Functional characterization of the beta2 integrins

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Functional Characterization of the β 2 Integrins

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at Nanyang Technological University

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at

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Abstract

α L β 2 (CD11a/CD18, LFA-1), α M β 2 (CD11b/CD18, Mac-1) and, α X β 2 (CD11c/CD18, p150, 95) are members of β 2 integrins involved in leukocyte function during immune and inflammatory responses. The first part of this study, the epitope of a β 2-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 α L β 2 affinity states required for ICAM-1 and ICAM-3 binding. Unlike ICAM-1 binding, which required only one activating agent, α L β 2/ICAM-3 binding required both Mg/EGTA and an activating mAb. This suggests that α L β 2 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 α L β 2 and α M β 2. We found that mutation of the isoleucine residue in the proposed "socket for isoleucine" in full-length α L I-domain does not lead to an active α L β 2, although mutation of the equivalent residue in α M I-domain does convey constitutive activity to α M β 2.

Finally, we showed that the src family kinase Hck co-capped selectively with α M β 2 but not α L β 2 or α X β 2. This was disrupted when the α M cytoplasmic tail was substituted with that of α L or α X. Co-capping was recovered by α L or α X cytoplasmic tail truncation or forced separation of the α and β cytoplasmic tails via salt-bridge disruption.

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Publication from other study

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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	deoxyguanine 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-hydroxyethyl]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	phenylmethanesulphonylfluoride
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 mediate 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, $\beta 1$ integrins are expressed on almost all cell types (Zutter and Santoro, 1990), whereas $\beta 2$ 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 $\alpha M\beta 2$ 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 $\alpha 2\beta 1$ 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 $\alpha V\beta 3$ (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	$\alpha_v\beta_3, \alpha_v\beta_5$
<i>Borrelia burgdorferi</i>	$\alpha_{IIb}\beta_3$
<i>Candida albicans</i>	$\alpha_M\beta_2$
Collagens	$\alpha_1\beta_1, \alpha_2\beta_1, \alpha_{11}\beta_1, \alpha_{16}\beta_3$
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	$\alpha_3\beta_1, \alpha_M\beta_2, \alpha_v\beta_3, \alpha_x\beta_2, \alpha_{IIb}\beta_3$
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	$\alpha_1\beta_1, \alpha_2\beta_1, \alpha_6\beta_1, \alpha_7\beta_1, \alpha_6\beta_4, \alpha_v\beta_3$
MAcCAM-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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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 $G\alpha/G\beta$ 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$, 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 α V β 3. (A) Ribbon drawing of crystallized α V β 3 [shown in blue (α V) and red (β 3)]. (B) Model of a straightened extracellular segment of α V β 3. (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 $\alpha V\beta 3$. 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 ($\alpha 1$, $\alpha 2$, $\alpha 10$, $\alpha 11$, 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 $\alpha L\beta 2$ 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 $\alpha 4$ 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 $\alpha\text{L}\beta 2$ and $\alpha\text{M}\beta 2$ (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 or aspartate acid from the ligand completes an octahedral coordination sphere around the metal. A characteristic 'DXSXS' motif (Asp¹⁴⁰-Gly-Ser-Gly-Ser in αM I-domain), located at the $\beta 1$ strand and the loop between $\beta 1$ and $\alpha 1$ was found to contribute to three of these sites. Ser¹⁴², Ser¹⁴⁴ and Thr²⁰⁹ (at the C-terminal end of the $\alpha 3$ - $\alpha 4$ loop) all coordinate directly. Asp¹⁴⁰ interacted via a water molecule, as did Asp²⁴² (at the start of the $\beta 4$ - $\alpha 5$ loop). Mn²⁺ and Mg²⁺ have been shown to enhance ligand binding, whereas high concentration of Ca²⁺ 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 "Mg²⁺ bound" and "Mn²⁺ bound" (Figure 1.5). In the "Mg²⁺ bound" form, two serines and a threonine make electrostatic bonds to the Mg²⁺ via their side chains; two water molecules also interact with Mg²⁺ and two aspartates make indirect contacts via water molecules. The lack of direct electrostatic bonds between aspartates and Mg²⁺ enhanced the electrophilicity of Mg²⁺ (Lee et al., 1995a). In addition, from the crystal structure of the "Mg²⁺ bound" I-domain, the side chain of a glutamate residue from a neighboring I-domain completes the coordination sphere, which is reminiscent of a liganded I-domain. In the "Mn²⁺ bound" form, the thereby direct electrostatic bond to the Thr²⁰⁹ is disrupted whilst a direct electrostatic bond to Asp²⁴² is formed, reducing

