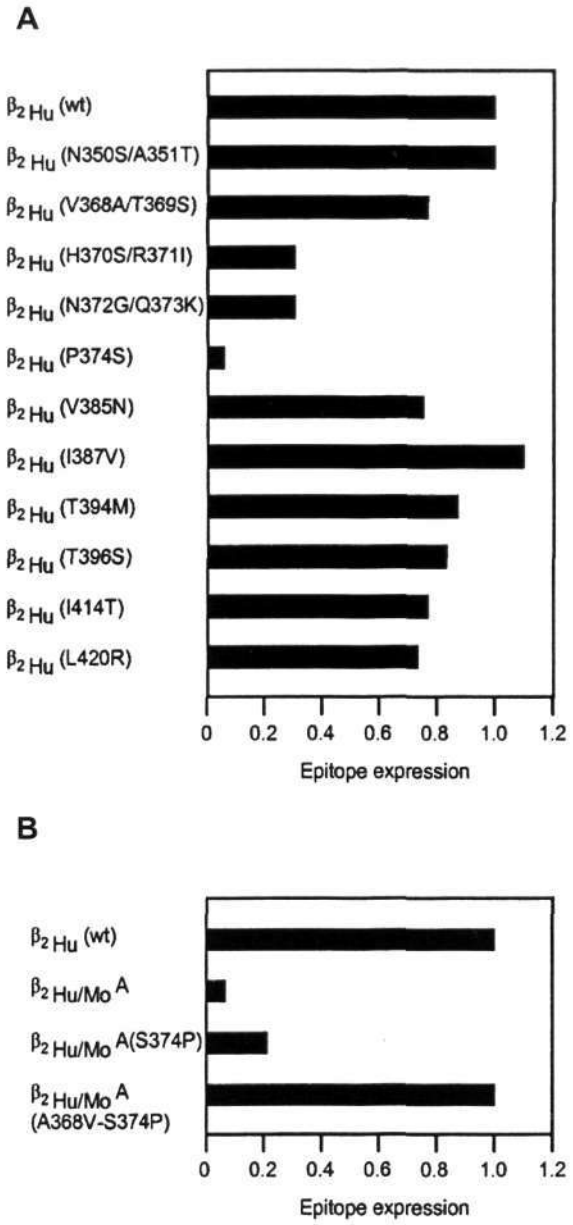

FIG 3.2. Epitope expression of mAb MEM148 on β2 knock-out and knock-in mutants. (A) β2 knock-out mutants were generated and stained for mAb MEM148 followed by flow cytometry analyses. The epitope expression of MEM148 is calculated by MEM148 EI/MEM48 EI (MEM48 is mapped to EGF-4 domain of β2 which is constitutively exposed) 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 β2 I-EGF 4 and β-tail domain (Lu et al., 2001c) and was included as a reference mAb because the epitope of MEM48 is absent in $\beta2_{Hu/Mo}A$. Epitope expression was shown based on expression index (EI).EI was calculated by % percentage cells gated positive multiply by geo-mean fluorescence

$\beta2_{Hu/Mo}$A. The expression of MEM148 epitope on $\beta2_{Hu/Mo}$ A(S374P) was low as compared with $\beta2_{Hu}$(wt) (Fig. 3.2B). However, transfectant bearing $\beta2_{Hu/Mo}$ A(A368V-S374P) fully restored the epitope of MEM148. Thus, it suggests that although human integrin β2 Pro^{374} is a critical residue, other residues, His^{370}, Arg^{371}, Asn^{372}, Gln^{373} are also required for the effective presentation of MEM148 epitope.

3.2.2 *Model of αLβ2 illustrating the MEM148 epitope*

Fine mapping of MEM148 epitope allows us to pinpoint its location in the quaternary structure of integrin. A model of integrin αLβ2 was generated by MODELLER (Tng et al., 2004) using αVβ3 structural coordinates as the template (Xiong et al., 2001) (Figure 3.3A). The structure of an intact I-domain-containing integrin is not solved; hence, the αL I-domain was excluded from the model. For clarity, the αL Calf-2, β2 PSI, I-EGFs, and β-tail domains were not included. The critical residue Pro^{374} of the mAb MEM148 epitope resides on the surface of the hybrid domain facing the αL subunit. In contrast, the epitope of mAb 7E4 (Val^{385}) (Tng et al., 2004) is located away from the αL subunit (Figure 3.3A, B). This could explain why mAb MEM148 fails to bind resting αLβ2; because if resting αLβ2 is in a severely bent conformation as proposed for αVβ3 (Xiong et al., 2001), the epitope of MEM148 will be shielded in this conformation (Figure 3.3C). Along the same line of reasoning, the binding of mAb MEM148 to an activated αLβ2 with an extended conformation is favorable and corroborates with previous observations (Tan et al., 2001; Drbal et al., 2001).

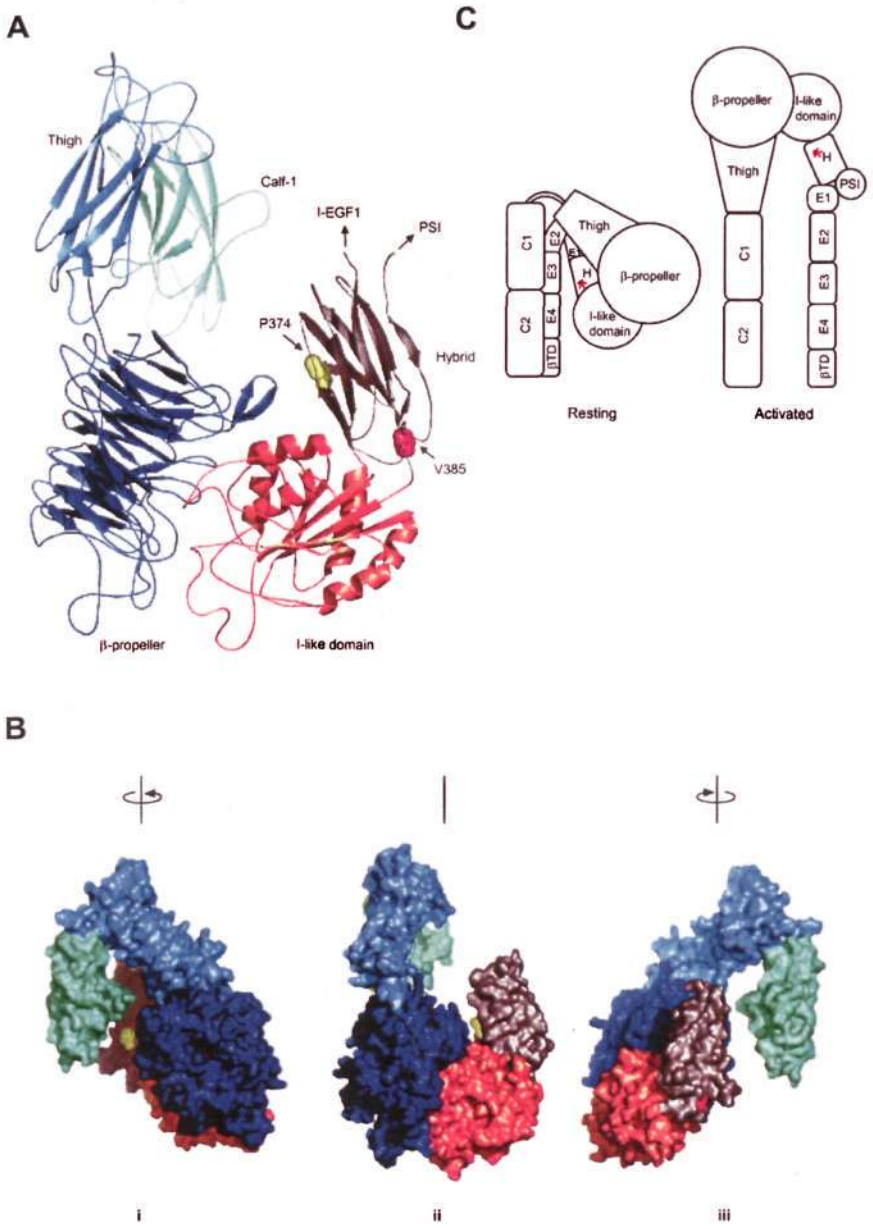

FIG 3.3. Model of the integrin αLβ2. (A) The model of αLβ2 was generated by the MODELLER using the αVβ3 structural coordinates (Xiong et al., 2001) as template. For clarity, only the β-propeller, thigh, Calf-1 of the αL subunit, and the I-like domain and hybrid domain of the β2 subunit are shown. The critical determinants Pro^{374} (*yellow*) for mAb MEM148 and Val^{385} (*red*) for mAb 7E4 (Tng et al., 2004) are indicated. (B) Surface representation with different rotation of the model. Pro^{374} (*yellow*) is located in the interface between the αL and β2 headpiece and between the αLβ2 headpiece and tailpiece interface. Val^{385} (*red*) is exposed. (C) Diagram illustrating the masking of Pro^{374} (*) in resting αLβ2 integrins and its exposure upon activation. The I-domain is not included. *C*, calf; *E*, I-EGF; *βTD*, β tail domain; *H*, hybrid domain.

3.2.3 *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^{2+}/EGTA (Tan et al., 2001). Divalent cations have a major influence on the α I-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 (Xiao et al., 2004). 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 determined whether Mg^{2+}/EGTA-treated αLβ2 can revert to its resting state using MEM148 as the reporter mAb. MOLT-4 cells were incubated in Mg^{2+}/EGTA containing RPMI and either MEM148 or KIM127 at 37 °C. The epitope of KIM127 resides in β2 I-EGF2, and like MEM148, its epitope expression depends on integrin activation (Lu et al., 2001c; Robinson et al., 1992). Significant staining was detected for both mAbs only in the presence of Mg^{2+}/EGTA (Figure 3.4A). The integrin αL-specific mAb MHM24 was included as a control. Next, cells in the presence of Mg^{2+}/EGTA were subsequently treated with EDTA to deplete existing Mg^{2+} 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.

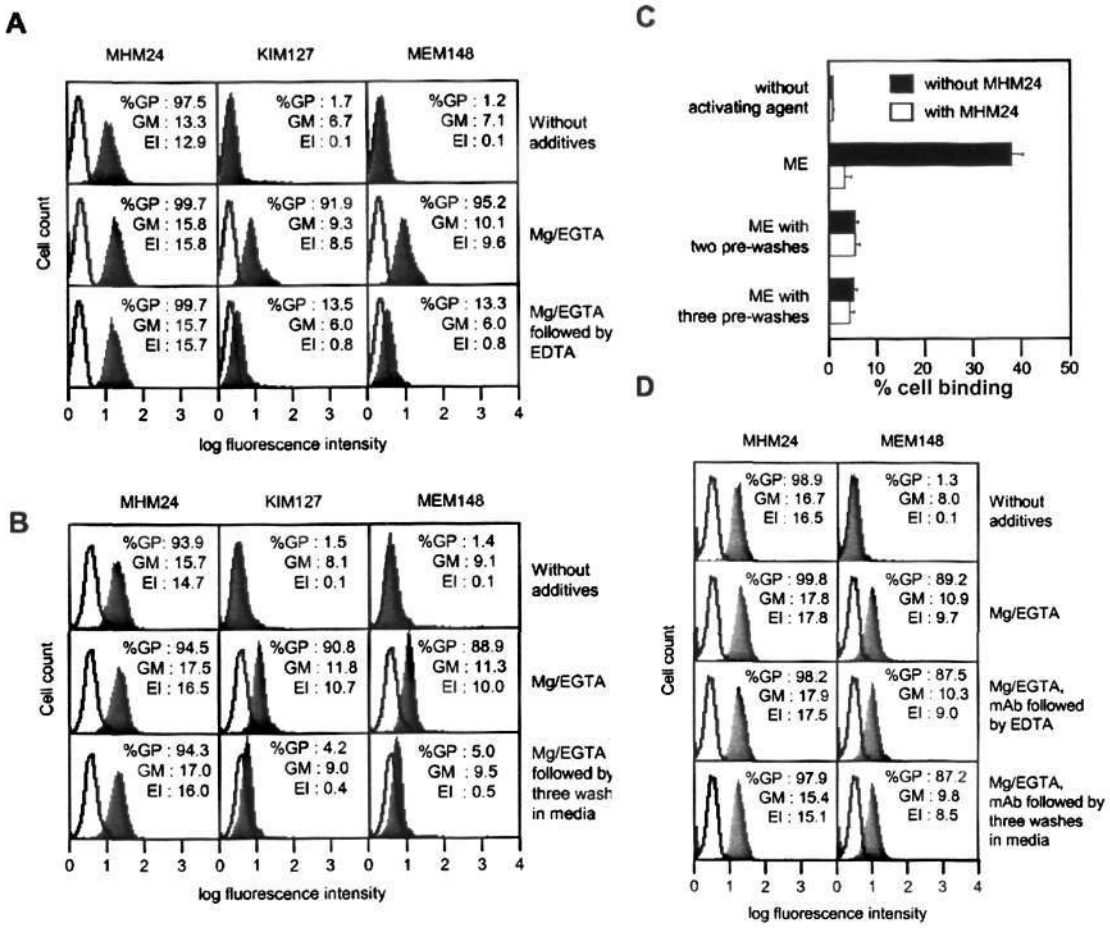

FIG 3.4. Analyses of integrin αLβ2 reversion from Mg^{2+}/EGTA-activated state to the resting state. (A) Flow cytometry analysis of mAb MEM148 epitope expression. MOLT-4 cells were stained with mAb KIM127 or MEM148 under different conditions at 37 °C; in the absence of additives, in the presence of Mg^{2+}/EGTA, or in the presence of Mg^{2+}/EGTA followed by EDTA treatment. All FITC-conjugated secondary antibody staining was performed at 4 °C. (B) In this experiment, instead of EDTA treatment, MOLT-4 cells treated with Mg^{2+}/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^{2+}/EGTA (*ME*). In separate samples, Mg^{2+}/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) The experiment was performed as in (A) and (B) except that cells were first treated with Mg^{2+}/EGTA in the presence of MEM148 and washed twice in media followed by EDTA treatment or further washes before secondary antibody staining and flow cytometry analyses. %*GP*, percentage cells gated positive; *GM*, geo-mean fluorescence; *EI*, expression index.

We next tested whether Mg^{2+}/EGTA-treated cells after washing in media could still express the epitopes of MEM148 and KIM127 (Figure 3.4B). Mg^{2+}/EGTA-treated cells were subjected to three washes in RPMI without additives followed by staining for either of the two mAbs. Low levels of MEM148 or KIM127 epitope expression were detected as compared with Mg^{2+}/EGTA-treated cells without washing.

Cell binding assay to ICAM-1 was investigated to determine whether Mg^{2+}/EGTA-treated cells followed by washing could still adhere to ICAM-1 (Figure 3.4C). In the presence of Mg^{2+}/EGTA, cells adhered significantly to ICAM-1. However, adhesion was minimal when cells were treated with Mg^{2+}/EGTA followed by two or three washes in RPMI wash buffer before allowing adherence to ICAM-1. Binding specificity was shown by including αL-specific function-blocking mAb MHM24. Together, our data suggest that reversion of Mg^{2+}/EGTA-activated αLβ2 to its resting conformation is possible when the activating divalent cation is depleted.

We furthered our analyses by changing the order of MOLT-4 treatment with different agents to test whether MEM148 remains bound to Mg^{2+}/EGTA-treated cells even in the presence of EDTA. Cells were first treated with Mg^{2+}/EGTA in the presence of MEM148 with subsequent treatment with EDTA or additional washes in media (Figure 3.4D). MEM148 staining was detected in both cases. This suggests that the αLβ2·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 αLβ2 ligand binding (Tng

et al., 2004; Rothlein et al., 1986). PMA also induced the expression of MEM148 epitope on myeloid cells; however, this is contributed by a proteolytically cleaved fragment of β2 unassociated with the αL, αM or αX subunit rather than the respective heterodimers (Drbal et al., 2001). To test whether PMA induced MEM148 epitope expression on MOLT-4 cells, MOLT-4 cells were surface labeled by sulfo-NHS-biotin (Pierce) at 0.5 mg/ml in PBS for 20 min on ice. Thereafter, cells were incubated in Mg/EGTA (5 mM $MgCl_2$ and 1.5 mM EGTA) or phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) with mAbs (irrelevant mAb, MHM23, or MEM148, in each condition, approximately $1x10^6$ cells were used) in RPMI media containing 5% (v/v) heat-inactivated fetal bovine serum and 10 mM HEPES (pH 7.4) for 30 min at 37 °C. Cells were washed twice with PBS and lysed in lysis buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40) with protease inhibitors followed by immunoprecipitation with rabbit anti-mouse IgG coupled to protein A-Sepharose beads (Sigma). Bound proteins were resolved by SDS-PAGE under reducing conditions and transferred onto Immobilon P membranes (Millipore, Bedford, MA), and protein bands were detected by ECL (GE Healthcare Amersham Biosciences) (Figure 3.5). 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^{2+}/EGTA or PMA treatment. MEM148 only immunoprecipitated αLβ2 when cells were treated with Mg^{2+}/EGTA but not with PMA. No corresponding protein bands were detected using irrelevant mAb, but αLβ2 bands were detected under all conditions using the heterodimer dependent β2-specific mAb MHM23. Truncated β2 (65-70 kDa) (Drbal et al., 2001) was not detected in MEM148 immunoprecipitation samples of PMA-

treated MOLT-4 cells. It is possible that the truncated αLβ2 is mainly expressed and is abundantly up-regulated on the surface of activated myeloid cells instead of lymphocytes (Drbal et al., 2001).

It is also interesting to note that PMA did not induce detectable expression of MEM148 epitope on αLβ2, although it was reported to promote αLβ2 ligand binding (Tng et al., 2004; Rothlein et al., 1986). It is possible that PMA promotes an active αLβ2 conformation that is different from that triggered by Mg^{2+}/EGTA for example. Alternatively, PMA increases the lateral mobility of αLβ2 on plasma membrane, allowing receptor clustering, which effects ligand binding (Kucik et al., 1996).

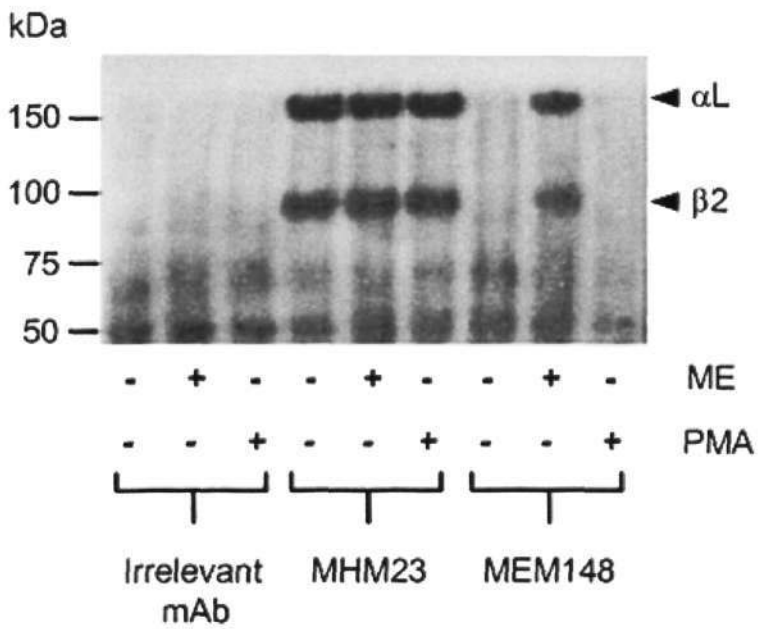

FIG 3.5. Effect of PMA on MEM148 staining to αLβ2. MOLT-4 cells were surface-biotinylated. Labeled cells were treated with Mg^{2+}/EGTA or PMA (100 ng/ml) in the presence of irrelevant mAb, MHM23 or MEM148. Cells were washed and lysed in lysis buffer followed by immunoprecipitation and ECL detection. Mg^{2+}/EGTA (ME). Equal amount of protein as determined by Bio-Rad protein assay kit of total cell lysate was loaded in each well.

3.2.4 *Binding of αLβ2 to immobilized ICAM-1 and ICAM-3 requires different affinity states*

To understand the molecular basis of αLβ2 activation with respect to ICAM-1 and ICAM-3 binding, we performed immobilized ligand binding assays using MOLT-4 cells. Cells were allowed to adhere to ICAMs in the presence of Mg^{2+}/EGTA, activating mAb, or a combination of both (Figure 3.6). MOLT-4 binding to ICAM-1 was minimal in the absence of activating agents. In the presence of Mg^{2+}/EGTA or any of the activating mAbs KIM185 or MEM48, binding to ICAM-1 was significantly augmented (Figure 3.6A). In contrast, in the presence of MEM148 alone, binding to ICAM-1 was minimal. Under the conditions of Mg^{2+}/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 αLβ2 on MOLT-4 cells because binding was effectively abrogated in the presence of function-blocking αL-specific mAb MHM24. It is, therefore, possible that activation of αLβ2 by either Mg^{2+}/EGTA or one of the activating mAbs may convert αLβ2 into an intermediate affinity state. Promotion of this population of αLβ2 into a higher affinity state can still be achieved by an additional activating agent.

ICAM-3 binding was carried out under similar conditions (Figure 3.6B). Binding to ICAM-3 was minimal in the presence of Mg^{2+}/EGTA or any of the mAbs. When Mg^{2+}/EGTA and one of the activating mAbs were included, binding was significantly augmented. This was in concordance with previous findings that mAbs KIM185 and KIM127 were required for αLβ2 COS-7 transfectants binding to ICAM-3 (Al-Shamkhani

et al., 1998). 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, whereas the other activating agent was Mg^{2+}/EGTA. Abrogation of binding by MHM24 demonstrated interaction specificity between αLβ2 and ICAM-3. Noteworthy, the combination of Mg^{2+}/EGTA with mAb MEM148 only had a marginal effect on αLβ2/ICAM-3 binding as compared with 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 a marginal effect on cell binding under all conditions (Figure 3.6C).

Binding of MEM148 to β2 hybrid domain is permissible on Mg^{2+}/EGTA-activated but not resting αLβ2. The ICAM-3 binding data suggest that Mg^{2+}/EGTA-activated αLβ2 adopts a conformation in which the epitope of MEM148 becomes exposed. The quaternary conformation adopted by Mg^{2+}/EGTA-activated αLβ2 may be similar to one of the intermediate affinity states proposed for β3 integrins (Xiao et al., 2004).

3.2.5 *Binding of αLβ2 to soluble ICAM-1 and ICAM-3*

If resting αLβ2 assumes a severely bent conformation like αVβ3, the accessibility of ligands to the α I-domain and β I-like domain on the cell surface is unfavorable because they are oriented toward the plasma membrane. It is necessary to further the analyses by using sICAMs binding assays (Figure 3.7) (Shimaoka et al., 2001; Yang et al., 2004). 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

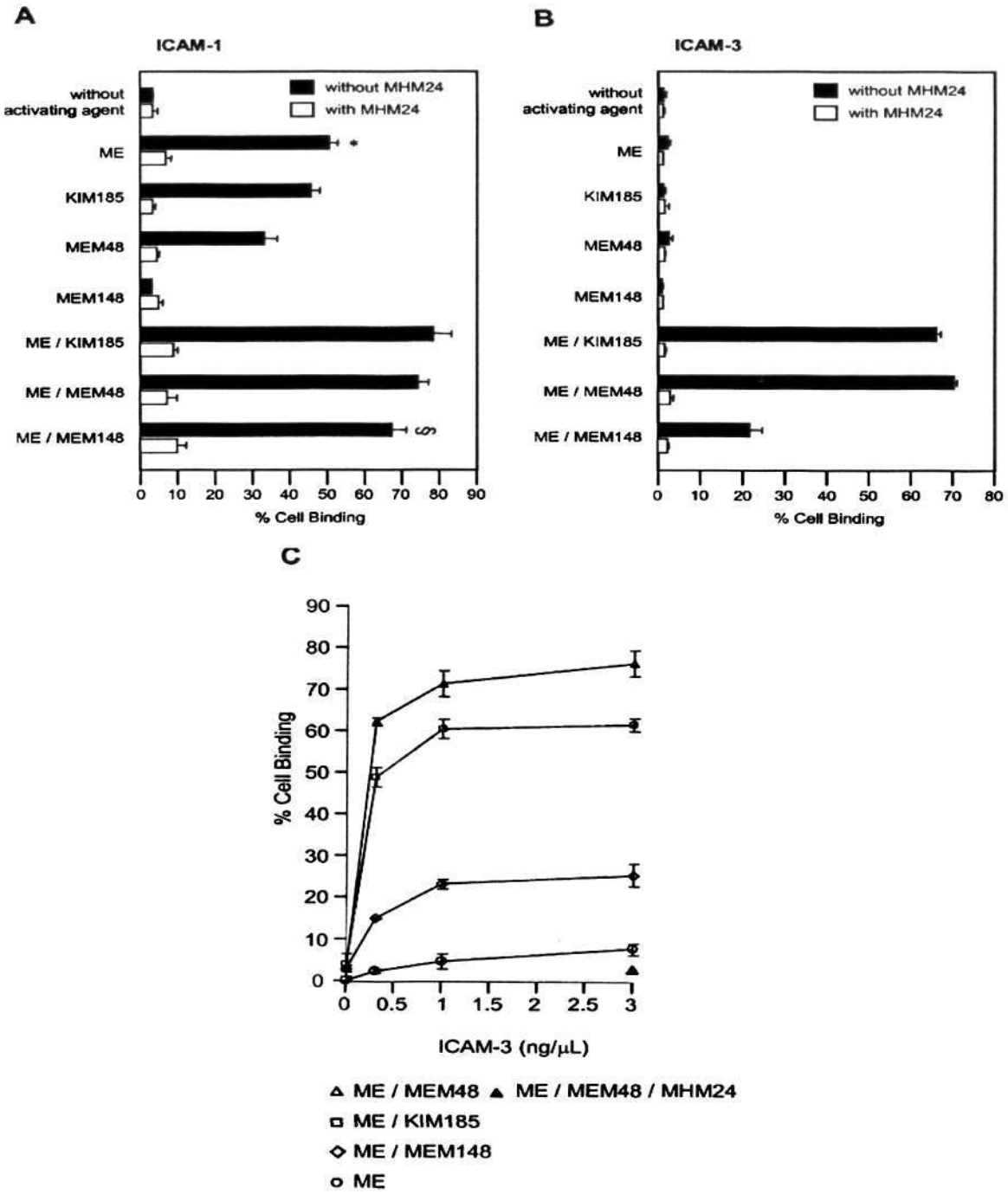

FIG 3.6. Binding of MOLT-4 cells to immobilized ICAMs. (A) MOLT-4 cells binding to ICAM-1-coated wells. Binding was specific because the addition of αL-specific mAb MHM24 abrogated binding under different conditions. (* and §, Student's *t* test, assuming unequal variance, $p < 0.01$). (B) MOLT-4 cells 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. ME, Mg^{2+}/EGTA.

cell surface. MOLT-4 cells bind to sICAM-1 when treated with Mg^{2+}/EGTA, KIM185, or MEM48 but not MEM148. Binding was increased in the presence of Mg^{2+}/EGTA with KIM185 or MEM48. In the presence of Mg^{2+}/EGTA and MEM148 binding was augmented as compared with Mg^{2+}/EGTA alone, which is similar to that observed under immobilized ICAM-1 binding assay (Figure 3.6A).

sICAM-3 binding to MOLT-4 cells was detected only when cells were treated with Mg^{2+}/EGTA together with KIM185 or MEM48. Similar to immobilized ICAM-3 binding assay, Mg^{2+}/EGTA with MEM148 could promote MOLT-4/sICAM-3 binding albeit at a lower level as compared with Mg^{2+}/EGTA with KIM185 or MEM48. Bindings were αLβ2-specific because they were effectively abrogated by MHM24. It was noted that there was a difference between immobilized and soluble ICAM assays. The percentage cell binding of Mg^{2+}/EGTA- and MEM48-treated MOLT-4 on ICAM-1, for example, was similar with that on ICAM-3 (Figure 3.6A and B). However, binding to sICAM-3 was much lower than sICAM-1 with Mg^{2+}/EGTA and MEM48 (Figure 3.7A and B). Such a difference was consistently detected in repeat experiments performed. It was also reported that sICAM-3 binding to purified αLβ2 was weaker as compared with sICAM-1 although the reason for these observations is unclear (Woska et al., 1998).