FIG 1.5. Structural comparisons of the α Mg²⁺ and Mn²⁺ I-domains. (A) The MIDAS motif in the Mg²⁺ form. (B) The MIDAS motif in the Mn²⁺ 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 “Mn²⁺ 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 Mn²⁺ 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 (β TD). 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 Trp²⁵. It contains four cysteine pairs connected in a 1-4, 2-8, 3-6, 5-7 pattern with the final 8th 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-

	2		3		4		5		6		7		8		EGF1	
	A		B		C		D		E		F		G		H	
1	GPNI	CTTRGV	SS	QQ	LAVSPM	---	---	---	---	---	---	---	---	---	---	---
3	DENR	CLKANA	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	---	QECTKFKV	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	---	NRCKKAPV	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	GLNI	CTSGSA	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	---	GALGGA	---	---	---	---	---	---	---	---	---	---	---	---	---	---
23	MLGS	QPA	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	EDNR	CASSNA	---	---	---	---	---	---	---	---	---	---	---	---	---	---

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 $\alpha V\beta 3$, 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 $\alpha 1$ helix, and the carbonyl oxygen of a methionine at the top of the $\alpha 7$ 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 $\alpha V\beta 3$, a metal ion was found at this site in the $\beta 3$ 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 suggests that the hybrid 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 $\alpha 7$ 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 (β TD). In the crystal structure, weak hydrophobic contacts exist between I-EGF4 and the β TD, indicating that these two domains may be flexibly linked (Arnaout, 2002). In β 3 integrins, β TD is not essential for cell surface expression of integrin dimer, and it contributes to restrain β 3 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 α 2, α 5, α 10 and β 1 and β 8 (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 $\beta 4$, 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 $\alpha\text{IIb}\beta_3$, results in a constitutively active integrin whereas cytoplasmic tail deletion that retain the 'GFFKR' sequence does not activate the integrin $\alpha\text{IIb}\beta_3$ (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 $\alpha\text{L}\beta_2$ clustering and make it constitutively active (van Kooyk et al., 1999). Mutation of either of the phenylalanines in the 'GFFKR' sequence in α_6 prevents its association with β_1 (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 $\alpha\text{IIb}\beta_3$ suggest that the Asp⁷²³ residue from this sequence in β_3 forms a salt bridge with the residue Arg⁹⁵⁵ found in the GFFKR sequence of the αIIb subunit cytoplasmic tail. This stabilizes the association of the membrane proximal regions and maintains $\alpha\text{IIb}\beta_3$ in a low affinity state (Huges et al., 1996). Furthermore, forced association of the cytoplasmic regions of $\alpha\text{L}\beta_2$ or $\alpha\text{M}\beta_2$ 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 α Ib or α 2 tails activate α Ib β 3 or α 2 β 1 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 α L β 2 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 α Ib β 3 integrin in fibroblastoid cells, while in β 2 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 α Ib β 3 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-1 α was reported to activate integrin α L β 2 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 $\alpha\text{IIb}\beta 3$ cytoplasmic tail complex. (A) Backbone ribbon diagram of the αIIb (red) and $\beta 3$ (blue) tail complex. The N-terminal helical structures terminate at P998 and K738 for the αIIb and $\beta 3$ 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 $\beta 3$ is disordered. (B) Electrostatic and (C) hydrophobic interactions in the $\alpha\text{IIb}\beta 3$ interface. Views (i) and (ii) illustrate 180° rotated views of the complex. The electrostatic interface consists of $\alpha\text{IIb}(\text{R995})$ – $\beta 3(\text{H722})$, $\alpha\text{IIb}(\text{R995})$ – $\beta 3(\text{D723})$ and $\alpha\text{IIb}(\text{R995})$ – $\beta 3(\text{E726})$. The hydrophobic interface consists of $\alpha\text{IIb}(\text{V990})$ – $\beta 3(\text{L718})$, $\alpha\text{IIb}(\text{V990})$ – $\beta 3(\text{I719})$, $\alpha\text{IIb}(\text{F992})$ – $\beta 3(\text{I721})$ and $\alpha\text{IIb}(\text{F992})$ – $\beta 3(\text{H722})$. (Adapted from Travis and Humphries, 2003).