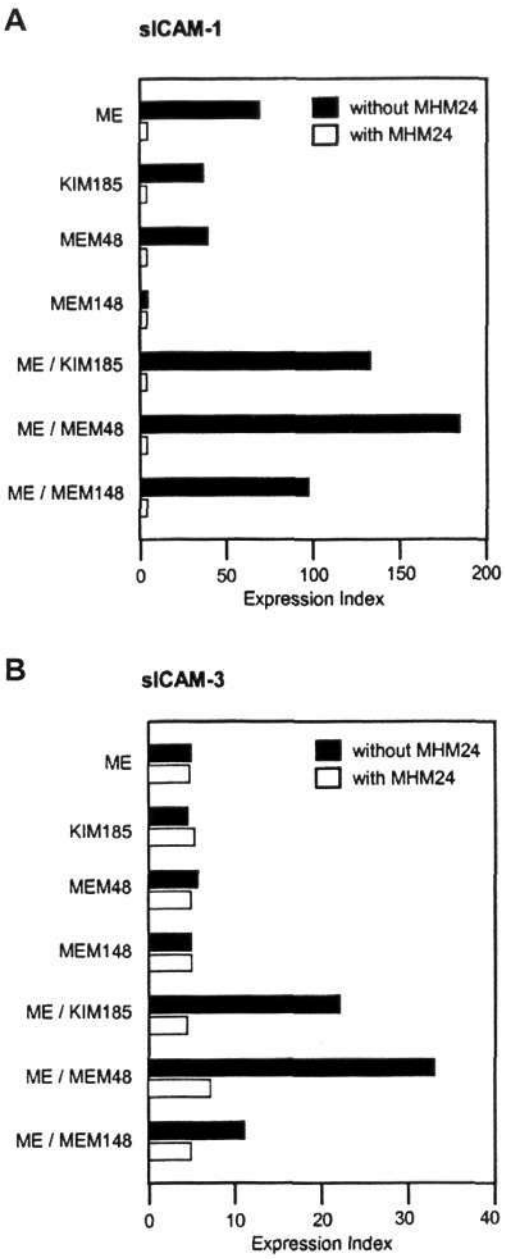

FIG 3.7. Binding of MOLT-4 cells to soluble ICAMs. MOLT-4 cells were allowed to bind to sICAM as described under "Material and Methods." The level of binding was determined by the intensity of fluorescence detected on the cell surface by flow cytometry. Fluorescence staining of MOLT-4 binding to sICAM in the absence of any additives was used as background for gating purposes. Binding was represented by the expression index calculated by percentage of cells gated positive multipy by geo-mean fluorescence. Binding was specific because the addition of αL-specific mAb MHM24 abolished binding under all conditions. ME, Mg^{2+}/EGTA. (A) MOLT-4 binding to sICAM-1. (B) MOLT-4 binding to sICAM-3. Results are representative of three independent experiments.

3.3 Discussion and conclusion

The headpiece of the integrin αLβ2 may be analogous to the integrin αIIβ3 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 (Xiao et al., 2004). The β2 I-like domain of integrin αLβ2 allosterically regulates ligand binding of the αL I-domain through binding of the β2 metal ion-dependent adhesion site (MIDAS) to Glu^{310}, found in the last helix of the αL I-domain. This was shown by replacing αL Glu^{310} with Cys and either β2 MIDAS Ala^{210} or Tyr^{115} to Cys, allowing a disulfide connection between the two domains (Yang et al., 2004). Downward displacement of this helix by β2 I-like domain with a "pull spring" motion triggers I-domain conversion from a closed to an open conformation ready for ligand binding (Yang et al., 2004). The hybrid domain is linked directly to the β I-like domain (Xiong et al., 2001). Previous study on αLβ2 hybrid domain maps the epitope of mAb 7E4 to Val^{385} and demonstrates the importance of the hybrid domain for the transfer of activation signal from β2 membrane proximal region to the αL I-domain (Tng et al., 2004). Preventing the movement of integrin α5β1 hybrid domain away from its β1 I-like domain by mAb SG/19 maintains α5β1 in low affinity for fibronectin, as determined by surface plasmon resonance (Luo et al., 2004).

Herein, we show that the binding determinant of mAb MEM148 consists of the critical residue Pro^{374} and residues His^{370}-Gln^{373} of the integrin β2 hybrid domain. Distinct from the exposed epitope Val^{385} recognized by mAb 7E4, these residues are hidden in the αLβ2 model using the bent αVβ3 as a template (Figure 3.3). It shares similarity to the epitopes,

recognized by mAbs 15/7 and HUTS-4, which are located on integrin β1 hybrid domain that are partly masked by integrin α5 β-propeller (Mould et al., 2003). Accessibility of mAb MEM148 to these 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 (Tan et al., 2001; Drbal et al., 2001). However, when exposed to Mg^{2+}/EGTA, αLβ2 can undergo a conformational change possibly via unbending, thereby exposing the epitope of MEM148.

Crystal structures of the integrins αIIbβ3 and αVβ3 with ligand mimetics, electron microscopy images of soluble integrin αVβ3 with cyclo-RGDfV peptide in the presence of Mn^{2+} or Ca^{2+}, and that of integrin α5β1 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 (Xiong et al., 2001; Xiao et al., 2004; Takagi et al., 2002; Takagi et al., 2003; Xiong et al., 2004). Based on these observations, it was proposed that integrin activation involves at least three affinity states (Xiao et al., 2004). 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 β I-like domain. Previously, it was reported that αLβ2 binding to ICAM-1 and ICAM-3 exhibit different affinities (de Fougerolles et al., 1992; Buckley et al., 1997; Woska et al., 1998). In this study we found that mAb MEM148 in combination with activating agents Mg^{2+}/EGTA further enhanced the affinity of αLβ2/ICAM-1 binding as compared with Mg^{2+}/EGTA alone. The observation could be attributed to the disruption of the extensive contacts between the αLβ2 headpiece-tailpiece interface when

in the presence of Mg^{2+} and with Ca^{2+} depleted. This would facilitate the unbending of αLβ2, hence allowing MEM148 to access the epitope masked previously.

From our αLβ2/ICAM-3 binding data, two activating agents are required to promote binding. Collectively, this implies that the conformation adopted by αLβ2 in the presence of Mg^{2+}/EGTA, mAb KIM185, or MEM48 represents an affinity state(s) capable of ICAM-1 but not ICAM-3 binding. The αLβ2 conformer in the presence of Mg^{2+}/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 enhance αLβ2/ICAM-1 binding and promote αLβ2/ICAM-3 binding. Indeed, a previous study showed that the mAb 7E4 binds to αLβ2 hybrid domain and prevents αLβ2 activation by mAb KIM185 but not Mg^{2+}/EGTA (Tng et al., 2004). The Mg/EGTA-activated αLβ2 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^{2+}/EGTA activation still did not favor αLβ2 binding of ICAM-3. We also found that the combination of Mg^{2+}/EGTA with mAb MEM148 did not promote effective αLβ2/ICAM-3 binding as opposed to Mg^{2+}/EGTA with mAb KIM185 or MEM48. It differs from that of ICAM-1 binding in which Mg^{2+}/EGTA and MEM148 had a cumulative effect. If mAb MEM148 further displaces the hybrid domain in Mg^{2+}/EGTA-activated αLβ2, resulting in a higher affinity state comparable with Mg^{2+}/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^{2+}/EGTA-activated conformation of αLβ2 by binding to its re-positioned hybrid domain.

It was hypothesized that other intermediates may exist during αVβ3 activation (Takagi et al., 2002). These intermediates may be depicted by half-bent structures with separation of their α and β subunits but still connected at their heads. Solution structure of intact integrin in Mg^{2+} only is lacking; nonetheless, it is possible that Mg^{2+}/EGTA-activated αLβ2 adopts a conformation akin to one of these intermediates. This may explain the reactivity of mAb MEM148 to Mg^{2+}/EGTA-activated αLβ2. The Mg^{2+}/EGTA-activated αLβ2 "intermediate" is also a dynamic structure that can be reverted to its resting bent conformation. We found that the reactivity of mAbs MEM148 and KIM127 was diminished in EDTA-treated αLβ2, which was beforehand activated by Mg^{2+}/EGTA. EDTA does not directly affect these mAbs because staining could be detected for single integrin β2 expressed on COS-7 cells in the presence of 5 mM EDTA (Tan et al., 2001). Because mAbs MEM148 (Tan et al., 2001) and KIM127 (Lu et al., 2001c) report β2 hybrid domain and I-EGF2 movement during αLβ2 activation respectively, our data favor a global conformation reversion to its resting state rather than a localized effect.

Structural data revealed an intermediate affinity αL I-domain (Leu^{161}-Cys/Phe^{299}-Cys lock) capable of ICAM-1 binding in the presence of Mg^{2+} (Shimaoka et al., 2003). The transition between low, intermediate, and high affinity conformations of αL I-domain revealed a ratchet-like movement of its β6- α7 loop (Shimaoka et al., 2003; Huth et al., 2000). Recently, molecular dynamics simulation reveals that intermediate affinity αL I-domain can be generated by applying a pulling-force on the α7 helix (Jin et al., 2004), which is allosterically regulated by the β2 I-like domain. It was demonstrated that Mg^{2+}/EGTA-treated αLβ2 could be further activated to bind ICAM-1 by the addition of activating mAb KIM185 or MEM48. Mg^{2+}/EGTA treatment would have converted αLβ2

into an intermediate affinity state. The addition of these mAbs would disrupt the interface between the αL and β2 leg pieces with eventual propagation of conformational change to the hybrid domain and β2 I-like domain. This allows further displacement of the I-domain α7 helix transforming the αLβ2 from an intermediate to a high affinity state for ICAM-1 binding.

Why does binding to ICAM-1 and ICAM-3 require different αLβ2 conformations? The difference may be contributed 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 ICAM-3 on the cell surface is similar, both having five C2-set IgSF domains. The binding sites in the ICAMs for αLβ2 also share similarity; Glu^{34} and Gln^{73} in IgSF domain 1 (D1) of ICAM-1 (Staunton et al., 1990) and the corresponding residues Glu^{37} and Gln^{75} in D1 of ICAM-3 (Klickstein et al., 1996; Sadhu et al., 1994; Holness et al., 1995). However, Lys^{39}, Met^{64}, Tyr^{66}, and Asn^{68} of ICAM-1 are important for binding to αL I-domain (Shimaoka et al., 2003), but Asn^{23}, Ser^{25}, and Phe^{54} of ICAM-3 are required for effective interaction (Klickstein et al., 1996). In addition, ICAM-3 IgSF domain 1 is extensively glycosylated as compared with ICAM-1 IgSF domain 1 (de Fougerolles et al., 1992). The binding sites of ICAM-1 and ICAM-3 on the αL I-domain have also been determined. Residues surrounding the αL MIDAS and those in proximity are found to be critical for ICAM-1 and ICAM-3 binding (Shimaoka et al., 2003; Song et al., 2005; Huang et al., 1995; Edwards et al., 1995). In another study, the Ile-Lys-Gly-Asn motif, located in the N terminus of the αL I-domain, was shown to be important for ICAM-3 but not ICAM-1 binding (van Kooyk et al., 1996). This is intriguing because the metal ion-dependent adhesion site is positioned at the top of the αL I-domain, whereas its N

terminus is located at its bottom. Recent crystal data of an engineered high affinity αL I-domain in complex with ICAM-3 D1 suggest that both ICAM-1 and ICAM-3 share a common docking mode with αL I-domain (Song et al., 2005). In this article, it was suggested that the differential binding may be contributed by: first, hydrophobicity at the binding surface (ICAM-1> ICAM-2> ICAM-3); second, glycosylation (ICAM-3>ICAM-1, and deglycosylation of ICAM-3 can increase affinity to αLβ2). However, the exact nature and differences of the interactions between intact ICAMs and αLβ2 require further investigations.

In conclusion, this study using β2 hybrid domain-specific reporter mAb MEM148 suggests the requirement of different affinity states for integrin αLβ2 binding to ICAM-1 and ICAM-3. A proposed model explaining these observations would be the displacement of the β2 hybrid domain in Mg^{2+}/EGTA-treated αLβ2, which renders αLβ2 capable of ICAM-1 binding. However, this movement did not generate an αLβ2 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 αLβ2 activation by structural analyses of intact αLβ2 with ICAMs under different conditions.

Chapter Four: Differential activation of αLβ2 and αMβ2 ligand binding domains

4.1 Introduction

The α subunits contain a β-propeller structure formed by seven repeating elements (Xiong et al., 2001). 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 (Plow et al., 2000). Crystal structures of recombinant I-domains of αL (Qu et al., 1995), αM (Lee et al., 1995a; Lee et al., 1995b), and αX (Vorup-Jensen et al., 2003) 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 (Qu et al., 1995; Lee et al., 1995b; Lee et al., 1995a; Vorup-Jensen et al., 2003; Huth et al., 2000).

Two conformations for integrin I-domains were identified when the integrin αM I-domain was crystallized (Lee et al., 1995a). 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 (Huth et al., 2000). 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 (Huth et al., 2000).

This isoleucine is invariant among the integrin α subunits (Figure 4.1A). 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 αMβ2 integrin (Xiong et al., 2000). The hydrophobic side chain finger of the isoleucine was described as fastened in the closed conformation by a conserved hydrophobic socket (Xiong et al., 2000). This "socket for isoleucine," or SILEN, was postulated to be a key component in allosteric regulation, controlling affinity and shape shifting in the I-domain of αMβ2. 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 (Vorup-Jensen et al., 2003).

It was reported previously that systematic truncation of integrin β2 leads to differential activation of αLβ2and αMβ2 ligand binding capacity (Tan et al., 2000). 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 (Yang et al., 2004) and dead-bolt (Xiong et al., 2003). However, revelation of integrin activation, involving the I-domain, is limited by the lack of structural data of 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 αLβ2 and αMβ2 ligand binding (Tan et al., 2000). Alternatively, the integrin I-domains may have regulatory mechanisms which are different. To distinguish between these two possibilities, SILEN isoleucine mutants were introduced into full-length αL and αM, as well as the isolated I-domain of αL and αM. The ligand binding activities of these mutants were analyzed.

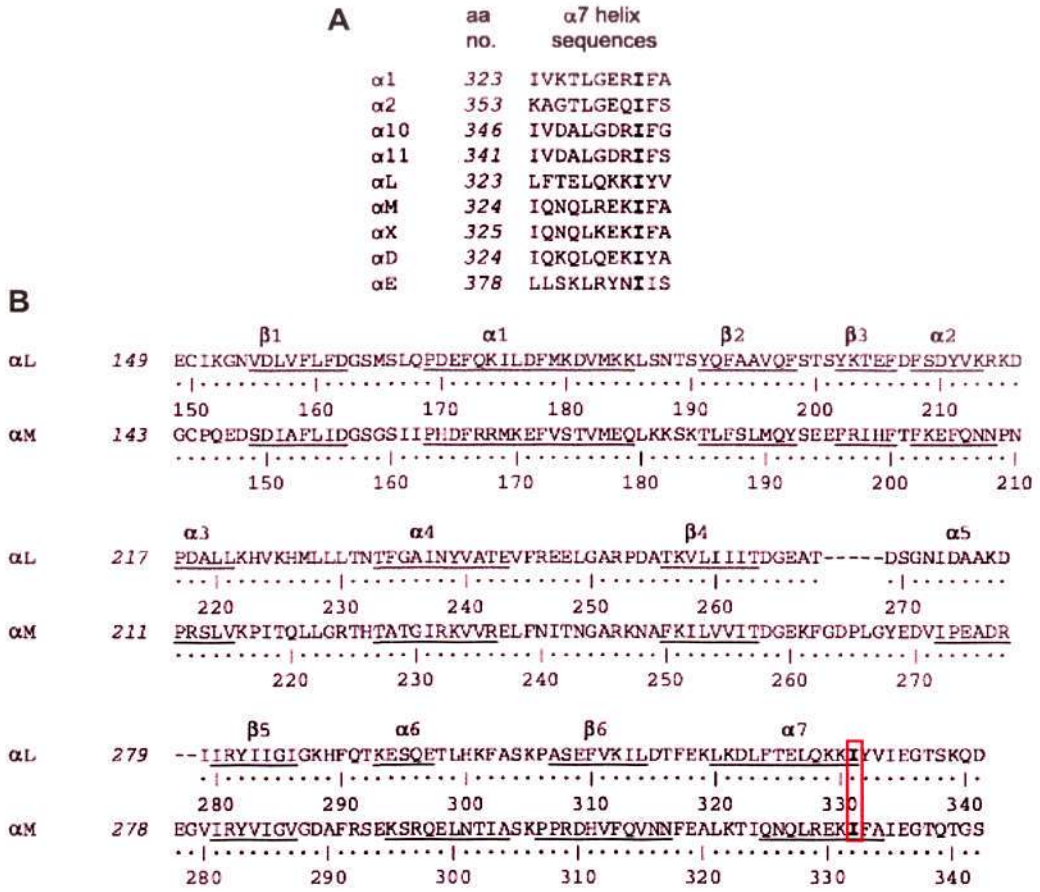

FIG 4.1. Sequence alignment of integrin α subunit I-domains. (A) Alignment of α7 helices of all known human integrin α I-domains. Amino acid numbers (aa no.) of the first residues in each α7 helix are indicated in italics (in all cases, the initiating methionine is numbered 1). The invariant isoleucine 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 (Thompson et al., 1994) was used to align the sequences.

4.2 Results

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

(The experiments in 4.2.1 and 4.2.2 were performed together with colleague Dr. Susannah E. Walters). 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 αMβ2 was determined by flow cytometry to be comparable (Figure 4.2A). The transfectants were assessed for their adhesion to denatured BSA (Davis, 1992) (Figure 4.2B). The mutant I332G in the αM subunit yielded αMβ2 that was active. Ligand binding could not be further activated in the presence of either of the activating antibodies KIM185 or MEM48.

4.2.2 *Mutation of I331G in the full-length integrin αL subunit has minimal effect on αLβ2 binding to ligand*

The experiment with the mutation in αLβ2 was repeated. 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. 4.3A). The transfectants were also assessed for their adhesion to ICAM-1 (Marlin et al., 1987) (Fig. 4.3B). 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^{2+}/EGTA, or both. Binding to ICAM-1 was specific since it was abrogated in the

presence of function-blocking αL-specific mAb MHM24.

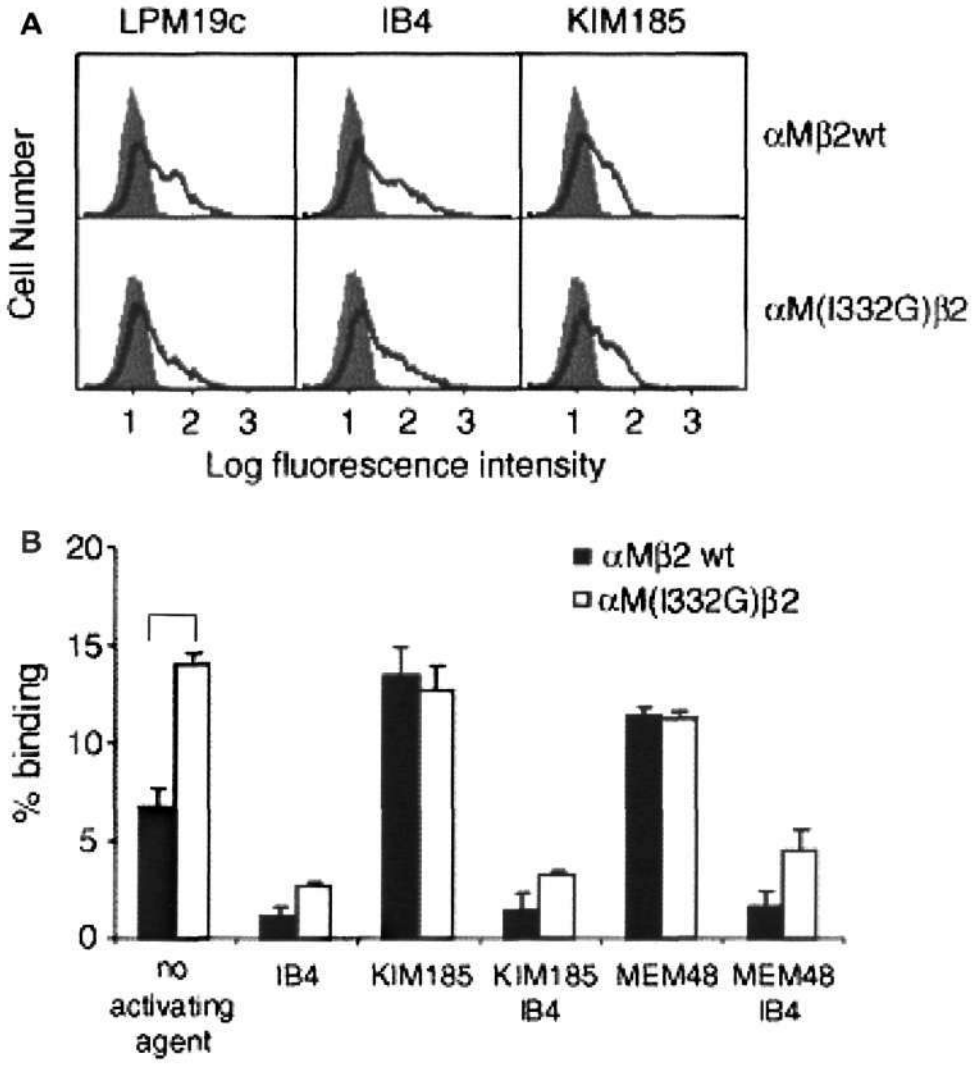

FIG 4.2. αM I332G variant is confirmed to be constitutively active in binding to αM ligand. (A) Cell surface expression of wild-type (wt) αMβ2 and αM(I332G)β2 as determined by flow cytometry. Background histograms (shaded) were obtained using the mAb MHM24. The mAbs LPM19c, IB4, and KIM185 were used 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.

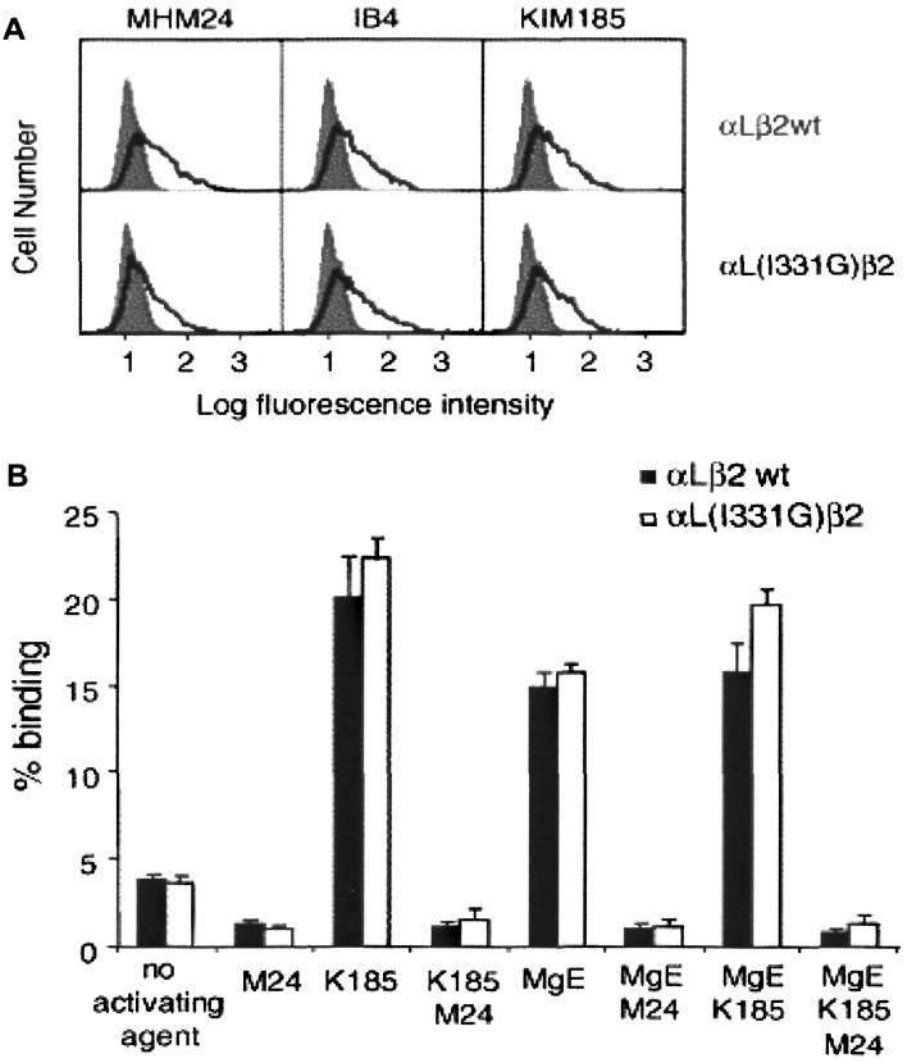

FIG 4.3. αL I331G mutation shows minimal difference when binding to ligand ICAM-1 compared to wild-type αL. (A) Cell surface expression of wild-type (wt) αLβ2 and αL(I331G)β2 as determined by flow cytometry. Background histograms (shaded) were obtained using the mAb LPM19c. The mAbs MHM24, IB4, and KIM185 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^{2+}/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).