Protein	Integrin chain	Binding site	Reference
calreticulin	α -tails	KXGFFKR	Coppolino et al, 1997
filamin	$\beta 1, \beta 2, \beta 7$	724-747	Sharma et al, 1995
α -actinin ,	$\beta 1, \beta 2$	736-746($\beta 2$) 762-774($\beta 1$)	Pavalko et al, 1998 Pavalko and LaRoche 1993
Rack -1	$\beta 1, \beta 2, \beta 3$	724-743($\beta 2$)	Sampath et al, 1998
14-3-3	$\beta 1$	776-790	Lilienthal and Chang, 1998
talin	$\beta 1A, \beta 1D, \beta 2, \beta 3, \beta 7$	membrane distal, several areas	Han et al, 2001 Knezevic et al, 1996
ILK	$\beta 1, \beta 2, \beta 3$	N.D.	Sampath et al, 1998, Pfaff et al, 1998, Calderwood et al, 2001
JAB-1	$\beta 2$	N.D.	Hannigan et al, 1996
FAK	$\beta 1, \beta 2, \beta 3$	membrane proximal β subunit	Bianchi et al, 2000
paxillin	$\beta 1, \beta 3, \alpha 4, \alpha 9$	membrane proximal, α subunit 983-991	Schaller et al, 1995, Tanaka et al, 1996 Liu et al, 1999, Young et al, 2001
Phospholipase C ψ	$\beta 1$	conserved membrane proximal region, α -chain GFFKR	Vossmeier et al, 2002
myosin	$\beta 3$	TyrP $\beta 3$	Jenkins et al, 1998
Shc	$\beta 3$	TyrP $\beta 3$	Cowan et al, 2000
DRAL/FHL2	$\alpha 3A, \alpha 3B, \alpha 7A, \beta$	β :C-tenninal NXXY α :12 aa next to membrane proximal	Wixler et al, 2000
cytohesin 1 and 3	$\beta 2$	723-725	Kolanus et al, 1996, Geier et al, 2000
$\beta 3$ endonexin	$\beta 3$	membrane distal	Shattil et al, 1995
ICAP-1	$\beta 1$	785-799	Chang et al, 1997 Zhang and Hemler, 1999, Degani et al, 2002
PI 3-kinase	$\beta 1$	pTyr-peptide	Johansson et al, 1994
CIB	αIIb	N.D.	Naik et al, 1997, Tsuboi, 2002
IRS-1	$\alpha V \beta 3$	N.D.	Vuori and Ruoslahti, 1994
melusin	$\beta 1A, \beta 1D$	membrane proximal	Brancaccio et al, 1999
MIBP	$\beta 1A, \beta 1B, \beta 1D$	membrane proximal	Li et al, 1999
c-src	$\beta 3$	C-terminal RGT	Arias-Salgado et al, 2005
Fyn	$\beta 3$	N.D.	Arias-Salgado et al, 2003
Hck	$\beta 1, \beta 2, \beta 3$	N.D.	Arias-Salgado et al, 2003
Lyn	$\beta 1, \beta 2, \beta 3$	N.D.	Arias-Salgado et al, 2003
c-Yes	$\beta 1, \beta 2, \beta 3$	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 $\beta 1$ and $\beta 3$ 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 a 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 $\alpha\text{IIb}\beta 3$ with c-Src (de Virgilio *et al.*, 2004). It was shown that c-Src can interact directly and specifically with the integrin $\beta 3$ 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-IB 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 $\beta 2$ 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 $\alpha L\beta 2$ integrin shed from human leukocytes during the cutaneous inflammatory response to the blistering agent cantharidin (Evans et al., 2006). Loss of adhesiveness upon $\alpha L\beta 2$ 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 $\alpha\text{IIb}\beta_3$ 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 integrin 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 $\alpha 7$ 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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 β TD locks the I-like domain in an inactive state in the native structure by preventing tertiary changes in its C-terminal $\alpha 7$ 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 β TD '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 $\alpha V\beta 3$ and $\alpha IIb\beta 3$, 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 represents 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 $\alpha\text{L}\beta 2$ and $\alpha\text{M}\beta 2$, 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 $\beta 6$ - $\alpha 7$ loop and upper portion of $\alpha 7$. These findings suggest that the intermediate conformation of the $\beta 6$ - $\alpha 7$ loop represents a discrete conformational state that could exist in intact integrins, and that the intermediate conformation could represent the second active state of $\alpha\text{L}\beta 2$ 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 $\beta 2$ subunit. Defects in both αIIb and $\beta 3$ have been associated with Glanzmann's Thrombasthenia (GT). Defects of the $\beta 4$ 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 defect	Symptom
Leukocyte adhesion deficiency (LAD) type I	$\beta 2$ -integrin subunit gene defect(Arnaout, 1990).	Recurrent bacterial and fungal infections, gingivitis, impaired wound healing and pus formation, and chronic leukocytosis
Glanzmann's thrombasthenia (GT)	$\alpha IIb\beta 3$ gene defect in either subunit (Bray, 1994).	Impaired platelet aggregation resulting in uncontrolled bleeding.
Junctional epidermolysis bullosa	Altered expression of $\alpha 6\beta 4$: A gene defect in the $\beta 4$ 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 heterogeneous molecular defects in the leukocyte $\beta 2$ 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 $\beta 2$ 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 $\beta 2$ 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). $\beta 2$ null mice exhibit a phenotype closely resembling human LAD-I (Sharfetter-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 $\beta 2$ -integrins in some circumstances. Inactivation of leukocyte integrin α -chains has revealed individual functions for $\alpha L\beta 2$ and $\alpha M\beta 2$ respectively. Neutrophil adhesion to endothelium occurs mainly via $\alpha L\beta 2$, and less of $\alpha M\beta 2$ (Ding et al., 1999). Leukocytes lacking $\alpha L\beta 2$ can still respond to viral infection, but T cell proliferation and cytotoxicity