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

These differences in the activity of αMβ2 and αLβ2 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} (Figure 4.1B) was engineered in fusion with a C-terminus coding for the c-myc tag and PDGF transmembrane and cytoplasmic tail in the pDisplay vector (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 (Figure 4.4A). Interestingly, transfectants expressing pDL-wt were found to be constitutively active and could bind ICAM-1 even in the absence of activating agents Mg^{2+}/EGTA (Figure 4.4B). Similar binding profiles were observed for pDL-I331G. In contrast, intact full-length αLβ2 could bind to ICAM-1 only in the presence of Mg^{2+}/EGTA. Furthermore, addition of Mg^{2+}/EGTA to the isolated I-domain constructs did not result in any further activation of the receptors, confirming their constitutive ligand-binding activity.

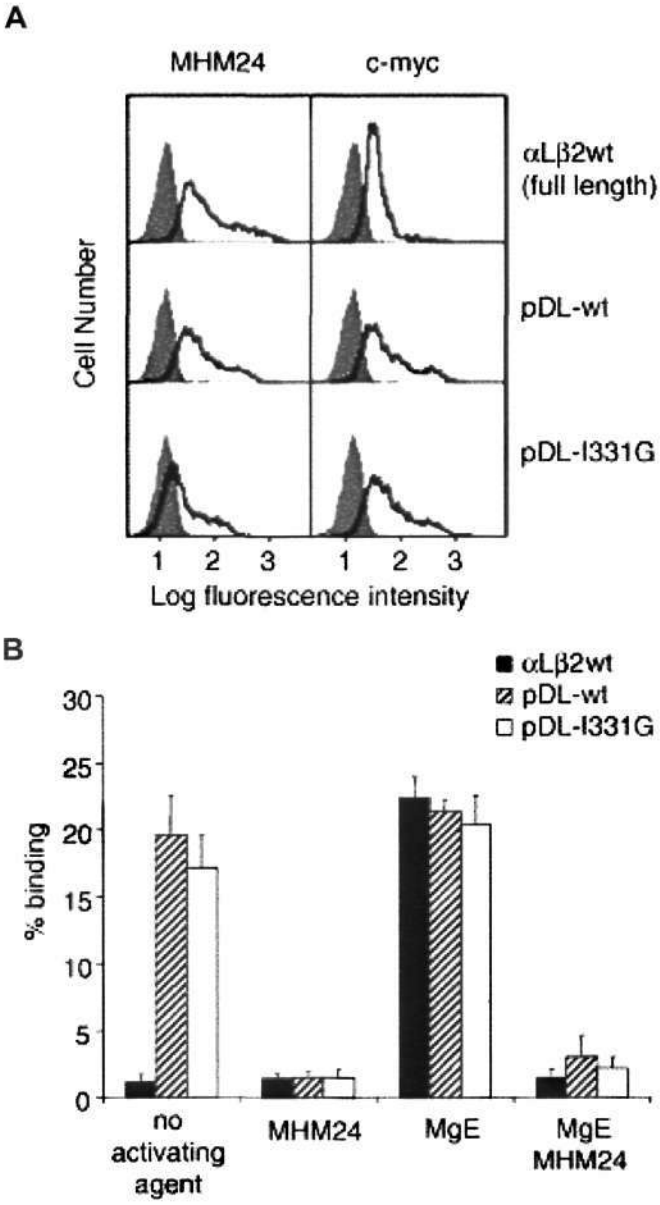

FIG 4.4. Isolated wild-type αL I-domain and mutant αL I331G show constitutive ligand-binding activity in 293T cells. (A) Cell surface expression was comparable as determined by FACS using the anti-c-myc antibody for the pDisplay constructs and MHM24 for the αL I-domain. (B) Isolated αL I-domain binding 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^{2+}/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^{2+}/EGTA. Addition of Mg^{2+}/EGTA to the isolated I-domain constructs did not result in any further activation of the receptors, confirming their constitutive ligand-binding activity.

4.2.4 *Binding activity at different coating levels 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 is added in each well). In the absence of Mg^{2+}/EGTA activation, wild-type αLβ2 showed minimal binding under all concentrations of ICAM-1 coated (Figure 4.5). The transfectants expressing either pDL-wt or pDisplay-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^{2+}/EGTA resulted in an adhesion profile similar to those of transfectants expressing the isolated I-domains.

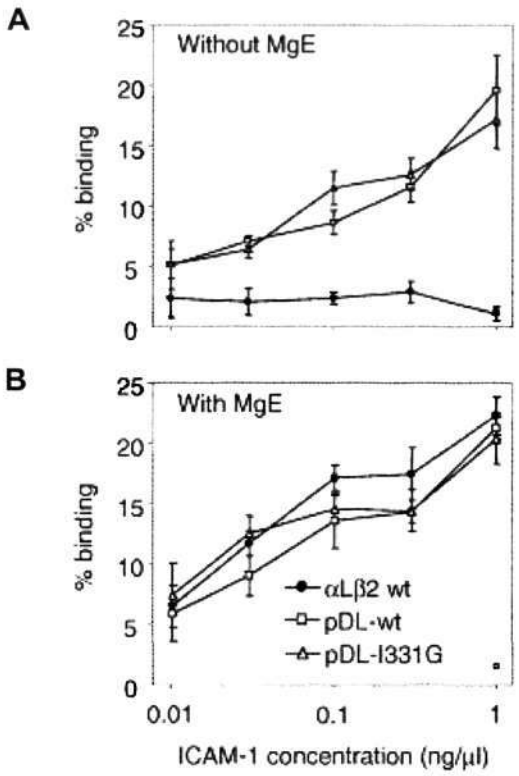

FIG 4.5. αL I-domain binding to ligand ICAM-1 at different concentrations. (A) In the absence of activation by Mg^{2+}/EGTA (MgE), the full-length wild-type αLβ2 showed minimal binding under all ICAM-1 concentrations. The binding for wild-type isolated I-domain and with the I331G mutation constructs 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 was blocked with MHM24 (○).

4.2.5 *Isolated wild-type αM I-domain and mutant I332G show similar binding activity to BSA as compared to full length αMβ2*

We furthered our study on binding property of isolated αM I-domain with or without isoleucine to glycine mutation. The cloning of isolated αM I-domain was similar to the generation of isolated αL I-domain but αM cDNA was used instead. In addition, αM I-domain having the I332G substitution was also constructed. These constructs will be referred to as pDL-wt(αM) and pDL-I332G(αM). 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 LPM19c recognizes the αM I-domain (Figure 4.6A). pDL-wt(αM) was found to have a similar binding profile to BSA as compared to full length αMβ2. A similar binding profile was also observed for pDL-I332G(αM) (Figure 4.6B).

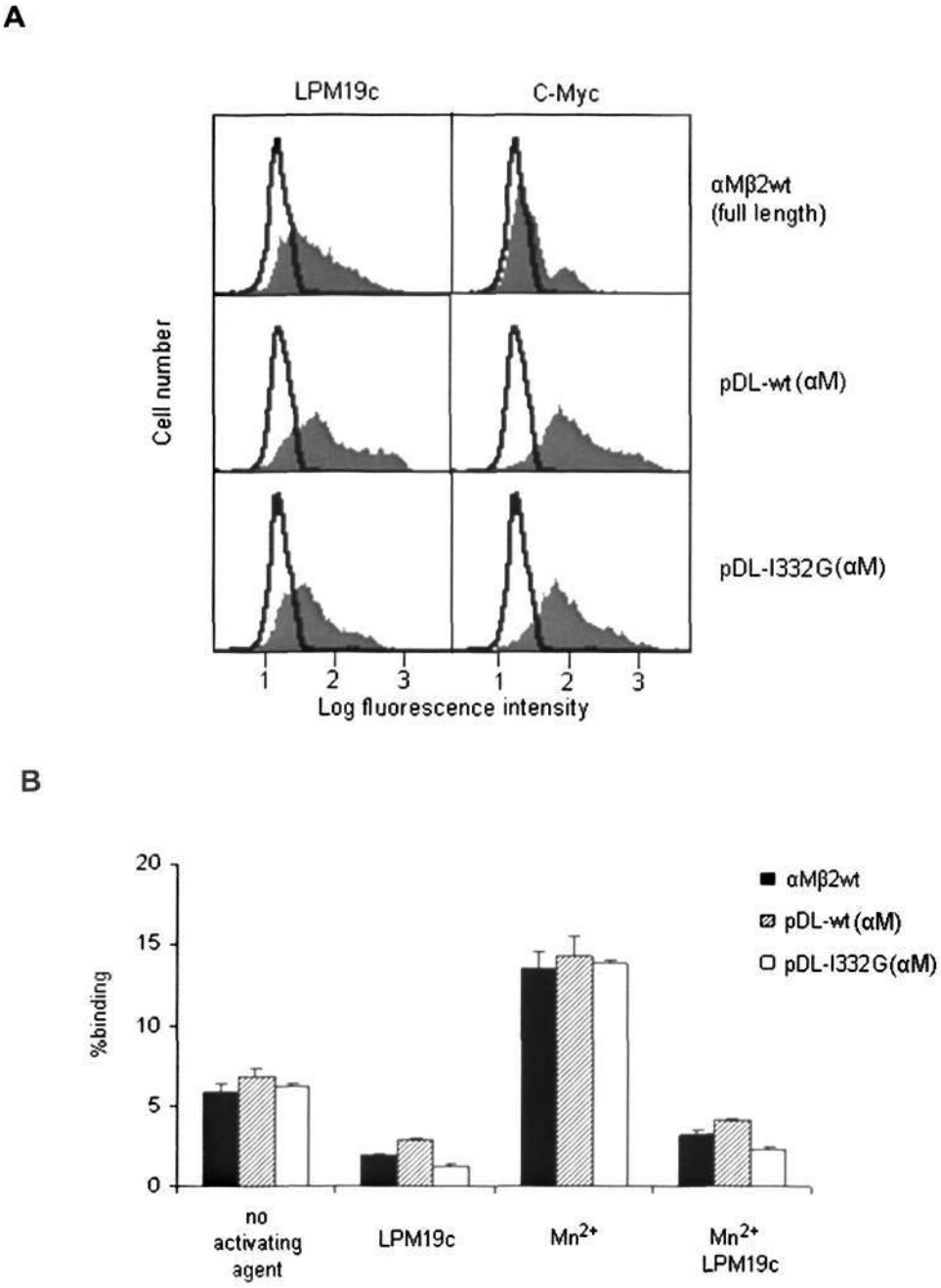

FIG 4.6. Isolated wild-type αM I-domain and mutant αM I332G show similar binding activity to BSA as compared to full length αMβ2 in 293T cells. (A) Cell surface expression was comparable as determined by FACS using the anti-c-myc antibody for the pDisplay constructs and LPM19c for the αM I-domain. (B) Isolated αM I-domain binding to BSA. pDisplay αM I-domain wild-type (pDL-wt(αM)) was found to have a similar binding profile to BSA as compared to full length αMβ2. A similar binding profile was also observed for pDL-I332G(αM).

4.3 Discussion and conclusion

There is much evidence to suggest that activation of integrins occurs through conformational change (Shimaoka et al., 2002b). Early crystal structures of the I-domain of integrin αM revealed two distinct conformations—a ligand-occupied 'open' form and an unoccupied 'closed' form (Lee et al., 1995a; Lee et al., 1995b). 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 (Emsley et al., 1997; Shimaoka et al., 2003), 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 (Figure 4.7) (Xiong et al., 2000). 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 (Xiong et al., 2000). 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.

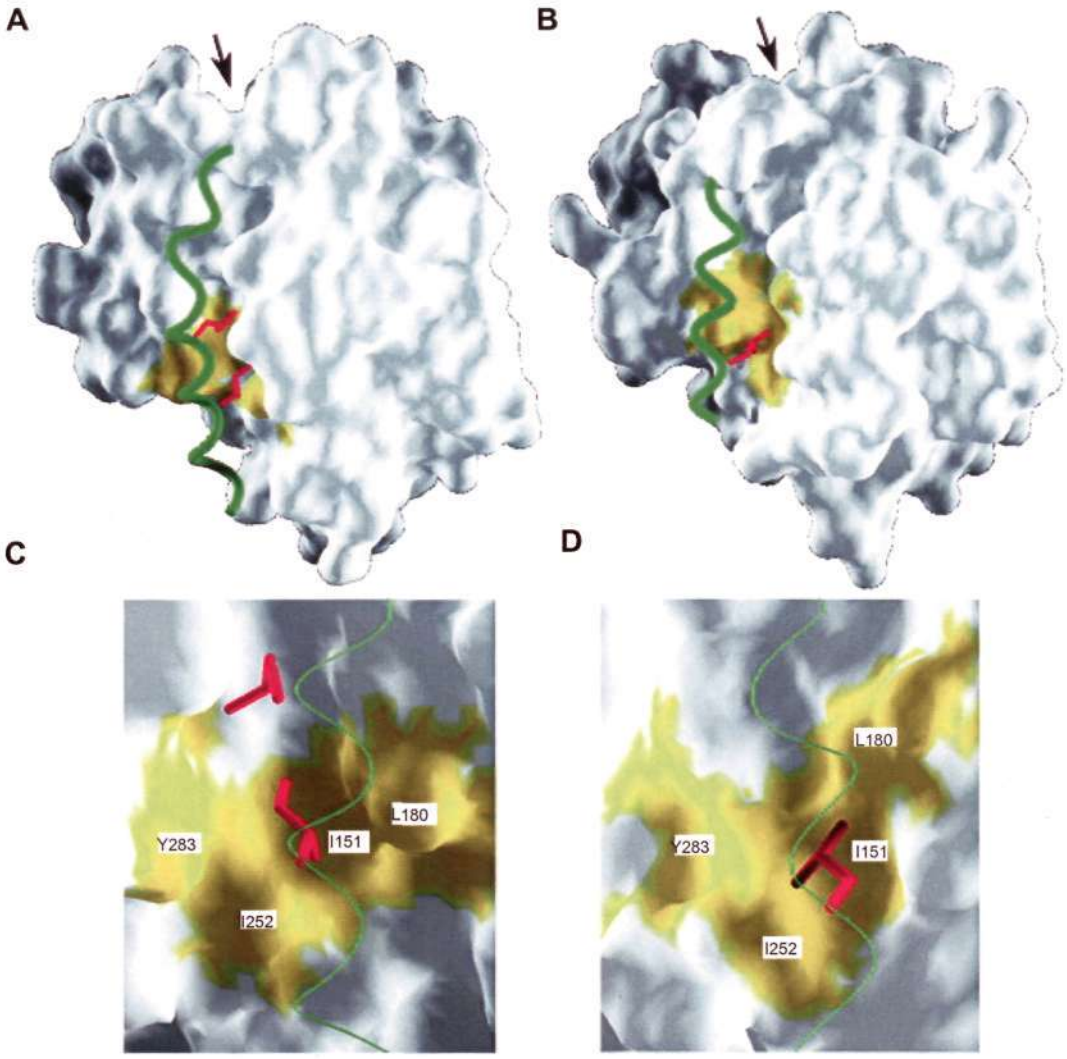

FIG 4.7. Ile^{332} coordination pocket. (A) and (B), surface representation of the αM I-domain crystal structure in its closed (A) and open (B) states, with the C-terminal α7 helix outlined *green*, and the socket for isoleucine (SILEN) residues (Ile^{151}, Leu^{180}, Ile^{252}, Tyr^{283}, lying within 4-Å radius from Ile332) shown in yellow. An arrow points to the MIDAS face at the top. As can be seen, MIDAS and SILEN lie on almost opposite ends of the I-domain structure. (C) and (D), magnified face view of SILEN in the closed (C) and open (D) conformations. (Adapted from Xiong et al., 2000).

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 αX I-domain-I333G variant was shown to have 200-fold higher affinity for iC3b as compared to wild-type αX I-domain (Vorup-Jensen et al., 2003). In a separate study, alanine scanning mutagenesis of the αL I-domain yielded many αLβ2 variants that showed higher ligand-binding capacity than the wild-type (Huth et al., 2000). 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. (Huth et al., 2000), it appears that although the αLβ2 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 reasoned that Ile^{331} is not the critical residue contributing to allosteric regulation of αLβ2 affinity.

Of interest, these 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 (Shimaoka et al., 2001). 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 (Lu et al., 2001d). In our hands, αL wild-type I-domain in pDisplay vector showed reproducible constitutive ICAM-1 adhesion using the same system on K562 cells (data from my colleague Cheng Ming). The similar results were obtained using 293T cells. For αM, constitutive binding to BSA can only be observed in full length αM(I332G)β2, but not in isolated αM I domain (wt and I332G). 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 (Tan et al., 2000).

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, suggest 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 αLβ2. More structural data of an I-domain-containing integrin are required to shed light on the mechanism of integrin activation involving the I-domain.

Chapter Five: Selective recruitment of Src family kinase Hck by leukocyte integrin αMβ2 but not αLβ2 or αXβ2

5.1 Introduction

Integrins mediate many biological processes (Hynes, 2002). Although they lack intrinsic enzymatic activities, integrins transduce intracellular signal events by recruiting signaling proteins to their cytoplasmic tails (Liu et al., 2000). In some integrins, several α subunits can pair with a common β subunit as exemplified in the leukocyte β2 integrins (αLβ2 CD11aCD18; αMβ2 CD11bCD18; αXβ2 CD11cCD18; αDβ2 CD11dCD18). Increasing evidence indicate that the α cytoplasmic tail determines integrin functional specialization. Integrin α2β1 (VLA-2) showed differential cell-migration on collagen and laminin when α2 cytoplasmic tail was replaced by that from integrin α5 or α4 (Chan et al., 1992). Integrin α4β1 (VLA-4) exhibited differential adhesion properties to ligand VCAM-1 under shear flow analyses when α4 tail was exchanged with that from integrin α2 or α5 (Kassner et al., 1993). The difference in α cytoplasmic tails of α4β1 and α5β1 may also account for the differential requirement of FAK (focal adhesion kinase) mediated signaling pathway (Hsia et al., 2005). There can be two possibilities accounting for signaling specificity amongst integrins having different α but the same β subunit. First, specific cytosolic proteins may interact with specific α cytoplasmic tails. For example, nischarin with α5 (Alahari et al., 2000), paxillin with α4 (Liu et al., 1999), calcium integrin binding protein (CIB) with αIIb, CD45 with αL (Geng et al., 2005), and T-cell protein tyrosine phosphatase (TCPTP) with α1 (Mattila et al., 2005) have been reported. Second, it is also possible that the different α cytoplasmic tails can exert different effects

on the binding of cytosolic proteins, compared to the β cytoplasmic tail. To date, this hypothesis has not been rigorously tested.

The Src family kinases (SFKs) are important for integrin downstream signaling (Shattil 2005). The SFKs member Hck (haematopoietic cell kinase) isoforms of 59 and 61 kDa have been described previously (Lichtenberg et al., 1992; Mohn et al., 1995). Hck along with c-Fgr and Lyn are expressed abundantly in neutrophils, monocytes, and macrophages (Thomas and Brugge, 1997). Murine neutrophils deficient in Hck and c-Fgr showed defective β2 integrin-mediated adhesion and respiratory burst in response to tumor necrosis factor (Lowell et al., 1996). Hck was reported to co-localize with αMβ2 around phagosome in αMβ2 transfectants (Astarie-Dequeker et al., 2002). In this part of study, we adopted a similar approach using αMβ2 transfectants to examine the effect of αMβ2 tail modifications on Hck recruitment. We showed that: (i) the co-capping of Hck to αMβ2 was abrogated when the αM tail was substituted with that of αL or αX; (ii) its restoration when this αL or αX tail was truncated or by disrupting the membrane proximal salt-bridge between the α and β2 tails and (iii) we identified a region within β2 tail that is required for αMβ2 and Hck co-capping. Together, these data suggest a primary role of the integrin α tail in regulating β2 tail interaction with cytosolic factors.

5.2 Results

5.2.1 Hck co-capped with αMβ2 but not αLβ2 or αXβ2

We examined Hck co-capping with the β2 integrins in human monocytic THP-1 cells. In our hands, suspension THP-1 cells express lower basal level of αXβ2 as compared to αLβ2 and αMβ2 (Figure 5.1A). THP-1 cells display macrophage-like differentiation in response to phorbol esters treatment (Auwerx, 1991). Therefore, THP-1 cells were treated with PMA to promote their differentiation into macrophage-like cells that were highly adherent and showed comparable expressions of the β2 integrins. Specific β2 integrins on PMA-treated THP-1 cells were cross-linked with relevant mAbs together with FITC-conjugated secondary antibody. Confocal microscopy revealed propensity and significant co-capping of Hck with αMβ2 but not αLβ2 or αXβ2 (Figure 5.1B). To verify Hck and αMβ2 co-capping specificity, CHO-K1 transfectants expressing αMβ2 and Hck or another SFK Lck, which is not known to interact with αMβ2, were examined. Importantly, Hck but not Lck co-capped with cross-linked αMβ2 (Figure 5.2A). However, Lck co-capped efficiently with its known interactor CD4 (Kim et al., 2003). Because the αMβ2-specific function-blocking mAb LPM19c was used in the cross-linking of αMβ2, we extended the analyses using the heterodimer-specific function-blocking mAb IB4 (Wright et al., 1983), the β2-specific activating mAb KIM185 (Andrew et al., 1993), and the β2-specific mAb KIM202 that is not known to activate or abrogate αMβ2 function (unpublished data from our lab) (Figure 5.2B). In all cases, Hck co-capping with αMβ2 was detected. It is therefore apparent that the association of Hck with αMβ2 is

constitutive. Of note, the SFK c-Src was shown to bind to platelet integrin αIIbβ3 constitutively (Arias-Salgado et al., 2003).

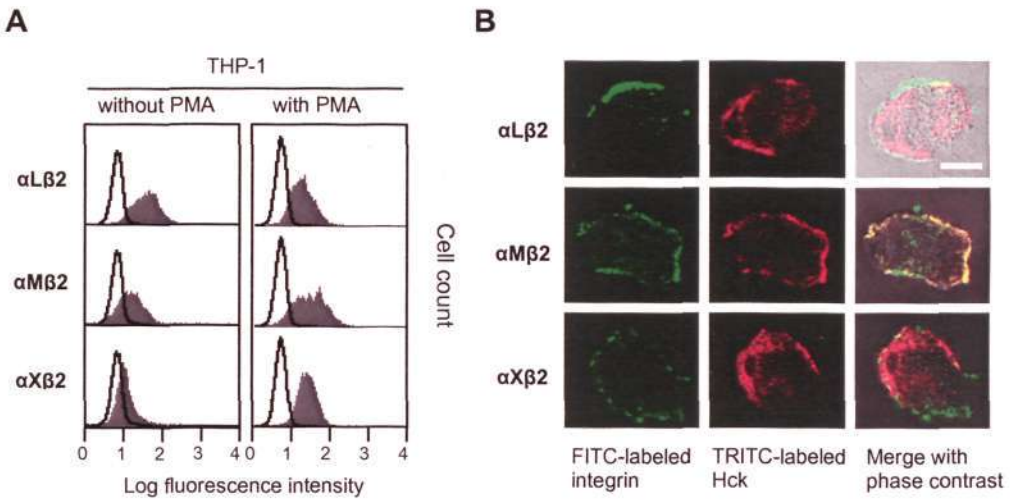

FIG 5.1. Specific co-localization of Hck with integrin αMβ2. (A) FACS analysis of PMA-treated THP-1 cells using integrin αLβ2 (MHM24), αMβ2 (LPM19c) and αXβ2 (KB43) specific mAb (shaded histograms). Background staining with irrelevant mAb (open histograms). (B) Co-localization of Hck with αMβ2 but not αLβ2 or αXβ2 in PMA-treated THP-1 cells. Representative images of three separate experiments were shown. This Integrins (green) and Hck (red). Staining was performed as described under Material and Methods. Bar, 10 μm.

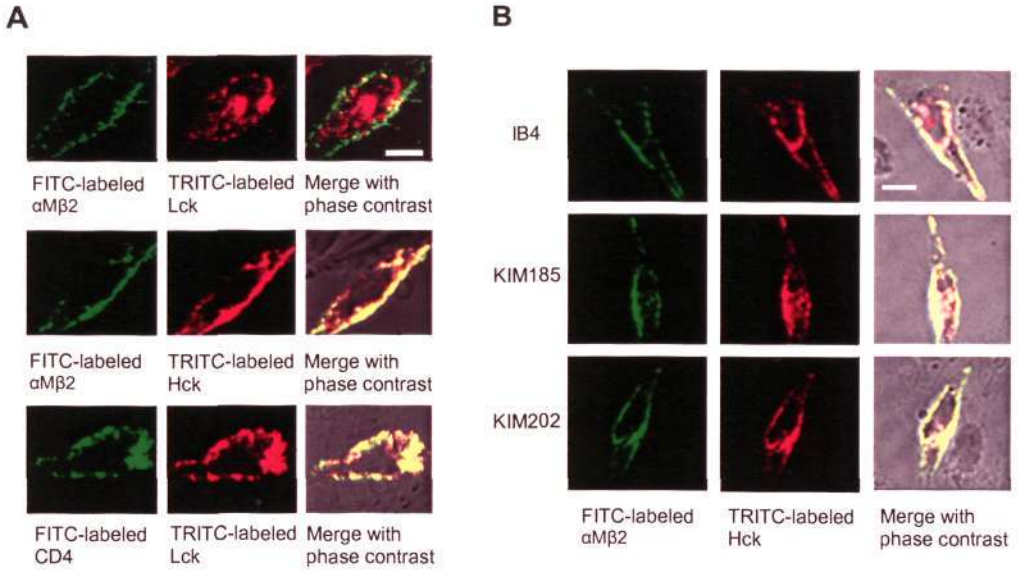

FIG 5.2. (A) Hck but not Lck co-capped with mAb LPM19c-crosslinked αMβ2 in CHO-K1 transfectants. Lck co-capped with CD4. Representative images of three separate experiments were shown (B) Co-capping of Hck with αMβ2 in CHO-K1 transfectants. αMβ2 was cross-linked with function-blocking heterodimer-specific mAb IB4, β2-specific activating mAb KIM185, or β2-specific mAb KIM202 that is non-function-blocking nor activating. Representative images are shown. Bar, 10 μm.

5.2.2 *Substitution of αM tail with αL or αX tail abrogated Hck recruitment to αMβ2-mediated phagosome*

The length and composition of the αL, αM and αX tails differ (Figure 5.3A). To test whether these variations could account for Hck and αMβ2 co-capping specificity, we generated tail variants of αM. The αM tail (Lys^{1114}-Gln^{1137}) was replaced with αL (Lys^{1087}-Asp^{1145}) or αX (Lys^{1110}-Lys^{1144}) tail to generate αML and αMX variants respectively (Figure 5.3B). CHO-K1 transfectants of αMLβ2 and αMXβ2 showed comparable expression levels to αMβ2 as determined by FACS (Figure 5.4A). The binding properties of these variants to αMβ2 ligand iC3b, a serum complement opsonin, were also comparable to αMβ2 transfectants (Figure 5.4B). Further, αMβ2, αMLβ2 and αMXβ2 transfectants can phagocytose OZ (Figure 5.5). Hck accumulation around OZ-containing phagosomes was reported in αMβ2 CHO-K1 transfectants (Astarie-Dequeker et al., 2002). To extend our study in the context of a biological process, co-localization of Hck with αMLβ2 and αMXβ2 during phagocytosis was examined. CHO-K1 transfected with Hck and αMβ2, αMLβ2 and αMXβ2 were allowed to phagocytose rhodamine red-labeled OZ following by immunofluorescence staining of Hck and confocal microscopy analyses (Fig. 5.6 C and D). Prominent Hck accumulation at the periphery of the phagocytosed-OZ was detected in αMβ2 transfectants but poorly in αMLβ2 or αMXβ2 transfectants.

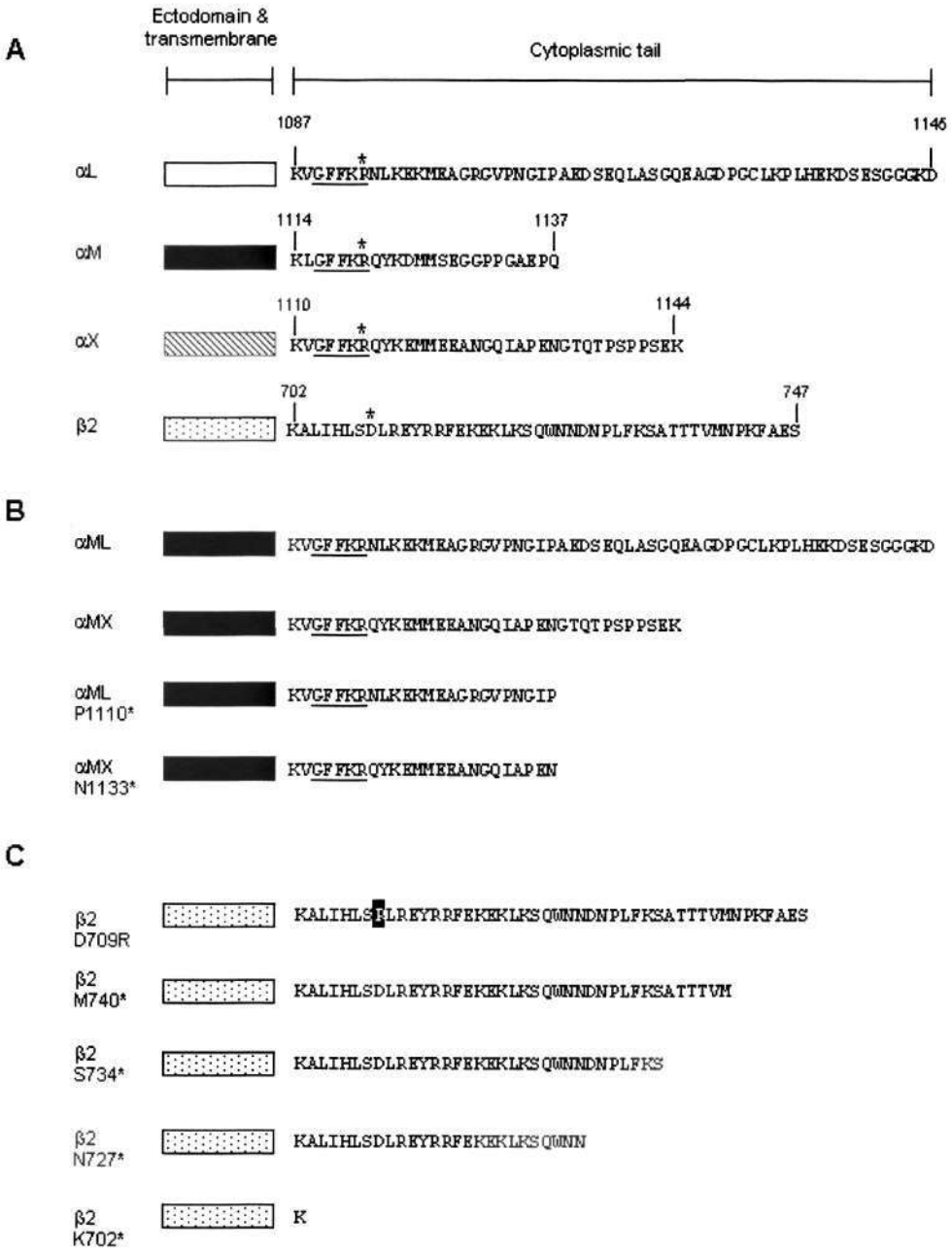

FIG 5.3. Schematic representation of integrin and mutants cytoplasmic tails. (A)The αL, αM, αX and β2 ectodomain and transmembrane (TM) regions are represented by different filled boxes. The Arg and Asp residues of the α and β tails respectively that are involved in salt-bridge formation are marked with asterisks. The conserved membrane proximal GFFKR motif is underlined. (B) The αMLβ2 or αMXβ2chimeras are illustrated by a black box followed by the αL or αX tail sequence respectively. The αMLP1110* and αMXN1133* are similarly illustrated but with corresponding deletions of the α cytoplasmic tails. (C) The β2 cytoplasmic tail that has its Asp (salt-bridge forming residue) substituted by Arg (black box) is shown. The other β2 cytoplasmic tail truncation mutants are illustrated.

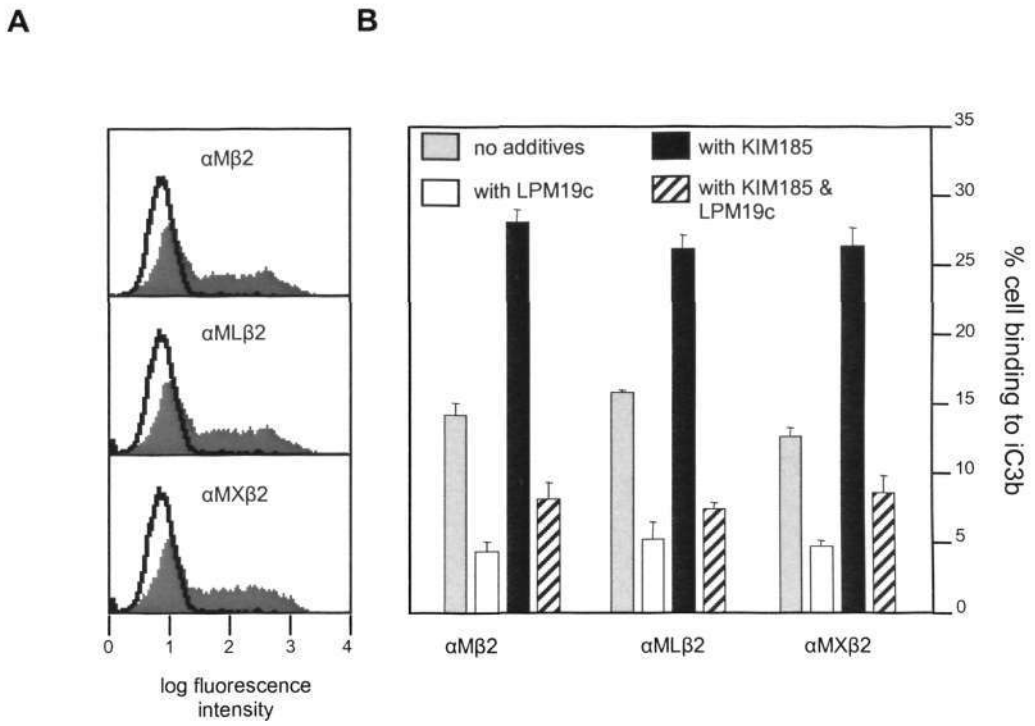

FIG 5.4. Effect of integrin α cytoplasmic tails on αMβ2 binding to iC3b. (A) Cell surface expression of αMβ2, αMLβ2, and αMXβ2 on CHO-K1 transfectants by FACS analyses using heterodimer specific mAb IB4 (shaded histograms). Background staining with irrelevant mAb (open histograms). (B) Adhesion of transfectants to iC3b. The integrin β2 specific activating mAb KIM185 was included. Specificity of adhesion was demonstrated by its abrogation in the presence of integrin αMβ2 function-blocking mAb LPM19c.

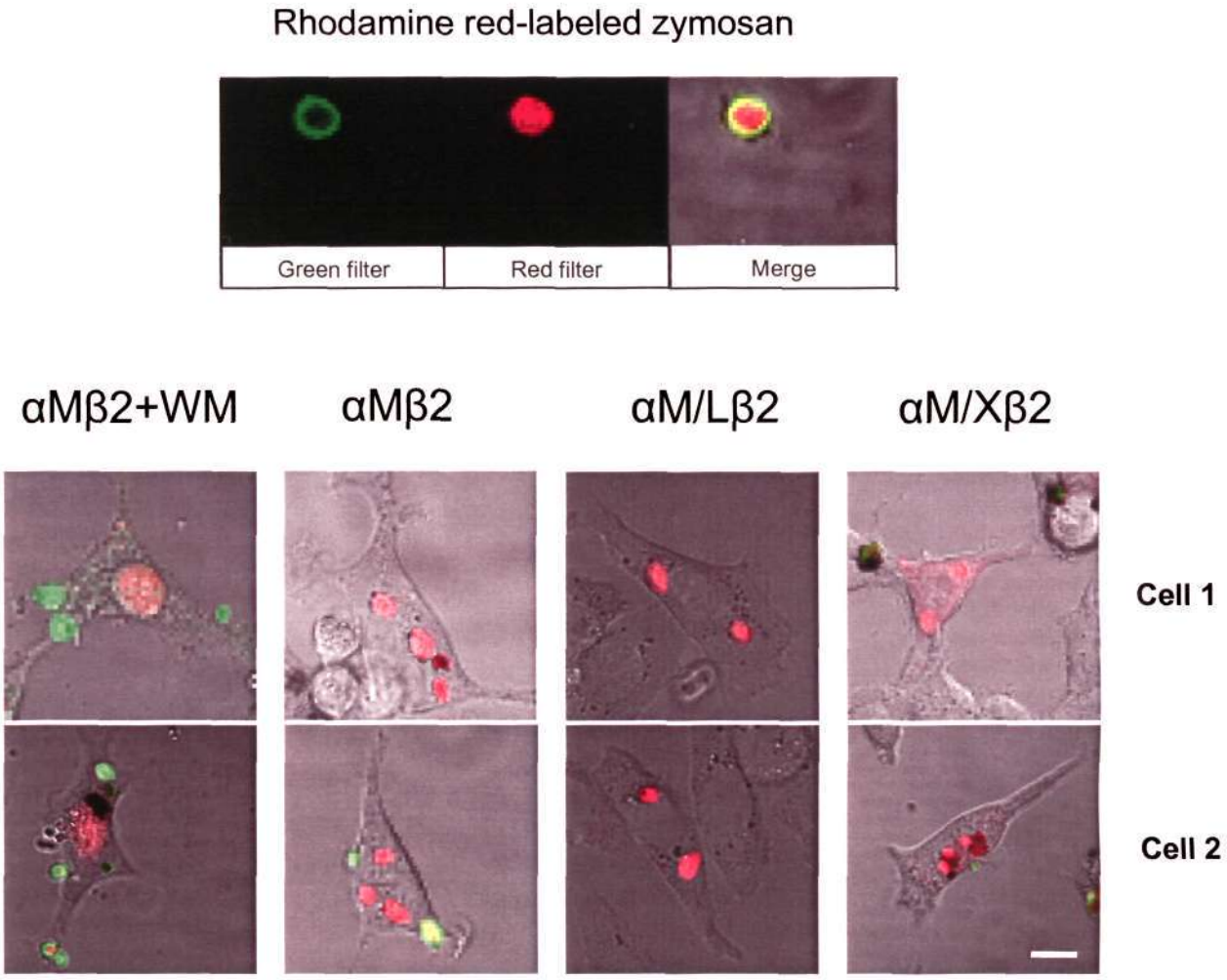

FIG 5.5. Opsonized zymosan (OZ) phagocytosed by αMβ2, αMLβ2, and αMXβ2 transfectants on 293T cells. Rhodamine red-labeled OZ (red) was first stained with rabbit anti-zymosan antibody and opsonized. 293T Transfectants were allowed to phagocytose OZ. Bound but not phagocytosed zymosans were stained with anti-rabbit IgG FITC antibody and showed green color. Inside OZ remained red. 100nM wortmannin (a potent and selective inhibitor of phosphatidylinositol 3-kinase) was added to αMβ2 during phagocytosis as a negative control. Bar, 10 μm.

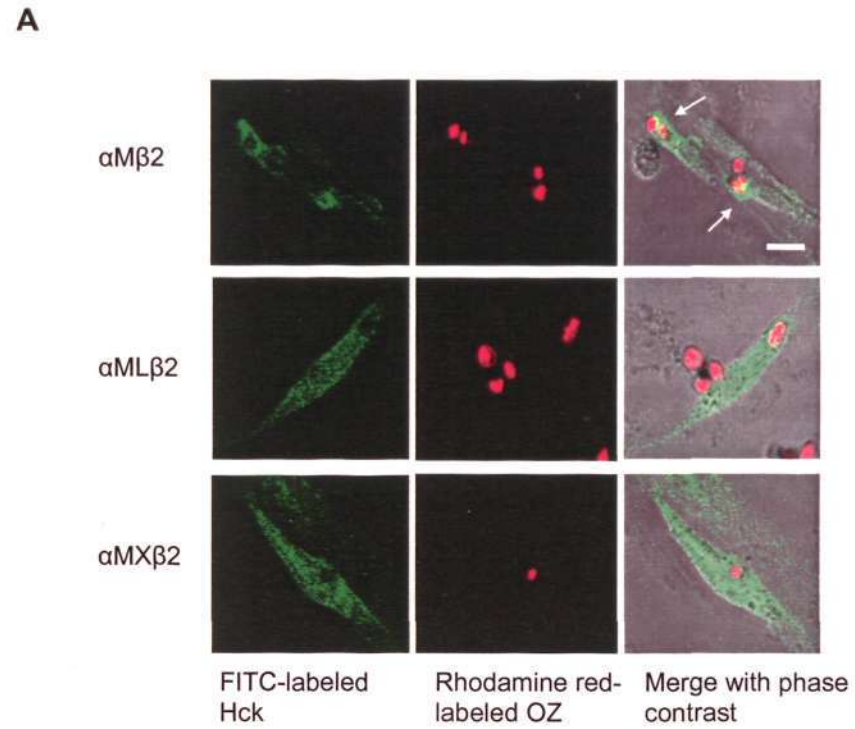

FIG 5.6 Effect of integrin α cytoplasmic tails on Hck clustering in OZ during phagocytosis mediated by αMβ2. (A) CHO-K1 transfectants were allowed to phagocytose rhodamine red-labeled OZ (red) followed by Hck detection (green). Clustering of Hck in the region of the phagocytosed OZ is indicated by white arrows. Staining was performed as described under experimental procedures. Representative images are shown. Bar, 10 μm. Representative images of three separate experiments were shown (B) % transfectants with Hck clustering around phagocytosed-OZ by counting an entire field. αMβ2 value was taken as 100%.

5.2.3 *Tail truncation or salt-bridge disruption restored αMLβ2 or αMXβ2 recruitment of Hck.*

Nuclear magnetic resonance studies reveal hydrophobic and electrostatic contacts between integrin αIIb and β3 tails (Vinogradova et al., 2000; Vinogradova et al., 2002). The proximity of αL and β2 tails is also evidenced by fluorescence resonance energy transfer (FRET) study (Kim et al., 2003). Hence, C-terminal truncated variants αMLP1110* and αMXN1133* were generated such that the length of their αL and αX cytoplasmic elements are comparable to that of the αM tail (Figure 5.3B). Significant Hck accumulation at the periphery of the phagocytosed-OZ could be detected in αMLP1110* β2 and αMXN1133* β2 transfectants (Figure 5.7).

A membrane proximal salt-bridge of the integrin tails is formed by a highly conserved Arg and an Asp in the α and β tails respectively (Hughes et al., 1996). Salt-bridge disruption activates integrin by promoting the separation of its tails (Kim et al., 2003). Therefore, β2D709R variant having its Asp^{709} substituted with Arg was generated (Fig. 5.3C). Marked accumulation of Hck around phagocytosed-OZ was detected in αMLβ2D709R and αMXβ2D709R transfectants (Figure 5.7).

The binding properties of αMβ2, αMLβ2, or αMXβ2 transfectants may be similar at high coating-concentration (7.5 μg/mL) of iC3b as shown in *Figure 5.4B*. However, at lower concentrations of iC3b, difference in binding capacities amongst these transfectants may account for the difference in subsequent Hck recruitment during phagocytosis of OZ as

observed for αMβ2, αMLβ2, and αMXβ2. To resolve this concern, we examined the binding of αMβ2, αMLβ2, and αMXβ2, αMβ2D709R, αMLβ2D709R, αMXβ2D709R, αMLP1110* β2, and αMXN1133* β2 transfectants on varying concentrations of iC3b (Figure 5.8). All variants had similar cell surface expression profiles as compared to that of αMβ2 (Figure 5.8A). Further, comparable iC3b-binding profiles were observed for αMβ2, αMLβ2, αMXβ2, αMLP1110* β2 and αMXN1133* β2 transfectants even at low concentration (0.3 μg/mL) of iC3b without or with activating mAb KIM185. The αMβ2D709R, αMLβ2D709R, αMXβ2D709R transfectants showed higher level of iC3b-binding as compared to αMβ2 in the absence of KIM185, which corroborates well with enhanced integrin activity as a result of salt-bridge disruption (Kim et al., 2003). Taken together, these data suggest that the lack of Hck accumulation at the periphery of the phagocytosed-OZ in αMLβ2, and αMXβ2 transfectants as compared to transfectants expressing αMβ2 and other variants could not be due to lower ligand-binding affinity but was attributed to intrinsic differences in the cytoplasmic tails as demonstrated in this study.

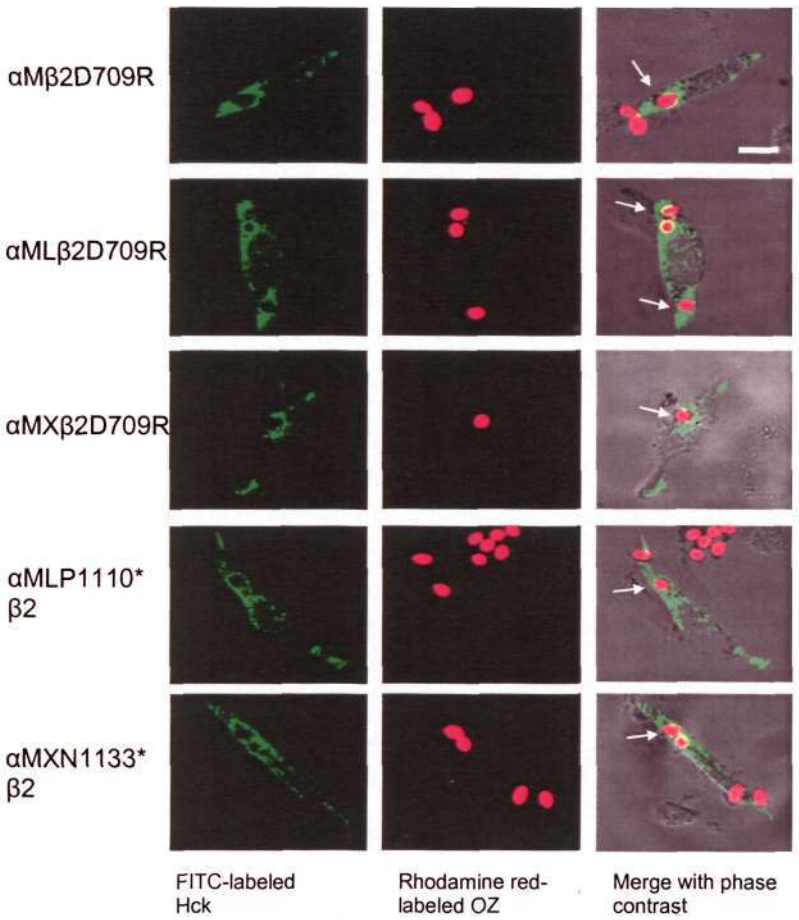

FIG 5.7. Recovery of Hck clustering to various transfectants. αMβ2D709R, αMLβ2D709R, αMXβ2D709R, αMLP1110* β2, and αMXN1133* β2 transfectants phagocytosed OZ with concomitant clustering of Hck. Representative images of two separate experiments are shown. Clustering of Hck in the region of the phagocytosed OZ is indicated by white arrows. Bar, 10 μm.

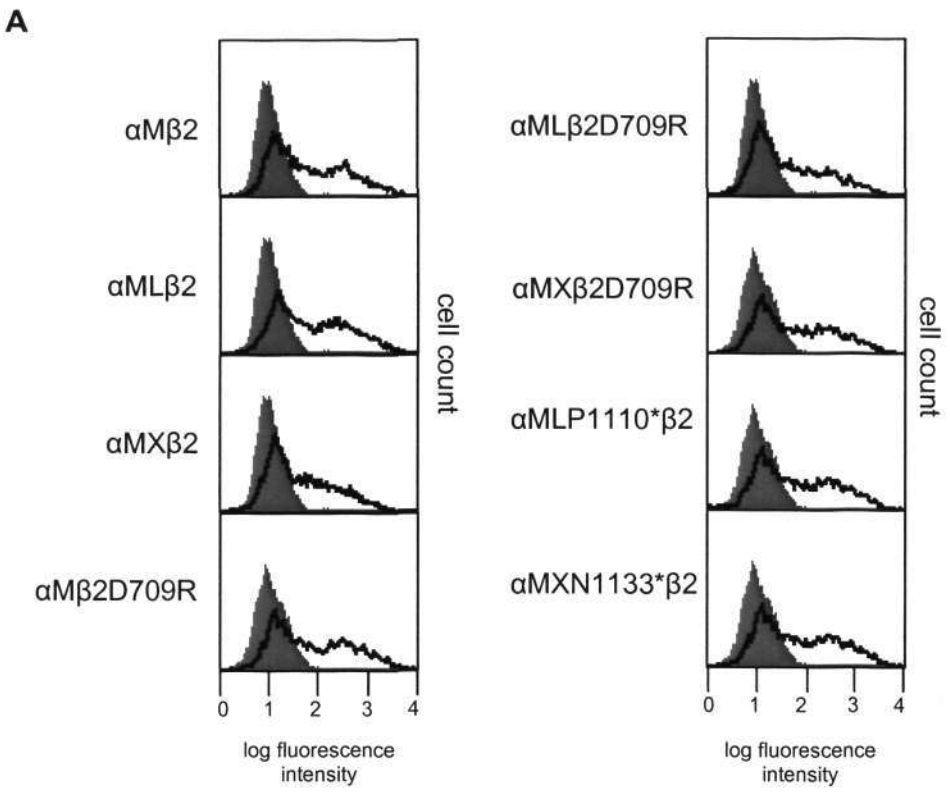

FIG 5.8. Expression and ligand-binding profiles of αMβ2 variants. (A) FACS analyses of CHO-K1 transfected with αMβ2 or variants using heterodimer-specific mAb IB4 (open histogram) and irrelevant mAb (shaded histogram).

B

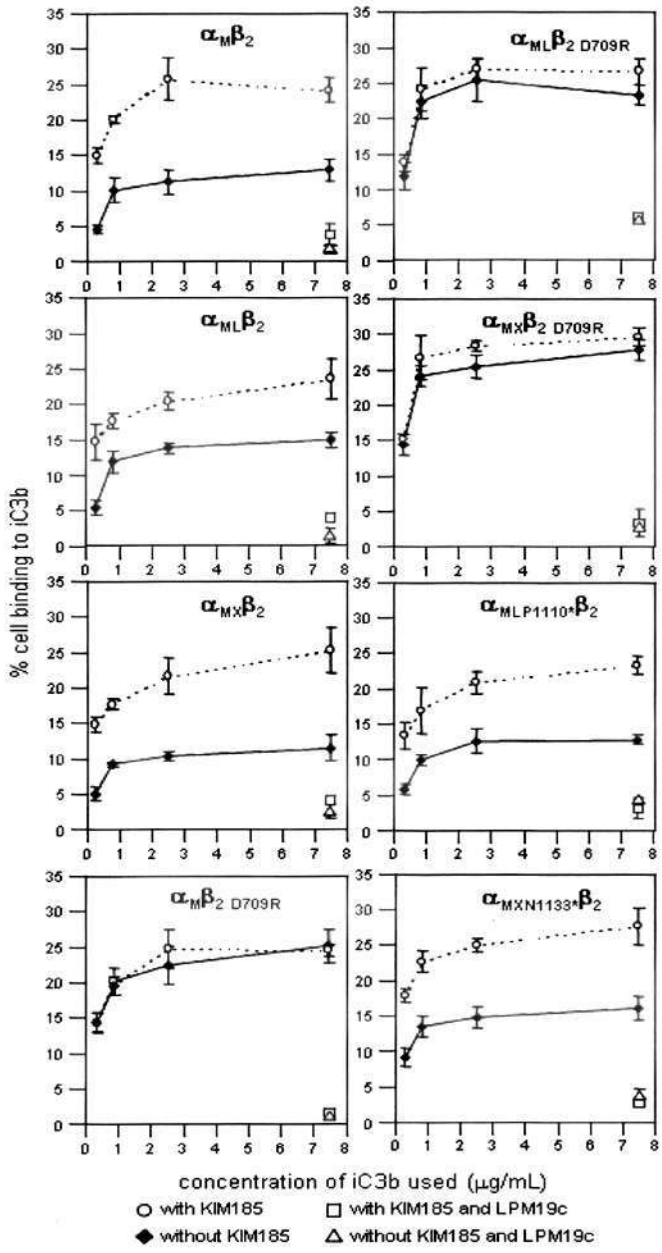

FIG 5.8. Expression and ligand-binding profiles of αMβ2 variants (continued). (B) CHO-K1 transfectants were allowed to adhere wells coated with different concentrations (0.3, 0.8, 2.5, and 7.5 μg/mL) of iC3b in the presence or absence of β2-activating mAb KIM185. Binding to iC3b was specific in all cases because binding of transfectants to the wells coated with the highest concentration of iC3b (7.5 μg/mL) was effectively abrogated in the presence of αMβ2 function-blocking mAb LPM19c.

5.2.4 *β2 tail sequence Asp^{728}-Ser^{734} is required for Hck recruitment*

Hck was reported to interact with β2 tail in pull-down analyses using platelet lysate and purified recombinant β2 tail protein (Arias-Salgado et al., 2003). However, we were unable to co-immunoprecipitate Hck with αMβ2 from lysates of THP-1 or transfectants although αMβ2 was effectively precipitated. What accounts for this discrepancy is unclear. It is possible that co-capping of αMβ2 and Hck may involve an adaptor protein that is susceptible to disruption under conditions of immunoprecipitation.

Notwithstanding, we generated truncated β2 tail variants (β2M740*, β2S734*, β2N727*, and β2K702*) with sequential deletion from their C-termini (Figure 5.3C) and examined their effects on the co-capping of αMβ2 and Hck in transfectants (Figure 5.9). αMβ2, αMβ2M740*, and αMβ2S734* co-capped with Hck. This was markedly diminished in αMβ2N727*, and αMβ2K702*. These data suggest that the β2 tail sequence Asp^{728}-Ser^{734} is required for Hck recruitment to αMβ2 in CHO-K1 transfectants.

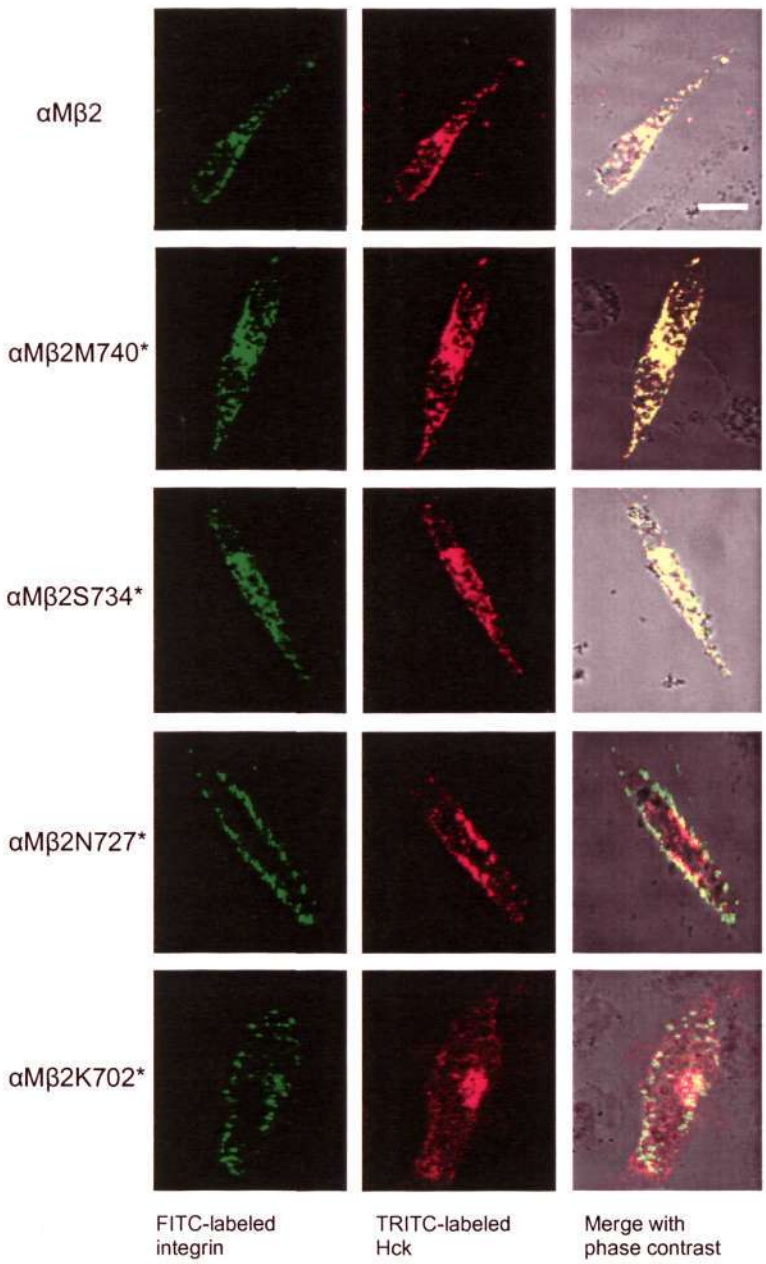

FIG 5.9. Defining the region in integrin β2 tail that is required for Hck recruitment. CHO-K1 cells were transfected with plasmids of α integrin, β integrin and Hck. Hck co-capped efficiently with αMβ2, αMβ2M740*, αMβ2S734* but not with αMβ2N727* and αMβ2K702* in CHO-K1 transfectants. Representative images of two separate experiments are shown. Bar, 10 μm.

5.3 Discussion and conclusion

Hck is involved in the activation of neutrophils and monocytes mediated by Fc receptor (Zhou et al., 1995; Durden et al., 1995). Hck is also implicated in integrin-mediated cellular functions. Macrophages from double knockout mice of Hck and another SFK c-Fgr, which shares overlapping functions (Lowell et al., 1994), exhibit impaired integrin-mediated signaling (Suen et al., 1999). Neutrophils from $hck^{-/-}fgr^{-/-}$ mice had impaired β2 integrin-induced respiratory burst (Lowell et al., 1996). Further, it was reported that Hck accumulates around phagosome that contains OZ in neutrophils (Mohn et al., 1995). Interestingly, Hck was shown to be activated in neutrophils and differentiated human promyelocytic NB4 cells that were allowed to phagocytosed OZ (Welch et al., 1997). There are also evidences suggesting a role of activated Hck in regulating αMβ2 mediated adhesion in neutrophils (Piccardoni et al., 2001; Totani et al., 2006). These data lend support to the importance of Hck in integrin-mediated functions of polymorphonuclear leukocytes.

Integrin triggers intracellular signaling cascades by the interaction of cytosolic proteins with its cytoplasmic tails. This study demonstrates a primary role of the α tails in determining Hck recruitment to the leukocyte integrin αMβ2 but not αLβ2 or αXβ2. What is the underlying mechanism? Specific binding of paxillin to the α4 tail affects focal adhesion kinase (FAK) phosphorylation and recruitment by integrin α4β1 (Schaller et al., 1995; Liu et al., 1999; Han et al., 2001). It is therefore possible that intrinsic variations in the length, composition, or modification such as phosphorylation status of the αL or αX tail as compared to αM may specify its association with different cytosolic proteins.

These factors, yet to be identified, prevent Hck recruitment by the β2 tail. Indeed, we observed that a reduction in length of the αL or αX tail in αMLβ2 or αMXβ2 made comparable to that of αM restored co-localization with Hck. However, we do not exclude the possibility of the α tail directly affecting the function of the β2 tail. The highly-charged cytoplasmic C-terminal half of αIIb is reported to regulate αIIbβ3 activity perhaps via direct inter-molecular interaction between its two tails (Vinogradova et al., 2000). Data on the restoration of Hck co-localization with salt-bridge disrupted variants αMLβ2D709R and αMXβ2D709R suggest that the close proximity and possibly interaction of the αL or αX with the β2 tail may prevent Hck recruitment to the β2 tail. Structural determination of these cytoplasmic tails, which is currently lacking for the β2 integrins, will be required to define these molecular mechanisms. Although we could not show direct interaction between Hck and the β2 tail as reported (Arias-Salgado et al., 2003), our data suggest that the region Asp^{728}-Ser^{734} of the β2 tail is required for effective Hck and αMβ2 co-capping. Interestingly, this region contains an NPLF motif that is highly conserved amongst several integrin β tails. Talin, a widely expressed cytoplasmic protein that serves to maintain the connectivity between integrins and actin cytoskeleton, binds to a corresponding motif in the β3 tail (Garcia-Alvarez et al., 2003). Talin head domain also initiates integrin activation by promoting cytoplasmic tail separation and salt-bridge disruption (Kim et al., 2003). Therefore, the interesting possibility of functional co-operativity between αMβ2, Hck, and talin warrants future investigations.

Chapter Six: Summary

The focus of this study is the β2 integrins. The β2 integrins are critical in the immune response for the extravasation of leukocytes from the circulation to sites of infection, activation of neutrophils and monocytes, the phagocytosis of pathogens and neutrophil apoptosis (Larson and Springer, 1990). Aforementioned, our interests lie in the understanding of the β2 integrin activation states for ligand binding and the role of its cytoplasmic domain in integrin functions.

αLβ2 integrin has been the therapeutic target in certain clinical trials (Leonardi et al., 2005). In psoriasis, an anti-αLβ2 antibody (Efalizumab), which can prevent αLβ2 binding to ICAM-1, interferes with the pathogenesis of psoriasis via inhibition of T-lymphocyte trafficking and T-lymphocyte activation and reactivation (Springer et al., 1987; Dustin et al., 1988; Werther et al., 1996; Krueger et al., 2000). It was noticed that this antibody may have side effects, such as headache (Gottlieb et al., 2000). Since this antibody is targeted to all αLβ2, not only those active αLβ2 but also resting ones, the treatment may be improved if an antibody which is specific for only active αLβ2 is used. Therefore, the understanding of the integrin activation states is very important. A few observations lend support to the different activation states for αLβ2. COS-7 cells expressing wild type αLβ2 did not adhere to ICAM-1 unless in the presence of either activating agent Mg^{2+}/EGTA or mAb KIM185. On the other hand, binding to ICAM-2 and ICAM-3 required the presence of both activation reagents (Al-shamkhani and Law, 1998). It was also reported that αLβ2 exists in distinct ICAM-specific activation states (Buckley et al., 1997). The

different activation states of integrin are coupled with its structural rearrangement. It has been reported that preventing the swing-out motion of the β subunit hybrid domain markedly attenuates integrin ligand binding (Mould et al., 2003). Crystal structure data of the hybrid domain, PSI domain and I-EGF1 suggest a rigid structure based on extensive contacts established in their interfaces (Shi et al., 2005; Xiao et al., 2004, Xiong et al., 2004). Taken together, the hybrid domain may act as a mechanical lever for signal propagation in integrin activation. In line with this, electron microscopy studies of integrin α5β1 in complex with ligand fibronectin exhibited hybrid domain displacement as compared to the unliganded receptor (Takagi et al., 2003).

The mAb MEM148 is mapped to the critical residue Pro^{374} and residues His^{370}-Gln^{373} of the integrin β2 hybrid domain. Unlike the exposed epitope Val^{385} recognised by mAb 7E4 (Tng et al., 2004), the epitope for MEM148 is hidden in the αLβ2 model, when using the bent αVβ3 as template (Figure 3.3). Therefore MEM148 could be used as a reporter mAb to detect β2 integrin which are activated. First, we performed a series of FACS analyses to show the αLβ2 integrin can shuttle between MEM148-staining (active) conformation and non MEM148-staining (inactive) conformation. Next, we tested the ligand-binding ability of αLβ2 to ICAM-1 and ICAM-3. Interestingly, we found that MEM148 itself cannot promote binding of αLβ2 to ICAM-1 but rather can stabilize the αLβ2 in a certain affinity state once it is already activated by activating agent Mg^{2+}/EGTA or mAb KIM185. This state is an "intermediate state" because it is able to bind to ICAM-1 but binding to ICAM-3 needs further activation. Another question emerges here, why does binding to ICAM-1 and ICAM-3 require different αLβ2

conformations? In chapter three, I have discussed that the difference may be contributed by dissimilar dissociation rates of the interactions and the conformations of the binding pockets. Other than that, different physiological functions of αLβ2/ICAM-1 and αLβ2/ICAM-3 interaction may also cause the difference in αLβ2 activation states requirements. ICAM-1 is thought to be important in leukocyte emigration through the endothelium into sites of immunological challenge (Butcher, 1991; Springer, 1994). We speculate that tight adhesion is disfavored because it may reduce cell motility and lead to impaired migration. Therefore, intermediate affinity may be suitable for αLβ2 in the process of migration. By contrast, ICAM-3 is the major αLβ2 ligand on resting T cells, on which ICAM-1 and ICAM-2 are either expressed at a very low level or not at all. Thus we propose that high affinity requirement for αLβ2 binding to ICAM-3 is essential to prevent cell-cell firm contact in resting T cells. While in the initial step of immune response, αLβ2 becomes active and the αLβ2/ICAM-3 adhesive event may facilitate interaction of T cells with antigen-presenting B cells for antigen presentation (de Fougerolles and Springer, 1992; Fawcett et al., 1992; Vazeux et al., 1992). Although the affinity requirements of αLβ2 binding to ICAM-1 and ICAM-3 are different, αLβ2/ICAM-1 and αLβ2/ICAM-3 binding are interrelated as in, (i) αLβ2/ICAM-3 interaction will enhance αLβ2/ICAM-1 binding; (ii) αLβ2/ICAM-3 adhesion is critical for αLβ2/ICAM-1 dependent T cell proliferation (Bleijs et al., 2000).

As MEM148 is a β2 specific mAb and has been used to study different activation states of αLβ2, we may also want to extend the study on other β2 integrin family members, such as αMβ2. Does αMβ2 also have more than one active conformation? It may not be

true that αMβ2 will behave exactly the same as αLβ2, because several studies have revealed that activation mechanism of αLβ2 and αMβ2 may not be the same (Tan et al., 2000; Walters et al., 2005).

In chapter four, we mainly compared the I-domain of αLβ2 and αMβ2 with respect to its ligand binding property. It was previously reported that when an invariant isoleucine in α subunit I-domain was mutated to glycine, both αLβ2 and αMβ2 became active in terms of ligand binding (Huth et al., 2000; Xiong et al., 2000). The hydrophobic side chain finger of the isoleucine was described as fastened in the closed conformation by a conserved hydrophobic socket (Xiong et al., 2000). This "socket for isoleucine," or SILEN, was postulated to be a key component in allosteric regulation, controlling affinity and shape shifting in the I-domain of αMβ2. By inference, the SILEN was suggested as the universal mechanism that regulates integrin conformation and function. However, in our hands, mutating this invariant isoleucine in αL to glycine does not generate an active αLβ2 to bind to ICAM-1, while the same mutation in αMβ2 does lead to αMβ2 activation. Furthermore, we found that the isolated I-domains of αL and αM show different affinity states for ligand binding. In brief, isolated αL I-domain exists in a constitutive active state and can bind to ICAM-1 without any addition of activating reagent; isolated I-domain of αM exists in a resting state and can bind to BSA only if activating reagents is added. This observation could not be due to cell line specificity, because same results were obtained in 293T cells and K562 cells. The reason for this apparent discrepancy remains to be investigated. We notice that, only αLβ2/ICAM-1 and αMβ2/BSA binding were analyzed in this part of study. Since αLβ2 and αMβ2 both have various native ligands, binding

assays using ligands other than ICAM-1 and BSA may help to give a better understanding of the regulation mechanism for αLβ2 and αMβ2. Nevertheless, our data revealed that the proposed 'SILEN' regulation of I-domain is applicable to specific I-domain containing integrins, e.g., αM, but may not serve as a universal 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 (Tan et al., 2000). In that report, deletions of β2 transmembrane and cytoplasmic domain will lead to activation of αLβ2 but not αMβ2. This should not happen if αLβ2 and αMβ2 share a common regulation mechanism for their I-domains. It is proposed that β2 subunit may have different roles in the function of αLβ2 and αMβ2. Differential activation of αLβ2 and αMβ2 I-domains may account for such difference.

It has been widely accepted that integrins can transduce signal bidirectionally across the cell membrane. "Out-side in" signaling refers to that type of integrin ligand-binding which triggers an intracellular signal cascade. The ectodomain of integrin plays a major role in this process and has been the main objective for my study in the first two parts. In the process of "in side-out signaling", integrin cytoplasmic tails play a key role in relaying signals across the plasma membrane. A pool of cytoplasmic protein interacting with the integrin cytoplasmic tails has been identified (Liu et al., 2000). We notice that β2 integrin family members share a common β subunit and specific α subunits. Will the different α subunit tail affect the interactions between the common β subunit tail and its cytosolic partners?

Increasing evidence indicate that the α cytoplasmic tail determines integrin functional specialization. Integrin α2β1 (VLA-2) showed differential cell-migration on collagen and laminin when α2 cytoplasmic tail was replaced by that from integrin α5 or α4 (Chan et al., 1992). Integrin α4β1 (VLA-4) exhibited differential adhesion properties to ligand VCAM-1 under shear flow analyses when α_4 tail was exchanged with that from integrin α2 or α5 (Kassner et al., 1993). The difference in α cytoplasmic tails of α4β1 and α5β1 may also account for the differential requirement of FAK mediated signaling pathway (Hsia et al., 2005). In a recent study, a dimerized β2 cytoplasmic tail model protein was shown to interact with several SFKs (Arias-Salgado et al., 2003). Since that model protein does not have α subunit tail, it is interesting to know whether α subunit tail will affect the interaction between β2 and SFKs. In the third part of the study, we found (i) the co-capping of Hck to αMβ2 was abrogated when the αM tail was substituted with that of αL or αX; (ii) its restoration when this αL or αX tail was truncated or by disrupting the membrane proximal salt-bridge between the α and β2 tails; (iii) the identification of a region within β2 tail that is required for αMβ2 and Hck co-capping. Together, these data suggest a potential role of the integrin α tail in regulating β2 tail interaction with cytosolic partners. Furthermore, there are several areas that need further investigation: (i) how does Hck and talin co-operatively affect αMβ2 function since they seem to interact with β2 tail through a similar region; (ii) integrin β tails do not have a proline rich region, which is a classic binding domain for SFKs SH3 domain. How SFKs still are able to interact with integrin β tails through their SH3 domain (Arias-Salgado et al., 2005)? (iii) Will the phosphorylation status of integrin cytoplasmic tail change after constitutive association with SFKs, and what is the effect if that change really takes place? These investigations

will lead to further understanding of integrin mediated signal transduction by interaction with its cytosolic partners.

References

Acevedo, A., del Pozo, M. A., Arroyo, A. G., Sanchez-Mateos, P., Gonzalez-Amaro, R., and Sanchez-Madrid, F. (1993). Distribution of ICAM-3-bearing cells in normal human tissues. Expression of a novel counter-receptor for LFA-l in epidermal Langerhans cells. Am. J. Pathol. *143*, 774-783.

Adair, B. D., and Yeager, M. (2002). Three-dimensional model of the human platelet integrin αIIbβ3 based on electron cryomicroscopy and x-ray crystallography. Proc. Natl. Acad. Sci. USA *99*, 14059-14064.

Akiyama, S. K. (1996). Integrins in cell adhesion and signaling. Hum. Cell. *9*, 181-186

Al-Shamkhani A., Law, S. K. (1998). Expression of the H52 epitope on the β2 subunit is dependent on its interaction with the α subunits of the leukocyte integrins LFA-1, Mac-1 and p150,95 and the presence of Ca2+. Eur. J. Immunol. *28*, 3291-3300.

Alahari, S. K., Lee, J. W., Juliano, R. L. (2000). Nischarin, a novel protein that interacts with the integrin α5 subunit and inhibits cell migration. J. Cell. Biol. *151*, 1141-1154.

Alon, R., and Etzioni, A. (2003). LAD-III, a novel group ofleukocyte integrin activation deficiencies. Trends. Immunol. *24*, 561-566.

Alonso, J. L., Essafi, M., Xiong, J. P., Stehle, T., and Arnaout, M. A. (2002). Does the integrin αA domain act as a ligand for its βA domain? Curr. Biol. *12*, R340-342.

Altieri, D. C., Bader, R., Mannucci, P. M., and Edgington, T. S. (1988a). Oligospecificity of the cellular adhesion receptor Mac-l encompasses an inducible recognition specificity for fibrinogen. J. Cell. Biol. *107,* 1893-1900.

Altieri, D. C., and Edgington, T. S. (1988). The saturable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-l receptor. J. Biol. Chem. *263*, 7007-7015.

Altieri, D. C., Morrissey, J. H., and Edgington, T. S. (1988b). Adhesive receptor Mac-1 coordinates the activation of factor X on stimulated cells of monocytic and myeloid differentiation: an alternative initiation of the coagulation protease cascade. Proc. Natl. Acad. Sci. USA *85*, 7462-7466.

Andrew, D., Shock, A., Ball, E., Ortlepp, S., Bell, J., and Robinson, M. (1993). KIM185, a monoclonal antibody to CD18 which induces a change in the conformation of CD18 and promotes both LFA-1- and CR3-dependent adhesion. Eur. J. Immunol. *23*, 2217-2222.

Andrew, D. P., Spellberg, J. P., Takimoto, H., Schmits, R., Mak, T. W., Zukowski, M. M.

(1998). Transendothelial migration and trafficking of leukocytes in LFA-1-deficient mice. Eur. J. Immunol. *28*. 1959-1969.

Anderson, D. C. and Springer, T. A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-I, LFA-l, and p150,95 glycoproteins. Annu. Rev. Med. *38*, 175-194.

Anderson, D. C. a. S., C.W. (2001). Leukocyte Adhesion Deficiencies. In The Metabolic and Molecular Basis of inherited Disease, C. R. Scriver, Beaudet, A.L., Sly, S., and Volle, D., ed. (New York, McGraw-Hill), 4829-4856.

Anderson, M. E., and Siahaan, T. J. (2003). Mechanism of binding and internalization of ICAM-l-derived cyclic peptides by LFA-l on the surface of T cells: a potential method for targeted drug delivery. Pharm. Res. *20,* 1523-1532.

Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H., Shattil, S. J. (2003). Src kinase activation by direct interaction with the integrin β cytoplasmic domain. Proc. Natl. Acad. Sci. USA *100*, 13298-13302.

Arias-Salgado, E. G., Lizano, S., Shattil, S. J., Ginsberg, M. H. (2005). Specification of the direction of adhesive signaling by the integrin β cytoplasmic domain. J. Biol. Chem. *280*, 29699-29707.

Armulik, A., Nilsson, I., von Heijne, G., and Johansson, S. (1999). Determination of the border between the transmembrane and cytoplasmic domains of human integrin subunits. J. Biol. Chem. *274*, 37030-37034.

Arnout, M. A. (2002). Integrin structure: new twists and turns in dynamic cell adhesion. Immunol. Rev. *186*, 125-140.

Asada, M., Furukawa, K., Kantor, C., Gahmberg, C. G., Kobata, A. (1991). Structural study of the sugar chains of human leukocyte cell adhesion molecules CD11/CD18. Biochemistry *30*, 1561-1571.

Astarie-Dequeker, C., Carreno, S., Cougoule, C. and Maridonneau-Parini, I. (2002) The protein tyrosine kinase Hck is located on lysosomal vesicles that are physically and functionally distinct from CD63-positive lysosomes in human macrophages. J. Cell. Sci. *115*, 81-89.

Auwerx, J. (1991) The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia *47*, 22-31.

Bailly, P., Tontti, E., Hermand, P., Cartron, J. P. and Gahmberg, C. G. (1995). The red cell LW blood group protein is an intercellular adhesion molecule which binds to CD11/CD18 leukocyte integrins. Eur. J. Immunol. *25*, 3316-3320.

Bainton, D. F., Miller, L. J., Kishimoto, T. K. and Springer, T. A. (1987). Leukocyte adhesion receptors are stored in peroxidase negative granules of human neutrophils. J. Exp. Med. *166*, 1641-1653.

Bajt, M. L., Ginsberg, M. H., Frelinger, A. L. 3rd, Berndt, M. C., Loftus, J. C. (1992). A spontaneous mutation of integrin αIIbβ3 (platelet glycoprotein IIb-IIIa) helps define a ligand binding site. J. Biol.Chem. *267*, 3789-3794.

Baker, E. K., Tozer, E. C., Pfaff, M., Shattil, S. J., Loftus, J. C., Ginsberg, M. H. (1997). A genetic analysis of integrin function: Glanzmann thrombasthenia in vitro. Proc. Natl. Acad. Sci. USA *94*, 1973-1978.

Barclay, A. N., Brown, Marion H., Law, S.K. Alex, McKnight, Andrew J., Tomlinson, Michael G., van der Merwe, P. Anton. (1997). The Leucocyte Antigen FactsBook, Second edn, Academic Press).

Bazzoni, G. and Hemler, M. E. (1998). Are changes in integrin affinity and conformation overemphasized? Trends In Biochemical Sciences *23*, 30-34.

Beckerle, M. C. (2001). Cell Adhesion. Frontiers in Molecular Biology *39*.

Beglova, N., Blacklow, S. C., Takagi, J. and Springer, T. A. (2002). Cysteine-rich module structure reveals a fulcrum for integrin rearrangement upon activation. Nat. Struct. Biol. *9*, 282-287.

Beller, D. I., Springer, T. A. and Schreiber, R. D. (1982). Anti-Mac-l selectively inhibits the mouse and human type three complement receptor. J. Exp. Med. *156*, 1000-1009.

Benimetskaya, L., Loike, J. D., Khaled, Z., Loike, G., Silverstein, S. C., Cao, L., Khoury, J., Cai, T. Q., and Stein, C. A. (1997). Mac-l (CD11b/CD18) is an oligodeoxynucleotide-binding protein. Nat. Med. *3*, 414-420.

Bennett, J. S. (2005). Structure and function of the platelet integrin αIIbβ3. J. Clin. Invest. 115, 3363-3369.

Berlin-Rufenach, C., Otto, F., Mathies, M., Westermann, J., Owen, M. J., Hamann, A., Hogg, N. (1999). Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. J. Exp. Med. *189*, 1467-1478.

Bilsland, C. A., Springer, T. A. (1994).Cloning and expression of the chicken CD18 cDNA. J. Leukoc. Biol. *55*, 501-506.

Bilsland, C. A., Diamond, M. S., and Springer, T. A. (1994). The leukocyte integrin p150,95 (CD11c/CD18) as a receptor for iC3b. Activation by a heterologous β subunit and localization of a ligand recognition site to the I domain. J. Immunol. *152*,

4582-4589.

Binnerts, M. E., van Kooyk, Y. (1999). How LFA-1 binds to different ligands. Immunol. Today. *20*, 240-245.

Binnerts, M. E., van Kooyk, Y., Simmons, D. L., Figdor, C. G. (1994). Distinct binding of T lymphocytes to ICAM-1, -2 or -3 upon activation of LFA-1. Eur. J. Immunol. *24*, 2155-2160.

Blackford, J., Reid, H. W., Pappin, D. J., Bowers, F. S. and Wilkinson, J. M. (1996). A monoclonal antibody, 3/22, to rabbit CD11c which induces homotypic T cell aggregation: evidence that ICAM-1 is a ligand for CD11c/CD18. Eur. J. Immunol. *26*, 525-531.

Bleijs, D. A., Binnerts, M. E., van Vliet, S. J., Figdor, C. G., van Kooyk, Y. (2000). Low-affinity LFA-1/ICAM-3 interactions augment LFA-1/ICAM-1-mediated T cell adhesion and signaling by redistribution of LFA-1. J. Cell. Sci. *113*, 391-400.

Blois, S., Tometten, M., Kandil, J., Hagen, E., Klapp, B. F.,Margni, R. A.,Arck, P. C. (2005). Intercellular adhesion molecule-1/LFA-1 cross talk is a proximate mediator capable of disrupting immune integration and tolerance mechanism at the feto-maternal interface in murine pregnancies. J. Immunol. *174*, 1820-1829.

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. (1988). The 2.0 A X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. EMBO. J. *7*, 2593-2599.

Bork, P., Doerks, T., Springer, T. A. and Snel, B. (1999). Domains in plexins: links to integrins and transcription factors. Trends. Biochem. Sci. *24*, 261-263.

Bouvard, D., Brakebusch, C., Gustafsson, E., Aszodi, A., Bengtsson, T., Berna, A., Fassler, R. (2001). Functional consequences of integrin gene mutations in mice. Circ. Res. *89*, 211-223.

Brugge, J. S., Erikson, R. L. (1977). Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature. *269*, 346-348.

Butcher, E. C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 67, 1033-1036.

Butta, N., Arias-Salgado, E. G., Gonzalez-Manchon, C., Ferrer, M., Larrucea, S., Ayuso, M. S., Parrilla, R. (2003). Disruption of the β3 663-687 disulfide bridge confers constitutive activity to β3 integrins. Blood *102*, 2491-2497.

Buckley, C. D., Ferguson, E. D., Littler, A. J., Bossy, D., Simmons, D. L. (1997). Role of ligands in the activation of LFA-1. Eur. J. Immunol. *27*, 957-962.

Bunting, M., Harris, E. S., McIntyre, T. M., Prescott, S. M. and Zimmerman, G. A. (2002). Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving β 2 integrins and selectin ligands. Curr. Opin. Hemato. *19*, 30-35.

Cain, J. A., Newman, S. L., Ross, G. D. (1987). Role of complement receptor type three and serum opsonins in the neutrophil response to yeast. Complement. *4*, 75-86.

Calderwood, D. A. (2004). Integrin activation. J. Cell. Sci. *117*, 657-666.

Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C. and Ginsberg, M. H. (2002). The phosphotyrosine binding-like domain of talin activates integrins. J. Biol. Chem. *277*, 21749-21758.

Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O. and Ginsberg, M. H. (1999). The Talin head domain binds to integrin β subunit cytoplasmic tails and regulates integrin activation. J. Biol. Chem. *274*, 28071-28074.

Calvete, J. J., Henschen, A. and Gonzalezrodriguez, J. (1991). Assignment Of Disulphide Bonds In Human Platelet GPIIIa -a Disulphide Pattern For the B-Subunits Of the Integrin Family. Biochemical Journal *274*, 63-71.

Campbell, J. J., Hedrick, J., Zlotnik, A., Siani, M. A., Thompson, D. A., Butcher, E. C. (1998). Chemokines and the arrest of lymphocytes rolling under flow conditions. Science *279*, 381-384.

Carman, C. V. and Springer, T. A. (2003). Integrin avidity regulation: are changes in affinity and conformation underemphasized? Curr. Opin. Cell. Biol. *15*, 547-556.

Carpen, O., Pallai, P., Staunton, D. E., Springer, T. A. (1992). Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and α-actinin. J. Cell. Biol. 118, 1223-1234.

Casasnovas, J. M., Springer, T. A., Liu, J. H., Harrison, S. C. and Wang, J. H. (1997). Crystal structure of ICAM-2 reveals a distinctive integrin recognition surface. Nature *387*, 312-315.

Casasnovas, J. M., Stehle, T., Liu, J. H., Wang, J. H. and Springer, T. A. (1998). A dimeric crystal structure for the N-terminal two domains of intercellular adhesion molecule-I. Proc. Natl. Acad. Sci. USA *95*, 4134-4139.

Chan, B. M., Kassner, P. D., Schiro, J. A., Byers, H. R., Kupper, T. S., Hemler, M. E. (1992). Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell *68*, 1051-1060.

Chan, J. R., Hyduk, S. J., Cybulsky, M. I. (2003). Detecting rapid and transient upregulation of leukocyte integrin affinity induced by chemokines and chemoattractants.

J. Immunol. Methods. *273*, 43-52.

Chen, W. J., Goldstein, J. L., Brown ,M. S. (1990). NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. *265*, 3116-3123.

Chigaev, A., Blenc, A. M., Braaten, J. V,, Kumaraswamy, N., Kepley, C. L., Andrews, R. P., Oliver, J. M., Edwards, B. S., Prossnitz, E. R., Larson, R. S., Sklar, L. A. (2001). Real time analysis of the affinity regulation of α4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn(2+) or antibody activation. J. Biol. Chem. *276*, 48670-48678.

Cierniewska-Cieslak, A., Cierniewski, C. S., Blecka, K., Papierak, M., Michalec, L., Zhang, L., Haas, T. A. and Plow, E. F. (2002). Identification and characterization of two cation binding sites in the integrin β3 subunit. J. Biol. Chem. *277*, 11126-11134.

Collett, M. S., Erikson, R. L. (1978). Protein kinase activity associated with the avian sarcoma virus src gene product. Proc. Natl Acad. Sci. USA. *75*, 2021-2024.

Colombatti, A., Bonaldo, P. (1991). The superfamily of proteins with von Willebrand factor type A-like domains: one theme common to components of extracellular matrix, hemostasis, cellular adhesion, and defense mechanisms. Blood *77*, 2305-2315.

Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J. Y., Butcher, E. C., Laudanna, C. (2000). Chemokines trigger immediate β2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. Immunity 13, 759-769.

Coombe, D. R., Watt, S. M. and Parish, C. R. (1994). Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. Blood *84*, 739-752.

Corbi, A. L., Larson, R. S., Kishimoto, T. K., Springer, T. A. and Morton, C. C. (1988). Chromosomal location of the genes encoding the leukocyte adhesion receptors LFA-1, Mac-1 and p150, 95. Identification of a gene cluster involved in cell adhesion. J. Exp. Med. *167*, 1597-1607.

Coxon, A., Rieu, P., Barkalow, F. J., Askari, S., Sharpe, A. H., von Andrian, U. H., Arnaout, M. A., Mayadas, T. N. (1996). A novel role for the β2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. Immunity *5*, 653-666.

Crowley, C. A., Curnutte, J. T., Rosin, R. E., Andre-Schwartz, J., Gallin, J. I., Klempner, M., Snyderman, R., Southwick, F. S., Stossel, T. P. and Babior, B. M. (1980). An inherited abnormality of neutrophil adhesion. Its genetic transmission and its association with a missing protein. N. Engl. J. Med. *302,* 1163-1168.

Davignon, D., Martz, E., Reynolds, T., Kurzinger, K. and Springer, T. A. (1981). Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from

Lyt-2,3 that participates in T lymphocyte-mediated killing. Proc. Natl. Acad. Sci. USA *78*, 4535-4539.

Davis, G. E. (1992). The Mac-1 and p150, 95 β2 Integrins Bind Denatured Proteins to Mediate Leukocyte Cell Substrate Adhesion. Experimental Cell Research *200,* 242-252.

de Fougerolles, A. R., Klickstein, L. B. and Springer, T. A. (1993). Cloning and expression of intercellular adhesion molecule 3 reveals strong homology to other immunoglobulin family counter-receptors for lymphocyte function-associated antigen 1. J. Exp. Med. *177*, 1187-1192.

de Fougerolles, A. R. and Springer, T. A. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J. Exp. Med. *175*, 185-190.

de Fougerolles, A. R., Stacker, S. A., Schwarting, R. and Springer, T. A. (1991). Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J. Exp. Med. *174*, 253-267.

De Melker, A. A., Kramer, D., Kuikman, I. and Sonnenberg, A. (1997). The two phenylalanines in the GFFKR motif of the integrin α6A subunit are essential for heterodimerization. Biochem. J. *328* (*Pt* 2), 529-537.

de Virgilio, M., Kiosses, W. B., Shattil, S. J. (2004). Proximal, selective, and dynamic interactions between integrin αIIbβ3 and protein tyrosine kinases in living cells. J. Cell. Biol. *165*, 305-311

Diacovo, T. G., deFougerolles, A. R., Bainton, D. F. and Springer, T. A. (1994). A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. J. Clin. Invest. *94*, 1243-1251.

Diamond, M. S., Alon, R., Parkos, C. A., Quinn, M. T. and Springer, T. A. (1995). Heparin is an adhesive ligand for the leukocyte integrin Mac-1 (CD11b/CD18). J. Cell. Biol. *130,* 1473-1482.

Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S. A., Garcia-Aguilar, J., Hibbs, M. L. and Springer, T. A. (1990). ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). J. Cell. Biol. *111*, 3129-3139.

Diamond, M. S., Staunton, D. E., Marlin, S. D. and Springer, T. A. (1991). Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell *65*, 961-971.

Ding, Z. M., Babensee, J. E., Simon, S. I., Lu, H., Perrard, J., L., Bullard, D., C., Dai, X. Y., Bromley, S. K., Dustin, M. L., Entman, M. L., Smith, C. W., Ballantyne, C. M.

(1999). Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. J. Immunol. *163*, 5029-5038.

Discipio, R. G., Daffern, P. J., Schraufstatter, I. U. and Sriramarao, P. (1998). Human polymorphonuclear leukocytes adhere to complement factor H through an interaction that involves αMβ2 (CD11b/CDI8). J. Immunol. *160,* 4057-4066.

Dong, Z. M., Gutierrez-Ramos, J. C., Coxon, A., Mayadas, T. N., Wagner, D.D. (1997). A new class of obesity genes encodes leukocyte adhesion receptors. Proc. Natl. Acad. Sci. USA *94*, 7526-7530.

Douglas, I. S., Leff, A. R. and Sperling, A. I. (2000). CD4+ T cell and eosinophil adhesion is mediated by specific ICAM-3 ligation and results in eosinophil activation. J. Immunol. *164*, 3385-3391.

Douglass, W. A., Hyland, R. H., Buckley, C. D., Al-Shamkhani, A., Shaw, J. M., Scarth, S. L., Simmons, D. L., Law, S. K. (1998). The role of the cysteine-rich region of the β2 integrin subunit in the leukocyte function-associated antigen-1 (LFA-1, αLβ2, CD11a/CD18) heterodimer formation and ligand binding. FEBS. Lett. 440, 414-418.

Drbal, K., Angelisova, P., Cerny, J., Hilgert, I., Horejsi, V. (2001). A novel anti-CD18 mAb recognizes an activation-related epitope and induces a high-affinity conformation in leukocyte integrins. Immunobiology *203*, 687-698.

Drbal, K., Angelisova, P., Hilgert, I., Cerny, J., Novak, P., Horejsi, V. (2001). A proteolytically truncated form of free CD18, the common chain of leukocyte integrins, as a novel marker of activated myeloid cells. Blood *98*, 1561-1566.

Du, X., Gu, M., Weisel, J. W., Nagaswami, C., Bennett, J. S., Bowditch, R., Ginsberg, M. H. (1993). Long range propagation of conformational changes in integrin αIIbβ3. J. Biol. Chem. *268*, 23087-23092

Durden, D.L. Kim, H.M., Calore, B., and Liu, Y. (1995) The Fc gamma RI receptor signals through the activation of hck and MAP kinase. J. Immunol. *154*, 4039-4047.

Dustin, M. L. Shaw, A. S. (1999). Costimulation: building an immunological synapse. Science *283*, 649-650.

Dustin, M. L. Springer, T. A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619-624.

Dustin, M. L., Springer, T. A. (1988). Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell. Biol. *107*, 321-331.

Dustin, M. L. (2001). Role of adhesion molecules in activation signaling in T lymphocytes. J. Clin. Immunol. *21*, 258-263.

Edwards, C. P., Champe, M., Gonzalez, T., Wessinger, M. E., Spencer, S. A., Presta, L. G., Berman, P. W., Bodary, S. C. (1995). Identification of amino acids in the CD11a I-domain important for binding of the leukocyte function-associated antigen-1 (LFA-1) to intercellular adhesion molecule-1 (ICAM-1). J. Biol. Chem. *270*, 12635-12640.

Eigenthaler, M., Hofferer, L., Shattil, S. J., Ginsberg, M. H. (1997). A conserved sequence motif in the integrin β3 cytoplasmic domain is required for its specific interaction with β3-endonexin. J. Biol. Chem.*272*, 7693-7698.

Einheber, S., Milner, T. A., Giancotti, F., Salzer, J. L. (1993). Axonal regulation of Schwann cell integrin expression suggests a role for α 6 β 4 in myelination. J. Cell Biol. *123*, 1223-1236.

Eliceiri, B. P. (2001). Integrin and growth factor receptor crosstalk. Circ. Res. *89*, 1104-1110.

Emsley, J., King, S. L., Bergelson, J. M. and Liddington, R. C. (1997). Crystal structure of the I domain from integrin α2β1. J. Biol. Chem. *272*, 28512-28517.

Emsley, J., Knight, C. G., Famdale, R. W., Barnes, M. J. and Liddington, R. C. (2000). Structural basis of collagen recognition by integrin α2β1. Cell *101,* 47-56.

Evans, B. J., McDowall, A., Taylor, P. C., Hogg, N., Haskard, D. O., Landis, R. C. (2006). Shedding of lymphocyte function-associated antigen-1 (LFA-1) in a human inflammatory response. Blood. 107, 3593-3599.

Fawcett, J., Holness, C. L., Needham, L. A., Turley, H., Gatter, K. C., Mason, D. Y., and Simmons, D. L. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-I, constitutively expressed on resting leukocytes. Nature *360,* 481-484.

Fine, J. D., Bauer, E. A., Briggaman, R. A., Carter, D. M., Eady, R. A. J., Esterly, N. B., Holbrook, K. A., Hurwitz, S., Johnson, L. and Lin, A. (1991). Revised clinical and laboratory criteria for subtypes of inherited epidermolysis bullosa. Am. Acad. Dermatol. *24*, 119-135.

Fuortes, M., Melchior, M., Han, H., Lyon, G. J., Nathan, C. (1999). Role of the tyrosine kinase pyk2 in the integrin-dependent activation of human neutrophils by TNF. J. Clin. Invest. *104*, 327-335.

Gahmberg, C. G. (1997). Leukocyte adhesion: CD11/CD18 integrins and intercellular adhesion molecules. Curr. Opin. Cell. Biol. *9*, 643-650.

Garcia-Alvarez, B., de Pereda, L. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H. and Liddington, R. C. (2003). Structural

determinants of integrin recognition by talin. Mol. Cell. *11*, 49-58.

Giancotti, F. G. and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028-1032.

Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H. and Liddington, R. C. (2003) Structural determinants of integrin recognition by talin. Mol. Cell. *11*, 49-58.

Geng, X., Tang, R. H., Law, S. K., Tan, S. M. (2005). Integrin CD11a cytoplasmic tail interacts with the CD45 membrane-proximal protein tyrosine phosphatase domain 1. Immunology *115*, 347-357.

Giagulli, C., Scarpini, E., Ottoboni, L., Narumiya, S., Butcher, E. C., Constantin, G., Laudanna, C. (2004). RhoA and zeta PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte in vivo homing. Immunity *20*, 25–35

Gil, S. G., Brown T. A., Ryan, M. C. and Carter W. G. (1994). Junctional epidermolysis bullosis: defects in expression of epiligrin/nicein/kalinin and integrin β4 that inhibit hemidesmosme formation. J. Invest. Dermatol. *103*, 31S-38S.

Gottlieb, A., Krueger, J. G., Bright, R., Ling, M., Lebwohl, M., Kang, S., Feldman, S., Spellman, M., Wittkowski, K., Ochs, H. D., Jardieu, P., Bauer, R., White, M., Dedrick, R., Garovoy, M. (2000). Effects of administration of a single dose of a humanized monoclonal antibody to CD11a on the immunobiology and clinical activity of psoriasis. J. Am. Acad. Dermatol. *42*, 428-435.

Gogstad, G. O., Hagen, I., Korsmo, R. and Solum, N. O. (1981). Characterization of the proteins of isolated human platelet a-granules. Evidence for a separate a-granule pool of the glycoproteins IIb and III. Biochem. Biophys. Acta. *670*, 150-162.

Goodman, T. G., Bajt, M. L. (1996). Identifying the putative metal ion-dependent adhesion site in the β2 (CD18) subunit required for αLβ2 and αMβ2 ligand interactions. J. Biol. Chem. *271*, 23729-23736.

Gottschalk, K. E. and Kessler, H. (2002). The structures of integrins and integrin ligand complexes: implications for drug design and signal transduction. Angew. Chem. Int. Ed. Engl. *41*, 3767-3774.

Griffiths, C. E., Railan, D., Gallatin, W. M. and Cooper, K. D. (1995). The ICAM-3/LFA-1 interaction is critical for epidermal Langerhans cell alloantigen presentation to CD4+ T cells. Br. J. Dermatol. *133*, 823-829.

Han, J., Liu, S., Rose, D. M., Schlaepfer, D. D., McDonald, H. and Ginsberg, M. H. (2001) Phosphorylation of the integrin α4 cytoplasmic domain regulates paxillin binding. J. Biol. Chem. *276*, 40903-40909.

Hantgan, R. R., Paumi, C., Rocco, M., Weisel, J. W. (1990). Effects of ligand-mimetic peptides Arg-Gly-Asp-X (X = Phe, Trp, Ser) on αIIbβ3 integrin conformation and oligomerization. Biochemistry *38*, 14461-14474.

Hayashi, A. H., Galliani, C. A. and Gillis, D. A. (1991). Congenital pyloric atresia and junctional epidermolysis bullosa: a report of long-term survival and a review of the literature. J. Ped. Surgery. *27*, 1341-1345.

Hayflick, J. S., Kilgannon, P. and Gallatin, W. M. (1998). The intercellular adhesion molecule (ICAM) family of proteins. New members and novel functions. Immunol. Res. *17*, 313-327.

Hayward, A. R., Harvey, B. A., Leonard, J., Greenwood, M. C., Wood, C. B. and Soothill, J. F. (1979). Delayed separation of the umbilical cord, widespread infections, and defective neutrophil mobility. Lancet *1*, 1099-1101.

Heiska, L., Kantor, C., Parr, T., Critchley, D. R., Vilja, P., Gahmberg, C. G., Carpen, O. (1996). Binding of the cytoplasmic domain of intercellular adhesion molecule-2 (ICAM-2) to α-actinin. J. Biol. Chem. *271*, 26214-26219.

Hermand, P., Huet, M., Callebaut, I., Gane, P., Ihanus, E., Gahmberg, C. G., Cartron, J. P. and Bailly, P. (2000). Binding sites of leukocyte β2 integrins (LFA-1, Mac-1) on the human ICAM-4/LW blood group protein. J. Biol. Chem. *275*, 26002-26010.

Hersmann, G. H., Kriegsmann, J., Simon, J., Huttich, C. and Brauer, R. (1998). Expression of cell adhesion molecules and cytokines in murine antigen-induced arthritis. Cell. Adhes. Commun. *6*, 69-82.

Hildreth, J. E., Gotch, F. M., Hildreth, P. D. and McMichael, A. J. (1983). A human lymphocyte-associated antigen involved in cell-mediated lympholysis. Eur. J. Immunol. *13*, 202-208.

Holness, C. L., Bates, P. A., Little, A. J., Buckley, C. D., McDowall, A., Bossy, D., Hogg, N., Simmons, D. L. (1995). Analysis of the binding site on intercellular adhesion molecule 3 for the leukocyte integrin lymphocyte function-associated antigen 1. J. Biol. Chem. *270*, 877-884.

Hogg, N., and Bates, P. A. (2000). Genetic analysis of integrin function in man: LAD-1 and other syndromes. Matrix. Biol .*19*, 211-222.

Hogg, N., Henderson, R., Leitinger, B., McDowall, A., Porter, J. and Stanley, P. (2002). Mechanisms contributing to the activity of integrins on leukocytes. Immunol. Rev. *186*,164-171.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C., Burridge, K. (1986). Interaction of plasma membrane fibronectin receptor with talin--a transmembrane linkage. Nature. *320*,

531-533.

Hsia, D. A., Lim, S. T., Bernard-Trifilo, J. A., Mitra, S. K., Tanaka, S., den Hertog, J., Streblow, D. N., Ilic, D., Ginsberg, M. H., Schlaepfer, D. D. (2005). Integrin α4β1 promotes focal adhesion kinase-independent cell motility via α4 cytoplasmic domain-specific activation of c-Src. Mol. Cell. Biol. *25*, 9700-9712.

Huang, C., Springer, T. A. (1995). A binding interface on the I domain of lymphocyte function-associated antigen-1 (LFA-1) required for specific interaction with intercellular adhesion molecule 1 (ICAM-1). J. Biol. Chem. *270*, 19008-19016.

Huang, C., Zang, Q., Takagi, J. and Springer, T. A. (2000). Structural and functional studies with antibodies to the integrin β 2 subunit. A model for the I-like domain. J. Biol. Chem. *275*, 21514-21524.

Hubbard, A. K. and Rothlein, R. (2000). Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. Free. Radic. Biol. Med. *28*, 1379-1386.

Hughes, P. E., DiazGonzalez, F., Leong, L., Wu, C. Y., McDonald, J. A., Shattil, S. J. and Ginsberg, M. H. (1996). Breaking the integrin hinge -A defined structural constraint regulates integrin signaling. J. Biol. Chem. *271*, 6571-6574.

Hughes, P. E., O'Toole, T. E., Ylanne, J., Shattil, S. J. and Ginsberg, M. H. (1995). The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. J. Biol. Chem. *270*, 12411-12417.

Humphries, M. J. (2000). Integrin structure. Biochem. Soc. Trans. *28*, 311-339.

Humphries, M. J., McEwan, P. A., Barton, S. J., Buckley, P. A., Bella, J. and Paul Mould, A. (2003). Integrin structure: heady advances in ligand binding, but activation still makes the knees wobble. Trends. Biochem. Sci. *28*, 313-320.

Huntington, N. D., Tarlinton, D. M. (2004). CD45: direct and indirect government of immune regulation. Immunol. Lett. *94*, 167-174.

Huth, J. R., Olejniczak, E. T., Mendoza, R., Liang, H., Harris, E. A., Lupher, M. L. Jr, Wilson, A. E., Fesik, S. W., Staunton, D. E. (2000). NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen 1 ligand binding. Proc. Natl. Acad. Sci. USA *97*, 5231-5236.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell *110*, 673-687.

Ingalls, R. R. and Golenbock, D. T. (1995). CD11c/CD18, a transmembrane signaling

receptor for lipopolysaccharide. J. Exp. Med. *181*, 1473-1479.

Janowski, R., Kozak, M., Jankowska, E., Grzonka, Z., Grubb, A., Abrahamson, M. and Jaskolski, M. (2001). Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping. Nat. Struct. Biol. *8*, 316-320.

Jin, M., Andricioaei, I., Springer, T. A. (2004).Conversion between three conformational states of integrin I domains with a C-terminal pull spring studied with molecular dynamics. Structure *12*, 2137-2147.

Kajiji, S., Tamura, R., N., Quaranta, V. (1989). A novel integrin (αE β4) from human epithelial cells suggests a fourth family of integrin adhesion receptors. EMBO. J. 8, 673-680.

Kamata, T., Wright, R., Takada, Y. (1995). Critical threonine and aspartic acid residues within the I domains of β 2 integrins for interactions with intercellular adhesion molecule 1 (ICAM-1) and C3bi. J. Biol. Chem. *270*, 12531-12535.

Kamata, T., Ambo, H., Puzon-McLaughlin, W., Tieu, K. K., Handa, M., Ikeda, Y. and Takada, Y. (2003). Critical Cys residues for regulation of integrin αIIbβ3 are clustered in the EGF domains of the β3 subunit. Biochem. J. *378*, 1079-1082.

Kassner, P. D., Hemler, M. E. (1993). Interchangeable α chain cytoplasmic domains play a positive role in control of cell adhesion mediated by VLA-4, a β 1 integrin. J. Exp. Med. *178*, 649-660.

Keizer, G. D., Te Velde, A. A., Schwarting, R., Figdor, C. G. and De Vries, J. E. (1987). Role of p150, 95 in adhesion, migration, chemotaxis and phagocytosis of human monocytes. Eur. J. Immunol. *17*, 1317-1322.

Kennel, S. J., Godfrey, V., Ch'ang, L. Y., Lankford, T. K., Foote, L. J., Makkinje, A. (1992). The β4 subunit of the integrin family is displayed on a restricted subset of endothelium in mice. J. Cell. Sci. *101*, 145-150.

Kennel, S. J., Foote, L. J., Cimino, L., Rizzo, M. G., Chang, L. Y., Sacchi, A. (1993). Sequence of a cDNA encoding the β 4 subunit of murine integrin. Gene. *130*, 209-216.

Kilgannon, P., Turner, T., Meyer, J., Wisdom, W. and Gallatin, W. M. (1998). Mapping of the ICAM-5 (telencephalin) gene, a neuronal member of the ICAM family, to a location between ICAM-l and ICAM-3 on human chromosome 19p13.2. Genomics *54*, 328-330.

Kim, M., Carman, C. V. and Springer, T. A. (2003). Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science *301,* 1720-1725.

Kishimoto, T. K., Hollander, N., Roberts, T. M., Anderson, D. C. and Springer, T. A.

(1987a). Heterogeneous mutations in the β subunit common to the LFA-l, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency. Cell *50,* 193-202.

Kim, P.W., Sun, Z.Y., Blacklow, S.C., Wagner, G. and Eck, M.J. (2003) A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. Science *301*, 1725-1728.

Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. and Springer, T. A. (1987b). Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell *48*, 681-690.

Klickstein, L. B., York, M. R., Fougerolles, A. R., Springer, T. A. (1996). Localization of the binding site on intercellular adhesion molecule-3 (ICAM-3) for lymphocyte function-associated antigen 1 (LFA-1). J. Biol. Chem. *271*, 23920-23927.

Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A., Soriano, P. (1999). Src family kinases are required for integrin but not PDGFR signal transduction. EMBO. J. *18*, 2459-2471.

Koivunen, E., Ranta, T. M., Annila, A., Taube, S., Uppala, A., Jokinen, M., van Willigen, G., Ihanus, E., and Gahmberg, C. G. (2001). Inhibition of β(2) integrin mediated leukocyte cell adhesion by leucine-leucine-glycine motif-containing peptides. J Cell Biol *153*, 905-916.

Kotovuori, P., Tontti, E., Pigott, R., Shepherd, M., Kiso, M., Hasegawa, A., Renkonen, R., Nortamo, P., Altieri, D. C. and Gahmberg, C. G. (1993). The vascular E-selectin binds to the leukocyte integrins CD11/CD 18. Glycobiology *3*, 131-136.

Kouns, W. C., Steiner, B., Kunicki, T. J., Moog, S., Jutzi, J., Jennings, L. K., Cazenave, J. P., Lanza, F. (1994). Activation of the fibrinogen binding site on platelets isolated from a patient with the Strasbourg I variant of Glanzmann's thrombasthenia. Blood *84*, 1108-1115.

Krueger, J., Gottlieb, A., Miller, B., Dedrick, R., Garovoy, M., Walicke, P. (2000). Anti-CD11a treatment for psoriasis concurrently increases circulating T-cells and decreases plaque T-cells, consistent with inhibition of cutaneous T-cell trafficking. J. Invest. Dermatol. *115*, 333.

Kucik, D. F., Dustin, M. L., Miller, J. M., Brown, E. J. (1996). Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. J. Clin. Invest. *97*, 2139-2144.

Lacour, J. P., Hoffman, P., Bastiani-Griffet, F., Boutte, P., Pisani, A. and Ortonne, J.-P. (1992). Lethal junctional epidermolysis bullosa with normal expression of BM600 and antro-pyloric atresia: a new variant of junctional epidermolysis bullosa? Eur. J. Pediatr. *151*, 252-257.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680-685.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E. and Sigler, P. B. (1996). The 2.0 A crystal structure of a heterotrimeric G protein. Nature *379*, 311-319.

Larson, R. S. and Springer, T. A. (1990). Structure and function of leukocyte integrins. Immunol. Rev. *114*, 181-217.

Larson, R. S., Corbi, A. L., Berman, L., Springer, T. A. (1989). Primary structure of the leukocyte function-associated molecule-1 α subunit: an integrin with an embedded domain defining a protein superfamily. J. Cell. Biol. *108*, 703-712

Law, S. K., Gagnon, J., Hildreth, J. E., Wells, C. E., Willis, A. C. and Wong, A. J. (1987). The primary structure of the β-subunit of the cell surface adhesion glycoproteins LFA-I, CR3 and p150, 95 and its relationship to the fibronectin receptor. EMBO. J. 6, 915-919.

Laudanna, C., Kim, J. Y., Constantin, G., Butcher, E. (2002). Rapid leukocyte integrin activation by chemokines. Immunol. Rev. *186*, 37-46.

Le Cabec, V., Carreno, S., Moisand, A., Bordier, C., Maridonneau-Parini, I. (2002). Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively. J. Immunol. *169*, 2003-2009.

Lee, J. O., Bankston, L. A., Arnaout, M. A. and Liddington, R. C. (1995a). Two conformations of the integrin A-domain (I-domain): a pathway for activation? Structure *3*, 1333-1340.

Lee, J. O., Rieu, P., Arnaout, M. A. and Liddington, R. (1995b). Crystal structure of the A domain from the α subunit of integrin CR3 (CD11b/CD18). Cell *80,* 631-638.

Leitinger, B. and Hogg, N. (2000). Effects of I domain deletion on the function of the β2 integrin lymphocyte function-associated antigen-1. Mol. Biol. Cell. *11*, 677-690.

Li, R., Babu, C. R., Valentine, K., Lear, L. D., Wand, A. J., Bennett, J. S. and DeGrado, W. F. (2002). Characterization of the monomeric form of the transmembrane and cytoplasmic domains of the integrin β3 subunit by NMR spectroscopy. Biochemistry *41*, 15618-15624.

Li, R., Mitra, N., Gratkowski, H., Vilaire, G., Litvinov, R., Nagasami, C., Weisel, J. W., Lear, J. D., DeGrado, W. F. and Bennett, J. S. (2003). Activation of integrin αIIbβ3 by modulation of transmembrane helix associations. Science *300*, 795-798.

Li, R., Nortamo, P., Valmu, L., Tolvanen, M., Huuskonen, J., Kantor, C. and Gahmberg, C. G. (1993). A peptide from ICAM-2 binds to the leukocyte integrin CD11a/CD18 and inhibits endothelial cell adhesion. J. Biol. Chem. *268*, 17513-17518.

Li, R., Rieu, P., Griffith, D. L., Scott, D. and Arnaout, M. A. (1998). Two functional states of the CD11b A-domain: correlations with key features of two Mn2+-complexed crystal structures. J. Cell. Biol. *143,* 1523-1534.

Lichtenberg, U., Quintrell, N., Bishop, J. M. (1992). Human protein-tyrosine kinase gene HCK: expression and structural analysis of the promoter region. Oncogene *7*, 849-858.

Liddington, R. C. (2002). Will the real integrin please stand up? Structure (Camb) *10,* 605-607.

Liu, S., Thomas, S. M., Woodside, D. G., Rose, D. M., Kiosses, W. B., Pfaff, M., Ginsberg, M. H. (1999). Binding of paxillin to α4 integrins modifies integrin-dependent biological responses. Nature *402*, 676-681.

Liu, S., Calderwood, D. A. and Ginsberg, M. H. (2000). Integrin cytoplasmic domain binding proteins. J. Cell..Sci. *113* , 3563-3571.

Loftus, J. C., O'Toole, T. E., Plow, E. F., Glass, A., Frelinger, A. L. 3rd, Ginsberg , M. H. (1990). A β3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science *249*, 915-918.

Loike, J. D., Sodeik, B., Cao, L., Leucona, S., Weitz, J. I., Detmers, P. A., Wright, S. D. and Silverstein, S. C. (1991). CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the A α chain of fibrinogen. Proc. Natl. Acad. Sci. USA *88*, 1044-1048.

Lowell, C.A., Soriano, P. and Varmus, H.E. (1994) Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes. Dev. *8*, 387-398.

Lowell, C. A., Fumagalli, L., Berton, G. (1996). Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. J. Cell. Biol. *133*, 895-910.

Lu, C., Ferzly, M., Takagi, J. and Springer, T. A. (200la). Epitope mapping of antibodies to the C-terminal region of the integrin β2 subunit reveals regions that become exposed upon receptor activation. J. Immunol. *166*, 5629-5637.

Lu, C., Shimaoka, M., Zang, Q., Takagi, J. and Springer, T. A. (2001b). Locking in alternate conformations of the integrin αLβ2 I domain with disulfide bonds reveals functional relationships among integrin domains. Proc. Natl. Acad. Sci. USA *98*, 2393-2398.

Lu, C., Takagi, J. and Springer, T. A. (2001c). Association of the membrane proximal

regions of the α and β subunit cytoplasmic domains constrains an integrin in the inactive state. J. Biol. Chem.*276*, 14642-14648.

Lu, C., Shimaoka, M., Ferzly, M., Oxvig, C., Takagi, J., Springer, T. A. (2001d). An isolated, surface-expressed I domain of the integrin αLβ2 is sufficient for strong adhesive function when locked in the open conformation with a disulfide bond. Proc. Natl. Acad. Sci. USA *98*, 2387-2392.

Lu, C. F. and Springer, T. A. (1997). The α subunit cytoplasmic domain regulates the assembly and adhesiveness of integrin lymphocyte function-associated antigen-1. J. Immunol. *159*, 268-278.

Lu, H., Smith, C. W., Perrard, J., Bullard, D., Tang, L., Shappell, S. B., Entman, M. L., Beaudet, A. L., Ballantyne, C. M. (1997). LFA-1 is sufficient in mediating neutrophil emigration in Mac-1-deficient mice. J. Clin. Invest. *99*, 1340-1350.

Lub, M., van Kooyk, Y., Figdor, C. G. (1995). Ins and outs of LFA-1. Immunol. Today. *16*, 479-483.

Luo, B. H., Springer, T. A. and Takagi, J. (2004). A specific interface between integrin transmembrane helices and affinity for ligand. PLoS. Biol. *2*, 776-786.

Luo, B. H., Carman, C. V., Takagi, J., Springer, T. A. (2005). Transmembrane domain helix packing stabilizes integrin αIIbβ3 in the low affinity state. J. Biol. Chem. *280*, 7294-7300.

Mankelow, T. J., Spring, F. A., Parsons, S. F., Brady, R. L., Mohandas, N., Chasis, J. A. and Anstee, D. J. (2004). Identification of critical amino-acid residues on the erythroid intercellular adhesion molecule-4 (ICAM-4) mediating adhesion to α V integrins. Blood *103,* 1503-1508.

Manser, E., Huang, H. Y., Loo, T. H., Chen, X. Q., Dong, J. M., Leung, T., Lim, L. (1997). Expression of constitutively active α-PAK reveals effects of the kinase on actin and focal complexes. Mol. Cell. Biol. 17, 1129-1143.

Marlin, S. D., and Springer, T. A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813-819.

Mattila, E., Pellinen, T., Nevo, J., Vuoriluoto, K., Arjonen, A., Ivaska, J. (2005). Negative regulation of EGFR signalling through integrin-α1β1-mediated activation of protein tyrosine phosphatase TCPTP. Nat. Cell. Biol. *7*, 78-85.

Masumoto, A., Hemler, M. E. (1993). Mutation of putative divalent cation sites in the α 4 subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and invasin. J. Cell. Biol. *123*, 245-253.

Michishita, M., Videm, V., Arnaout, M. A. (1993). A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell *72*, 857-867.

Micklem, K. J. and Sim, R. B. (1985). Isolation of complement-fragment-iC3b binding proteins by affinity chromatography. The identification of p150, 95 as an iC3b binding protein. Biochem. J. *231*,233-236.

Miller, J. L., Castella, A. (1982). Platelet-type von Willebrand's disease: characterization of a new bleeding disorder. Blood *60*, 790-794.

Miller, J., Knorr, R., Ferrone, M., Houdei, R., Carron, C. P. and Dustin, M. L. (1995). Intercellular adhesion molecule-1 dimerization and its consequences for adhesion mediated by lymphocyte function associated-1. J. Exp. Med. *182*, 1231-1241.

Miller, L. J., Bainton, D. F., Borregaard, N. and Springer, T. A. (1987). Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J. Clin. Invest. *80*, 535-544.

Miranti, C. K., Brugge, J. S. (2002) Sensing the environment: a historical perspective on integrin signal transduction. Nat. Cell. Biol. *4*, E83-90.

Mizgerd, J. P., Kubo, H., Kutkoski, G. J., Bhagwan, S. D., Scharffetter-Kochanek, K., Beaudet, A. L., Doerschuk, C. M. (1997). Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. J. Exp. Med. *186*, 1357-1364.

Mizuno, T., Yoshihara, Y., Inazawa, J., Kagamiyama, H. and Mori, K. (1997). cDNA cloning and chromosomal localization of the human telencephalin and its distinctive interaction with lymphocyte function-associated antigen-1. J. Biol. Chem. *272*, 1156-1163.

Mocsai, A., Zhou, M., Meng, F., Tybulewicz, V. L., Lowell, C. A. (2002). Syk is required for integrin signaling in neutrophils. Immunity *16*, 547-558.

Mohn, H., Le Cabec, V., Fischer, S., Maridonneau-Parini, I. (1995). The src-family protein-tyrosine kinase p59hck is located on the secretory granules in human neutrophils and translocates towards the phagosome during cell activation. Biochem. J. *309*, 657-665.

Monboisse, J. C., Garnotel, R., Randoux, A., Dufer, J., Borel, J. P. (1991). Adhesion of human neutrophils to and activation by type-I collagen involving a β2 integrin. J. Leukoc. Biol .*50*, 373-380.

Mould, A. P., Askari, J. A., Aota, S., Yamada, K. M., Irie, A., Takada, Y., Mardon, H. J. and Humphries, M. J. (1997). Defining the topology of integrin α5β1-fibronectin interactions using inhibitory anti-α5 and anti-β1 monoclonal antibodies. Evidence that the synergy sequence of fibronectin is recognized by the amino-terminal repeats of the α5 subunit. J. Biol. Chem. *272*, 17283-17292.

Mould, A. P., Barton, S. J., Askari, J. A., Craig, S. E. and Humphries, M. J. (2003a). Role of ADMIDAS cation-binding site in ligand recognition by integrin α5 β1. J. Biol. Chem. *278*, 51622-51629.

Mould, A. P., Symonds, E. J., Buckley, P. A., Grossmann, J. G., McEwan, P. A., Barton, S. J., Askari, J. A., Craig, S. E., Bella, J. and Humphries, M. J. (2003b). Structure of an integrin-ligand complex deduced from solution x-ray scattering and site-directed mutagenesis. J. Biol. Chem. *278*, 39993-39999.

Mould, A. P., Humphries, M. J. (2004) Regulation of integrin function through conformational complexity: not simply a knee-jerk reaction? Curr. Opin. Cell. Biol. *16*, 544-551.

Moyle, M., Foster, D. L., McGrath, D. E., Brown, S. M., Laroche, Y., De Meutter, J., Stanssens, P., Bogowitz, C. A., Fried, V. A., Ely, J. A., Soul H. R. and Vlasuk G. P. (1994). A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. J. Biol. Chem. *269*, 10008-10015.

Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J., Wright, S. D. (1989). Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. J. Cell. Biol. 109, 1341-1349.

Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S. and Yamada, K. M. (1988). Electron microscopy and structural model of human fibronectin receptor. EMBO. J. *7*, 4093-4099.

Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., Yamada, K. M. (1988). Electron microscopy and structural model of human fibronectin receptor. EMBO. J. *7*, 4093-4099.

Nham, S. U. (1999). Characteristics of fibrinogen binding to the domain of CD11c, an α subunit of p150, 95. Biochem. Biophys. Res. Commun. *264*, 630-634.

Nolte, M., Pepinsky, R. B., Venyaminov, S. Y., Koteliansky, V., Gotwals, P. J. and Karpusas, M. (1999). Crystal structure of the α1β1 integrin I-domain: insights into integrin I-domain function. Febs. Letters. *452*, 379-385.

Nortamo, P., Li, R., Renkonen, R., Timonen, T., Prieto, J., Patarroyo, M. and Gahmberg, C. G. (1991). The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines. Eur. J. Immunol. *21*, 2629-2632.

Noti, J. D. (2002). Expression of the myeloid-specific leukocyte integrin gene CD11d during macrophage foam cell differentiation and exposure to lipoproteins. Int. J. Mol. Med. *10,* 721-727.

Noti, J. D., Johnson, A. K. and Dillon, J. D. (2000). Structural and functional characterization of the leukocyte integrin gene CD11d. Essential role of Spl and Sp3. J. Biol. Chem. *275*, 8959-8969.

Obergfell, A., Eto, K., Mocsai, A., Buensuceso, C., Moores, S. L., Brugge, J. S., Lowell, C. A., Shattil, S. J. (2002). Coordinate interactions of Csk, Src, and Syk kinases with [α]IIb[β]3 initiate integrin signaling to the cytoskeleton. J. Cell. Biol. *157*, 265-275.

Otey, C. A., Pavalko, F. M., Burridge, K. (1990). An interaction between α-actinin and the β 1 integrin subunit in vitro. J. Cell. Biol. *111*, 721-729.

O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F. and Ginsberg, M. H. (1991). Modulation of the affinity of integrin αIIbβ 3 (GPIIb-IIIa) by the cytoplasmic domain of α IIb. Science *254*, 845-847.

Ozawa, M., Ringwald, M., Kemler, R. (1990). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc. Natl. Acad. Sci. USA. *87*, 4246-4250.

Ozawa, M., Baribault, H., Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J. *8*, 1711-1717.

Parise, L. V., Phillips, D. R. (1985). Platelet membrane glycoprotein IIb-IIIa complex incorporated into phospholipid vesicles. Preparation and morphology. J. Biol. Chem. *260*, 1750-1756.

Parise, L. V., Helgerson, S. L., Steiner, B., Nannizzi, L., and Phillips, D. R. (1987). Synthetic peptides derived from fibrinogen and fibronectin change the conformation of purified platelet glycoprotein IIb-IIIa. J. Biol. Chem. *262*, 12597-12602.

Pardi, R., Inverardi, L., Rugarli, C., Bender, J. R. (1992). Antigen-receptor complex stimulation triggers protein kinase C-dependent CD11a/CD18-cytoskeleton association in T lymphocytes. J. Cell. Biol. *116*, 1211-1220.

Patarroyo, M., Beatty. P. G., Fabre, J. W., Gahmberg, C. G. (1985). Identification of a cell surface protein complex mediating phorbol ester-induced adhesion (binding) among human mononuclear leukocytes. Scand. J. Immunol. *22*, 171-182.

Pavalko, F. M., LaRoche, S. M. (1993). Activation of human neutrophils induces an interaction between the integrin β 2-subunit (CD18) and the actin binding protein α-actinin. J. Immunol. *151*, 3795-3807.

Yan, B. and Smith, J. W. (2001). Mechanism of integrin activation by disulfide bond reduction. Biochemistry *40*, 8861-8867.

Yang, W., Shimaoka, M., Salas, A., Takagi, J. and Springer, T. A. (2004). Intersubunit signal transmission in integrins by a receptor-like interaction with a pull spring. Proc. Natl. Acad. Sci. USA *101*, 2906-2911.

Ylanne, J., Chen, Y., Otoole, T. E., Loftus, J. C., Takada, Y. and Ginsberg, M. H. (1993). Distinct Functions Of Integrin-A and Integrin-B Subunit Cytoplasmic Domains In Cell Spreading and Formation Of Focal Adhesions. J. Cell. Biol. *122*, 223-233.

Ylanne, J., Huuskonen, J., O'Toole, T. E., Ginsberg, M. H., Virtanen, I., Gahmberg, C. G. (1995). Mutation of the cytoplasmic domain of the integrin β3 subunit. Differential effects on cell spreading, recruitment to adhesion plaques, endocytosis, and phagocytosis. J. Biol. Chem. *270*, 9550-9557.

Yoshihara, Y., Mori, K. (1994). Telencephalin: a neuronal area code molecule? Neurosci. Res. *21*, 119-124.

Yuan, Q., Jiang, W. M., Leung, E., Hollander, D., Watson, J. D., Krissansen, G. W. (1992). Molecular cloning of the mouse integrin β7 subunit. J Biol Chem *267*, 7352-7358.

Zang, Q. and Springer, T. A. (2001). Amino acid residues in the PSI domain and cysteine-rich repeats of the integrin β2 subunit that restrain activation of the integrin α(X)β(2). J. Biol. Chem. *276*, 6922-6929.

Zhou, M. J., Lublin, D. M., Link, D. C. and Brown, E. J. (1995). Distinct tyrosine kinase activation and Triton X-100 insolubility upon Fc gamma RII or Fc gamma RIIIB ligation in human polymorphonuclear leukocytes. Implications for immune complex activation of the respiratory burst. J. Biol. Chem. *270*, 13553-13560.

Zutter, M. M., Santoro, S. A. (1990). Widespread histologic distribution of the α2β1 integrin cell-surface collagen receptor. Am. J. Pathol. *137*, 113-120.

Pavalko, F. M., Schneider, G., Burridge, K., Lim, S. S. (1995). Immunodetection of α-actinin in focal adhesions is limited by antibody inaccessibility. Exp. Cell Res. *217*, 534-540.

Peter K., O'Toole, T. E. (1995). Modulation of cell adhesion by changes in αLβ2 (LFA-1, CD11a/CD18) cytoplasmic domain/cytoskeleton interaction. J. Exp. Med. *181*, 315-326.

Piccardoni, P., Sideri, R., Manarini, S., Piccoli, A., Martelli, N., de Gaetano, G., Cerletti, C. and Evangelista, V. (2001) Platelet/polymorphonuclear leukocyte adhesion: a new role for SRC kinases in Mac-1 adhesive function triggered by P-selectin. Blood *98*, 108-16.

Plow, E. F., Haas, T. A., Zhang, L., Loftus L. and Smith J. W. (2000). Ligand binding to integrins. J. Biol. Chem. *275*, 21785–21788.

Peterson, J. A., Visentin, G. P., Newman, P. J., Aster, R.H. (1998). A recombinant soluble form of the integrin αIIbβ3 (GPIIb-IIIa) assumes an active, ligand-binding conformation and is recognized by GPIIb-IIIa-specific monoclonal, allo-, auto-, and drug-dependent platelet antibodies. Blood 92, 2053-2063.

Phillips, D. R., Charo, I. F., Scarborough, R. M. (1991). GPIIb-IIIa: the responsive integrin. Cell *65*, 359-362.

Phillips, M. L., Schwartz, B. R., Etzioni, A., Bayer, R., Ochs, H. D., Paulson, J. C. and Harlan, J. M. (1995). Neutrophil adhesion in leukocyte adhesion deficiency syndrome type 2. J. Clin. Invest. *96*, 2898-2906.

Phillips, R. J., Aplin, J. D. and Lake, B. D. (1994). Antigenic expression of the integrin α6β4 in junctional epidermolysis bullosa. Histopathology *24*, 571-576.

Plescia, J. and Altieri, D. C. (1996). Activation of Mac-l (CD11b/CD18)-bound factor X by released cathepsin G defines an alternative pathway of leucocyte initiation of coagulation. Biochem. J. *319*, 873-879.

Ponting, C. P., Schultz, J., Copley, R. R., Andrade, M. A., Bork, P. (2000). Evolution of domain families. Adv. Protein. Chem. *54*, 185-244.

Pyszniak, A. M., Welder, C. A., Takei, F. (1994). Cell surface distribution of high-avidity LFA-1 detected by soluble ICAM-1-coated microspheres. J. Immunol. *152*, 5241-5249.

Qin, J., Vinogradova, O. and Plow, E. F. (2004). Integrin bidirectional signaling: a molecular view. PLoS. Biol. *2*, 726-729.

Qu, A. and Leahy, D. J. (1995). Crystal structure of the I-domain from the CD11a/CD18 (LFA-l, αLβ2) integrin. Proc. Natl. Acad. Sci. USA *92*, 10277-10281.

Rabb, H., Michishita, M., Sharma, C. P., Brown, D., Arnaout, M. A. (1993). Cytoplasmic tails of human complement receptor type 3 (CR3, CD11b/CD18) regulate ligand avidity and the internalization of occupied receptors. J. Immunol. *151*, 990-1002.

Ridley, A. J. (2001). Rho GTPases and cell migration. J Cell Sci *114*, 2713-2722.
Reilly, P. L., Woska, J. R., Jr., Jeanfavre, D. D., McNally, E., Rothlein, R. and Bormann, B. J. (1995). The native structure of intercellular adhesion molecule-l (ICAM-l) is a dimer. Correlation with binding to LFA-1. J. Immunol. *155*, 529-532.

Robinson, M. K., Andrew, D., Rosen, H., Brown, D., Ortlepp, S., Stephens, P., Butcher, E. C. (1992). Antibody against the Leu-CAM β-chain (CD18) promotes both LFA-1- and CR3-dependent adhesion events. J. Immunol. *148*, 1080-1085.

Roos, D. and Law, S. K. (2001). Hematologically important mutations: leukocyte adhesion deficiency. Blood. Cells. Mol. Dis. *27*, 1000-1004.

Rosenkranz, A. R., Coxon, A., Maurer, M., Gurish, M. F., Austen, K. F., Friend, D. S., Galli, S. J., Mayadas, T. N. (1998). Impaired mast cell development and innate immunity in Mac-1 (CD11b/CD18, CR3)-deficient mice. J. Immunol. *161*, 6463-6467.

Ross, G. D., Cain, J. A., Myones, B. L., Newman, S. L. and Lachmann, P. J. (1987). Specificity of membrane complement receptor type three (CR3) for β-glucans. Complement *4*, 61-74.

Rothlein, R., Springer, T. A. (1986). The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J. Exp. Med. *163*, 1132-1149.

Russell, D. G. and Wilhelm, H. (1986). The involvement of the major surface glycoprotein (gp63) of Leishmania promastigotes in attachment to macrophages. J. Immunol. *136*, 2613-2620.

Sadhu, C., Lipsky, B., Erickson, H. P., Hayflick, J., Dick, K. O., Gallatin, W. M., Staunton, D. E. (1994). LFA-1 binding site in ICAM-3 contains a conserved motif and non-contiguous amino acids. Cell. Adhes. Commun. *2*, 429-440.

Sampath, R., Gallagher, P. J. and Pavalko, F. M. (1998). Cytoskeletal interactions with the leukocyte integrin β2 cytoplasmic tail. Activation-dependent regulation of associations with talin and α-actinin. J. Bio.l Chem. *273*, 33588-33594.

Sanchez-Madrid, F., del Pozo, M. A. (1999). Leukocyte polarization in cell migration and immune interactions. EMBO. J. *18*, 501-511.

Savage, B., Ruggeri, Z. M. (1991). Selective recognition of adhesive sites in surface-bound fibrinogen by glycoprotein IIb-IIIa on nonactivated platelets. J. Biol. Chem. *266,* 11227-11233.

Savage, B., Shattil, S. J., Ruggeri, Z. M. (1992) Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin αIIbβ3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. J. Biol. Chem. *267,* 11300-11306.

Scharffetter-Kochanek, K., Lu, H., Norman, K., van Nood, N., Munoz, F., Grabbe, S., McArthur, M., Lorenzo, I., Kaplan, S., Ley, K., Smith, C. W., Montgomery, C. A., Rich, S., Beaudet, A. L. (1998). Spontaneous skin ulceration and defective T cell function in CD18 null mice. J. Exp. Med. *188*, 119-131.

Schaller, M. D., Otey, C. A., Hildebrand, J. D. and Parsons, J. T. (1995) Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. J. Cell. Biol. *130*, 1181-1187.

Schlaepfer, D. D., Hauck, C. R. and Sieg, D. J. (1999). Signaling through focal adhesion kinase. Prog. Biophys. Mol. Biol. *71*, 435-478.

Schmits, R., Kundig, T. M., Baker, D. M., Shumaker, G., Simard, J. J., Duncan, G., Wakeham, A., Shahinian, A., van der Heiden, A., Bachmann, M. F., Ohashi, P. S., Mak, T. W., Hickstein, D. D. (1996). LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. J. Exp. Med. *183*, 1415-1426.

Schober, J. M., Chen, N., Grzeszkiewicz, T. M., Jovanovic, I., Emeson, E. E., Ugarova, T. P., Ye, R. D., Lau, L. F., Lam, S. C. (2002). Identification of integrin α(M)β(2) as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. Blood *99*, 4457-4465.

Schwartz, M. A. (2001). Integrin signaling revisited. Trends. Cell. Biol. *11*, 466-470.

Schwartz, M. A. and Ginsberg, M. H. (2002). Networks and crosstalk: integrin signalling spreads. Nat. Cell. Biol. *4*, E65-68.

Sengelov, H., Kjeldsen, L., Diamond, M. S., Springer, T. A. and Borregaard, N. (1993). Subcellular localization and dynamics of Mac-l (αMβ2) in human neutrophils. J. Clin. Invest. *92*, 1467-1476.

Shamri, R., Grabovsky, V., Gauguet, J. M., Feigelson, S., Manevich, E., Kolanus, W., Robinson, M. K., Staunton, D. E., von Andrian, U. H.,Alon, R. (2005). Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. Nat. Immunol. *6*, 497-506.

Sharma, C. P., Ezzell, R. M. and Arnaout, M. A. (1995). Direct interaction of filamin (ABP-280) with the β 2-integrin subunit CD18. J. Immunol. *154*, 3461-3470.

Shattil, S. J. (2005). Integrins and Src: dynamic duo of adhesion signaling. Trends .Cell. Biol. *15*, 399-403.

Shi, M., Sundramurthy, K., Liu, B., Tan, S. M., Law, S. K., Lescar, J. (2005). The crystal structure of the plexin-semaphorin-integrin domain/hybrid domain/I-EGF1 segment from the human integrin β2 subunit at 1.8-A resolution. J. Biol. Chem .*280*, 30586-30593.

Shier, P., Otulakowski, G., Ngo, K., Panakos, J., Chourmouzis, E., Christjansen, L., Lau, C. Y., Fung-Leung,W. P. (1996). Impaired immune responses toward alloantigens and tumor cells but normal thymic selection in mice deficient in the β2 integrin leukocyte function-associated antigen-1. J. Immunol. *157*, 5375-5386.

Shier, P., Ngo, K., Fung-Leung, W. P. (1999). Defective CD8+ T cell activation and cytolytic function in the absence of LFA-1 cannot be restored by increased TCR signaling. J. Immunol. *163*, 4826-4832.

Shimaoka, M., Lu, C., Palframan, R. T., von Andrian, U. H., McCormack, A., Takagi, J., Springer, T. A. (2001). Reversibly locking a protein fold in an active conformation with a disulfide bond: integrin αL I domains with high affinity and antagonist activity in vivo. Proc. Natl. Acad. Sci. USA *98*, 6009-6014.

Shimaoka, M., Lu, c., Salas, A., Xiao, T., Takagi, J. and Springer, T. A. (2002a). Stabilizing the integrin α M inserted domain in alternative conformations with a range of engineered disulfide bonds. Proc. Natl. Acad. Sci. USA *99*, 16737-16741.

Shimaoka, M., Takagi, J. and Springer, T. A. (2002b). Conformational regulation of integrin structure and function. Annu. Rev. Biophys. Biomol. Struct. *31*, 485-516.

Shimaoka, M., Xiao, T., Liu, J. H., Yang ,Y., Dong, Y., Jun, C. D., McCormack, A., Zhang, R., Joachimiak, A., Takagi, J., Wang, J. H., Springer, T. A. (2003). Structures of the αL I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. Cell *112*, 99-111.

Simon, D. I., Chen, Z., Xu, H., Li, C. Q., Dong, J., McIntire, L. Y., Ballantyne, C. M., Zhang, L., Furman, M. I., Berndt, M. C. and Lopez, J. A. (2000a). Platelet glycoprotein ibα is a counterreceptor for the leukocyte integrin Mac-l (CD11b/CD18). J. Exp. Med. *192*, 193-204.

Simon, D. I., Wei, Y., Zhang, L., Rao, N. K., Xu, H., Chen, Z., Liu, Q., Rosenberg, S. and Chapman, H. A. (2000b). Identification of a urokinase receptor-integrin interaction site. Promiscuous regulator of integrin function. J. Biol. Chem. *275*, 10228-10234.

Sims, P. J., Ginsberg, M. H., Plow, E. F. and Shattil, S. J. (1991). Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. J. Biol. Chem. *266*, 7345-7352.

Simsek, S., Heyboer, H., de Bruijne-Admiraal, L. G., Goldschmeding, R., Cuijpers, H. T., von dem Borne, A. E. (1993). Glanzmann's thrombasthenia caused by homozygosity for a splice defect that leads to deletion of the first coding exon of the glycoprotein IIIa mRNA. Blood *81*, 2044-2049.

Sligh, J. E., Jr., Ballantyne, C. M., Rich, S. S., Hawkins, H. K., Smith, C. W., Bradley, A. and Beaudet, A. L. (1993). Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA *90,* 8529-8533.

Solomon, E., Palmer, R. W., Hing, S. and Law, S. K. (1988). Regional localization of CD18, the β-subunit of the cell surface adhesion molecule LFA-l, on human chromosome 21 by in situ hybridization. Ann Hum Genet *52*, 123-128.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E. and Sigler, P. B. (1996). Crystal structure of a G-protein β gamma dimer at 2.1A resolution. Nature *379*, 369-374.

Song, G., Lazar, G. A., Kortemme, T., Shimaoka, M., Desjarlais, J. R., Baker, D., Springer, T. A. (2006). Rational design of intercellular adhesion molecule-1 (ICAM-1) variants for antagonizing integrin lymphocyte function-associated antigen-1-dependent adhesion. J. Biol. Chem. *281*, 5042-5049.

Song, G., Yang, Y., Liu, J. H., Casasnovas, J. M., Shimaoka, M., Springer, T. A., Wang, J. H. (2005).An atomic resolution view of ICAM recognition in a complex between the binding domains of ICAM-3 and integrin αLβ2. Proc. Natl. Acad. Sci. USA. 102(9), 3366-3371.

Sonnenberg, A., Linders, C. J., Daams, J. H., Kennel, S. J. (1990). The α 6 β1 (VLA-6) and α 6β4 protein complexes: tissue distribution and biochemical properties. J. Cell Sci. *96*, 207-217.

Soriano, S. G., Lipton, S. A., Wang, Y. F., Xiao, M., Springer, T. A., Gutierrez-Ramos, J. C. and Hickey, P. R. (1996). Intercellular adhesion molecule-1-deficient mice are less susceptible to cerebral ischemia-reperfusion injury. Ann. Neuro.*139*, 618-624.

Spinardi, L., Einheber, S., Cullen, T., Milner, T. A., Giancotti, F. G. (1995). A recombinant tail-less integrin β 4 subunit disrupts hemidesmosomes, but does not suppress α 6 β 4-mediated cell adhesion to laminins. J. Cell Biol. *129*, 473-487.

Springer, T. A. (1990). Leucocyte adhesion to cells. Scand. J. Immunol. *32*, 211-216.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301-314.

Springer, T. A. (1997). Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. Proc. Natl. Acad. Sci. USA *94*, 65-72.

Springer, T. A., Jing, H., Takagi, J. (2000). A novel Ca2+ binding β hairpin loop better resembles integrin sequence motifs than the EF hand. Cell *102*, 275-277.

Springer, T. A., Dustin, M. L., Kishimoto, T. K. and Marlin, S. D. (1987). The lymphocyte function-associated LFA-l, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Annu. Rev. Immunol. *5*, 223-252.

Stacker, S. A. and Springer, T. A. (1991). Leukocyte integrin p150, 95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. J. Immunol. *146*, 648-655.

Staquet, M. J., Peguet, J., Jacquet, C., Dezutter-Dambuyant, C. and Schmitt, D. (1995). Expression of ICAM-3 on human epidermal dendritic cells. Immunobiology *192*, 249-261.

Staunton, D. E., Dustin, M. L. and Springer, T. A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-l homologous to ICAM-l. Nature *339*, 61-64.

Staunton, D. E., Dustin, M. L., Erickson, H. P., Springer, T. A. (1990). The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell *61*, 243-254.

Stefanidakis, M., Bjorklund, M., Thanus, E., Gahmberg, C. G. and Koivunen, E. (2003). Identification of a negatively charged peptide motif within the catalytic domain of progelatinases that mediates binding to leukocyte β2 integrins. J. Biol. Chem. *278*, 34674-34684.

Stefansson, A., Armulik, A., Nilsson, I., Von Heijne, G. and Johansson, S. (2004). Determination of N- and C-terminal Borders of the Transmembrane Domain of Integrin Subunits. J. Biol. Chem. *279*, 21200-21205.

Stewart, M. P., Cabanas, C. and Hogg, N. (1996). T cell adhesion to intercellular adhesion molecule-l (ICAM-l) is controlled by cell spreading and the activation of integrin LFA-1. J. Immunol. *156*, 1810-1817.

Stewart, M. P., McDowall, A. and Hogg, N. (1998). LFA-l-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. J. Cell. Biol. *140,* 699-707.

Stewart, M. P., Hogg, N. (1996). Regulation of leukocyte integrin function: affinity vs. avidity. J. Cell. Biochem. *61*, 554-561

van Strijp, J. A., Russell, D. G., Tuomanen, E., Brown, E. J., Wright, S. D. (1993). Ligand specificity of purified complement receptor type three (CD11b/CD18, α m β 2, Mac-1). Indirect effects of an Arg-Gly-Asp (RGD) sequence. J. Immunol. *151*, 3324-3336

Suen, P. W., Ilic, D., Caveggion, E., Berton, G., Damsky, C. H. and Lowell, C. A. (1999) Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages. J. Cell. Sci. *112*, 4067-78.

Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H. and Calderwood, D. A. (2003). Talin binding to integrin β tails: a final common step in integrin activation. Science *302*, 103-106.

Takagi, J., Isobe, T., Takada, Y. and Saito, Y. (1997). Structural Interlock between Ligand-Binding Site and Stalk-Like Region of ß1 Integrin Revealed by a Monoclonal Antibody Recognizing Conformation-Dependent Epitope. J. Biochem. (Tokyo) *121*, 914-921

Takagi, J., Beglova, N., Yalamanchili, P., Blacklow, S. C. and Springer, T. A. (2001a). Definition of EGF-like, closely interacting modules that bear activation epitopes in integrin β subunits. Proc. Natl. Acad. Sci. USA *98*, 11175-11180.

Takagi, J., DeBottis, D. P., Erickson, H. P. and Springer, T. A. (2002a). The role of the specificity-determining loop of the integrin β subunit I-like domain in autonomous expression, association with the α subunit, and ligand binding. Biochemistry *41*, 4339-4347.

Takagi, J., Erickson, H. P. and Springer, T. A. (2001b). C-terminal opening mimics 'inside-out' activation of integrin α5β1. Nat. Struct. Biol. *8*, 412-416.

Takagi J, Springer TA. (2002). Integrin activation and structural rearrangement. Immunol. Rev. 186, 141-163.

Takagi, J., Petre, B. M., Walz, T. and Springer, T. A. (2002b). Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell *110*, 599-511.

Takagi, J., Strokovich, K., Springer, T. A., Walz, T. (2003). Structure of integrin α5β1 in complex with fibronectin. EMBO. J. 22, 4607-46015.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. Curr. Opin. Cell Biol. *7*, 619-627.

Talamas-Rohana, P., Wright, S. D., Lennartz, M. R. and Russell, D. G. (1990). Lipophosphoglycan from Leishmania mexicana promastigotes binds to members of the CR3, p150, 95 and LFA-1 family of leukocyte integrins. J. Immunol. *144*, 4817-4824.

Tamkun, J. W., DeSimone, D. W., Fonda, D., Patel, R. S., Buck, C., Horwitz, A. F. and Hynes, R. O. (1986). Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell *46*, 271-282.

Tan, S. M., Hyland, R. H., Al-Shamkhani, A., Douglass, W. A., Shaw, J. M., Law, S. K. (2000). Effect of integrin β2 subunit truncations on LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) assembly, surface expression, and function. J. Immunol. *165*, 2574-2581.

Tan, S. M., Robinson, M. K., Drbal, K., van Kooyk, Y., Shaw, J. M., Law, S. K. (2001). The N-terminal region and the mid-region complex of the integrin β2 subunit. J. Biol. Chem. *276*, 36370-36376.

Tan, S. M., Walters, S. E., Mathew, E. C., Robinson, M. K., Drbal, K., Shaw, J. M. and Law, S. K. (2001). Defining the repeating elements in the cysteine-rich region (CRR) of the CD18 integrin β2 subunit. FEBS. Lett. *505*, 27-30.

Tan, S. M. and Law, S. K. (2006). Intgerins: the dynamic cell adhesion molecules. Biomolecular Frontiers *1*, 9-22.

Teunissen, M. B., Koomen, C. W. and Bos, J. D. (1995). Intercellular adhesion molecule-3 (CD50) on human epidermal Langerhans cells participates in T-cell activation. J. Invest. Dermatol. *104*, 995-998.

Thomas, S. M., and Brugge, J. S. (1997) Cellular functions regulated by Src family kinases. Annu. Rev. Cell. Dev. Biol. *13*, 513-609.

Thompson, H. L., Matsushima, K. (1992). Human polymorphonuclear leucocytes stimulated by tumour necrosis factor-α show increased adherence to extracellular matrix proteins which is mediated via the CD11b/18 complex. Clin. Exp. Immunol. *90*, 280-285.

Thompson, J. D., Higgins, D. G., Gibson, T. J. (2004). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic. Acids. Res. *22*, 4673-4680.

Tian, L., Kilgannon, P., Yoshihara, Y., Mori, K., Gallatin, W. M., Carpen, O. and Gahmberg, C. G. (2000a). Binding of T lymphocytes to hippocampal neurons through ICAM-5 (telencephalin) and characterization of its interaction with the leukocyte integrin CD11a/CD18. Eur. J. Immunol. *30*, 810-818.

Tian, L., Nyman, H., Kilgannon, P., Yoshihara, Y., Mori, K., Andersson, L. C., Kaukinen, S., Rauvala, H., Gallatin, W. M. and Gahmberg, C. G. (2000b). Intercellular adhesion molecule-5 induces dendritic outgrowth by homophilic adhesion. J. Cell. Biol. *150,* 243-252.

Tian, L., Yoshihara, Y., Mizuno, T., Mori, K. and Gahrnberg, C. G. (1997). The neuronal glycoprotein telencephalin is a cellular ligand for the CD11a/CD18 leukocyte integrin. J. Immunol. *158*, 928-936.

Tng, E., Tan, S. M., Ranganathan, S., Cheng, M., Law, S. K. (2004) The integrin αLβ2 hybrid domain serves as a link for the propagation of activation signal from its stalk regions to the I-like domain. J. Biol. Chem. *279*, 54334-54339.

Totani, L., Piccoli, A., Manarini, S., Federico, L., Pecce, R., Martelli, N., Cerletti, C., Piccardoni, P., Lowell, C. A., Smyth, S. S., Berton, G. and Evangelista, V. (2006) Src-family kinases mediate an outside-in signal necessary for β_2 integrins to achieve full activation and sustain firm adhesion of polymorphonuclear leucocytes tethered on E-selectin. Biochem. J. *396*, 89-98.

Tuckwell, D. (1999). Evolution ofvon Willebrand factor A (VWA) domains. Biochem. Soc. Trans. *27*, 835-840.

Tuckwell, D. S. and Humphries, M. J. (1997). A structure prediction for the ligandbinding region of the integrin β subunit: Evidence for the presence of a von Willebrand factor A domain. FEBS. Letters. *400*, 297-303.

Tuckwell, D. S., Reid, K. B., Barnes, M. 1., and Humphries, M. J. (1996). The Adomain of integrin α2 binds specifically to a range of collagens but is not a general receptor for the collagenous motif. Eur. J. Biochem. *241*, 732-739.

Ueda, T., Rieu, P., Brayer, J. and Amaout, M. A. (1994). Identification of the complement iC3b binding site in the β2 integrin CR3 (CD11b/CD18). Proc. Natl. Acad. Sci. USA *91*, 10680-10684.

Ugarova, T. P. and Yakubenko, V. P. (2001). Recognition of fibrinogen by leukocyte integrins. Ann. NY. Acad. Sci. *936*, 368-385.

Ulmer, T. S., Yaspan, B., Ginsberg, M. H. and Campbell, I. D. (2001). NMR analysis of structure and dynamics of the cytosolic tails of integrin αIIbβ3 in aqueous solution. Biochemistry *40*, 7498-7508.

Ustinov, V. A. and Plow, E. F. (2002). Delineation of the key amino acids involved in neutrophil inhibitory factor binding to the I-domain supports a mosaic model for the capacity of integrin αMβ2 to recognize multiple ligands. J. Biol. Chem. *277*, 18769-18776.

Van der Vieren, M., Crowe, D. T., Hoekstra, D., Vazeux, R., Hoffman, P. A., Grayson, M. H., Bochner, B. S., Gallatin, W. M. and Staunton, D. E. (1999). The leukocyte integrin αDβ2 binds VCAM-l: evidence for a binding interface between I-domain and VCAM-l. J. Immunol. *163*, 1984-1990.

Van der Vieren, M., Le Trong, H., Wood, C. L., Moore, P. F., St John, T., Staunton, D. E. and Gallatin, W. M. (1995). A novel1eukointegrin, αDβ2, binds preferentially to ICAM-3. Immunity *3*, 683-690.

van Kooyk, Y., Binnerts, M. E., Edwards, C. P., Champe, M., Berman, P. W., Figdor, C. G, Bodary, S. C. (1996). Critical amino acids in the lymphocyte function-associated antigen-1 I domain mediate intercellular adhesion molecule 3 binding and immune function. J. Exp. Med. *183*, 1247-1252.

van Kooyk, Y., van Vliet, S. J. and Figdor, C. G. (1999). The actin cytoskeleton regulates LFA-1 ligand binding through avidity rather than affinity changes. J. Biol. Chem. *274*, 26869-26877.

van Kooyk, Y., Figdor, C. G. (2000). Avidity regulation of integrins: the driving force in leukocyte adhesion. Curr. Opin. Cell. Biol. *12*, 542-547.

van Noesel, C., Miedema, F., Brouwer, M., de Rie, M. A., Aarden, L. A., van Lier, R. A. (1988). Regulatory properties of LFA-1 α and β chains in human T-lymphocyte activation. Nature *333*, 850-852

Van Seventer, G. A., Shimizu, Y., Horgan, K. J., Shaw, S. (1990). The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J. Immunol. *144*, 4579-4586.

Van Strijp, J. A., Russell, D. G., Tuomanen, E., Brown, E. J., and Wright, S. D. (1993). Ligand specificity of purified complement receptor type three (CD11b/CD18, αM β2, Mac-1). Indirect effects of an Arg-Gly-Asp (RGD) sequence. J. Immunol .*151*, 3324-3336.

Vazeux, R., Hoffman, P. A., Tomita, J. K., Dickinson, E. S., Jasman, R. L., St John, T., Gallatin, W. M. (1992). Cloning and characterization of a new intercellular adhesion molecule ICAM-R. Nature 360, 485-488.

Vidal., F., Aberdam, D., Miquel, C. O., Christiano, A. M., Pulkkinen, L., Uitto, J., Ortonne, J.-P. and Meneguzzi, G. (1995). Integrin β4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. Nature.Genet. *10*, 229-234.

Vinogradova, O., Haas, T., Plow, E. F. and Qin, J. (2000) A structural basis for integrin activation by the cytoplasmic tail of the αIIb-subunit. Proc. Natl. Acad. Sci. USA *97*, 1450-1455.

Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., Qin, J. (2002). A structural mechanism of integrin α(IIb)β(3) "inside-out" activation as regulated by its cytoplasmic face. Cell *110,* 587-597.

Vinogradova, O., Vaynberg, J., Kong, X., Haas, T. A., Plow, E. F., and Qin, J. (2004). Membrane-mediated structural transitions at the cytoplasmic face during integrin activation. Proc. Natl. Acad. Sci. USA *101*, 4094-4099.

Vorup-Jensen, T., Ostermeier, C., Shimaoka, M., Hommel, U., Springer, T. A. (2003). Structure and allosteric regulation of the αXβ2 integrin I domain. Proc. Natl. Acad. Sci. USA *100*, 1873-1878.

Voss, L. M., Abraham, R. T., Rhodes, K. H., Schoon, R. A., Leibson, P. J. (1991). Defective T-lymphocyte signal transduction and function in leukocyte adhesion deficiency. J. Clin. Immunol. *11*, 175-183.

Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G. and Sprang, S. R. (1995). The structure of the G protein heterotrimer Gi α1 β1 gamma 2. Cell *83*, 1047-1058.

Walzog, B., Schuppan, D., Heimpel, C., Hafezi-Moghadam, A., Gaehtgens, P., Ley, K. (1995). The leukocyte integrin Mac-1 (CD11b/CD18) contributes to binding of human granulocytes to collagen. Exp. Cell. Res. *218*, 28-38.

Wang, Z., Leisner, T. M., Parise, L. V. (2003). Platelet α2β1 integrin activation: contribution of ligand internalization and the α2-cytoplasmic domain. Blood 102, 1307-1315.

Wary, K. K., Mariotti, A., Zurzolo, C. and Giancotti, F. G. (1998). A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell *94*, 625-634.

Weber, M. (1987). Hemidesmosome defiency of gastro-intestinal mucosa demonstrated in a child with Herlitz syndrome and pyloric atresia. Acta. Derm. Venereol (Stockh) *67*, 360-362.

Weisel, J. W., Nagaswami, C., Vilaire, G. and Bennett, J. S. (1992). Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. J. Biol. Chem. *267*, 16637-16643.

Weitzman, J. B., Wells, C. E., Wright, A. H., Clark, P. A. and Law, S. K. (1991). The gene organisation of the human β2 integrin subunit (CD18). FEBS. Lett. *294*, 97-103.

Welder, C. A., Lee, D. H., Takei, F. (1993). Inhibition of cell adhesion by microspheres coated with recombinant soluble intercellular adhesion molecule-1. J. Immunol. *150*, 2203-2210.

Welch, H., and Maridonneau-Parini, I. (1997). Hck is activated by opsonized zymosan and A23187 in distinct subcellular fractions of human granulocytes. J. Biol. Chem. *272*, 102-109.

Werther, W. A., Gonzalez, T. N., O'Connor, S. J., McCabe, S., Chan, B., Hotaling, T., Champe, M., Fox, J. A., Jardieu, P. M., Berman, P. W., Presta, L. G. (1996). Humanization of an anti-lymphocyte function-associated antigen (LFA)-1 monoclonal antibody and reengineering of the humanized antibody for binding to rhesus LFA-1. J. Immunol. *157*, 4986-4995.

Wencel-Drake, J. D.,Plow, E. F., Kunicki, T. J., Woods, V. L., Keller, D. M. and Ginsberg, M. H. (1986). Localization ofinternal pools ofmembrane glycoproteins involved in platelet adhesive responses. Am. J. Pathol. *124*, 324-334.

Wencel-Drake, J. D., Boudignon-Proudhon, C., Dieter, M. G., Criss, A. B. and Parise, L. V. (1996). Internalization of bound fibrinogen modulates platelet aggregation. Blood *87*, 602-612.

Wilcox, D. A., Paddock, C. M., Lyman, S., Gill, J. C., Newman, P. J. (1995). Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. Role of the GPIIb amino terminus in integrin subunit association. J. Clin. Invest. *95*, 1553-1560.

Williams, M. J., Hughes, P. E., O'Toole, T. E. and Ginsberg, M. H. (1994). The inner world of cell adhesion: integrin cytoplasmic domains. Trends Cell Biol *4*, 109-112.
Wong, D. A., Davis, E. M., LeBeau, M. and Springer, T. A. (1996). Cloning and chromosomal localization of a novel gene-encoding a human β2-integrin α subunit. Gene *171*, 291-294.

Woodside, D. G., Obergfell, A., Leng, L., Wilsbacher, J. L., Miranti, C. K., Brugge, J. S., Shattil, S. J. and Ginsberg, M. H. (2001). Activation of Syk protein tyrosine kinase through interaction with integrin β cytoplasmic domains. Curr. Biol. *11*, 1799-1804.

Woska, J. R. Jr, Morelock, M. M,, Jeanfavre, D. D., Caviness, G. O., Bormann, B. J., Rothlein, R. (1998). Molecular comparison of soluble intercellular adhesion molecule (sICAM)-1 and sICAM-3 binding to lymphocyte function-associated antigen-1. J. Biol. Chem. *273*, 4725-4733.

Wright, S. D. and Jong, M. T. (1986). Adhesion-promoting receptors on human macrophages recognize Escherichia coli by binding to lipopolysaccharide. J. Exp. Med. *164*, 1876-1888.

Wright, S. D., Rao, P. E., Van Voorhis, W. C., Craigmyle, L. S., Iida, K., Talle, M. A., Westberg, E. F., Goldstein, G. and Silverstein, S. C. (1983) Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. Proc. Natl. Acad. Sci. USA *80*, 5699-5703.

Xiao, J. H., Davidson, I., Matthes, H., Garnier, J. M., Chambon, P. (1991). Cloning, expression, and transcriptional properties of the human enhancer factor TEF-1. Cell. *65*, 551-568.

Xiao, T., Takagi, J., Coller, B. S., Wang, J. H., Springer, T. A. (2004). Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature *432*, 59-67.

Xie, C., Shimaoka, M., Xiao, T., Schwab, P., Klickstein, L. B., Springer, T. A. (2004). The integrin α-subunit leg extends at a Ca2+-dependent epitope in the thigh/genu interface upon activation. Proc. Natl. Acad. Sci. USA 101, 15422-15427.

Xie, J., Li, R., Kotovuori, P., Vermot-Desroches, C., Wijdenes, J., Arnaout, M. A., Nortamo, P. and Gahmberg, C. G. (1995). Intercellular adhesion molecule-2 (CD102) binds to the leukocyte integrin CD11b/CD18 through the A domain. J. Immunol. *155*, 3619-3628.

Xiong, J. P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L. and Arnaout, M. A. (2001). Crystal structure of the extracellular segment of integrin αV β3. Science *294*, 339-345.

Xiong, J. P., Stehle, T., Goodman, S. L. and Amin Arnaout, M. (2004). A novel adaptation of the integrin PSI domain revealed from its crystal structure. J. Biol. Chem. *279*, 40252-40254.

Xiong, J. P., Li, R., Essafi, M., Stehle, T., Arnaout, M. A. (2000) An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b A-domain. J. Biol. Chem. *275*, 38762-38767.

Xiong, J. P., Stehle, T., Goodman, S. L. and Arnaout, M. A. (2003a). Integrins, cations and ligands: making the connection. J. Thromb. Haemost. *1*, 1642-1654.

Xiong, J. P., Stehle, T., Goodman, S. L. and Arnaout, M. A. (2003b). New insights into the structural basis of integrin activation. Blood *102*, 1155-1159.

Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S., Arnaout, M. A. (2002). Crystal structure of the extracellular segment of integrin αV β3 in complex with an Arg-Gly-Asp ligand. Science *296*, 151-155.

Xu, H., Gonzalo, J. A., St Pierre, Y., Williams, I. R., Kupper, T. S., Cotran, R. S., Springer, T. A. and Gutierrez-Ramos, J. C. (1994). Leukocytosis and resistance to septic shock in intercellular adhesion molecule I-deficient mice. J. Exp. Med. *180*, 95-109.

Yakubenko, V. P., Lishko, V. K., Lam, S. C. and Ugarova, T. P. (2002). A molecular basis for integrin αMβ2 ligand binding promiscuity. J. Biol. Chem. *277*, 48635-48642.

Yalamanchili, P., Lu, C., Oxvig, C. and Springer, T. A. (2000). Folding and function of I domain-deleted Mac-l and lymphocyte function-associated antigen-1. J. Biol. Chem. *275*, 21877-21882.