in response to alloantigen are severely impaired, implicating additional roles for $\alpha L\beta 2$ (Schmits et al., 1996; Shier et al., 1996). $\alpha L\beta 2$ 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). $\alpha M\beta 2$ 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 $\alpha IIb\beta 3$, which functions as a receptor for fibrinogen, von Willebrand factor and perhaps other ligands (Plow et al., 2000). $\alpha IIb\beta 3$ 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 $\alpha\text{IIb}\beta 3$ on the surface platelets. The molecular defect responsible for the disease may either be on the α or the β subunit, resulting in impaired $\alpha\text{IIb}\beta 3$ 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 $\alpha\text{IIb}\beta 3$ are found on the cell surface, but functional analysis revealed an inability to bind ligand. This point mutation lies in the $\beta 3$ -MIDAS motif recognized as important in ligand binding (Bajt et al., 1992; Goodman and Bajt, 1996).

Molecular analysis of other GT $\beta 3$ 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 $\alpha 6\beta 4$ has been shown in several PA-JEB patients (Philips et al., 1994) whereas in one patient no $\beta 4$ was detectable at all (Gil et al., 1994). In support of this finding, mutations in the $\beta 4$ gene in PA-JEB have been identified that result in a reduced expression of the $\alpha 6\beta 4$ integrin (Vidal et al., 1995).

1.7 The $\beta 2$ integrins

The $\beta 2$ integrin subfamily has four members: $\alpha L\beta 2$ (CD11a/CD18, Leukocyte Function-associated Antigen -1 or LFA-1), $\alpha M\beta 2$ (CD11b/CD18, Complement Receptor 3 or Mac-1), $\alpha X\beta 2$ (CD11c/CD18, CR4 or p150, 95), and $\alpha D\beta 2$ (CD11d/CD18). The expression of these integrins is restricted to leukocytes. The gene encoding $\beta 2$ 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).

$\beta 2$ 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, $\alpha 6 \beta 4$ 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 $\alpha \text{IIb} \beta 3$ on unstimulated platelets adhere to immobilized fibrinogen (Savage and Ruggeri 1991).

1.7.1 $\alpha \text{L} \beta 2$

$\alpha \text{L} \beta 2$ is expressed on all leukocytes (Barclay, 1997). Anti- $\alpha \text{L} \beta 2$ 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 $\alpha \text{L} \beta 2$ 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 Siahhaan, 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 α L β 2 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 α L β 2 than the transmembrane-lacking monomeric form (Miller et al., 1995; Reilly et al., 1995). Through interactions with α L β 2 and α M β 2, 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 α L β 2 (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 Fougères 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 α L β 2 (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 (Hermans et al., 2000). Selective binding of ICAM-4 to different integrins may be important for a variety of normal erythrocyte functions and is also relevant to the pathology of thrombotic disorders and vasoocclusive events in sickle cell diseases (Mankelw 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 α L β 2 (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 $\alpha M\beta 2$

$\alpha M\beta 2$ 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 $\alpha M\beta 2$ (Yakubenko et al., 2002).

$\alpha M\beta 2$ 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). $\alpha M\beta 2$ 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 $\alpha M\beta 2$ 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 $\alpha M\beta 2$ 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, $\alpha M\beta 2$ 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 $\alpha M\beta 2$ 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 $\alpha M\beta 2$ mediates cell spreading on ligand-coated surfaces, led to the finding that $\alpha M\beta 2$ binds denatured protein such as denatured BSA (Davis, 1992).

Despite the known ligand binding promiscuity of $\alpha M\beta 2$, 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 $\beta 2$ 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 $\alpha M\beta 2$ (Koivunen et al., 2001).

It has been suggested that the ability of $\alpha M\beta 2$ 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 (Lys²⁴⁵-Arg²⁶¹) might constitute a consensus binding region (Yakubenko et al., 2002).

1.7.3 $\alpha X\beta 2$

$\alpha X\beta 2$ is expressed mainly on myeloid cells, with high levels on tissue macrophages. Its tissue distribution overlaps with that of $\alpha M\beta 2$ 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 $\alpha D\beta 2$

$\alpha D\beta 2$ is expressed predominantly on myelomonocytic cells, particularly on macrophage foam cells and splenic red pulp macrophages (Notu, 2002). $\alpha D\beta 2$ is the most recently discovered member of the $\beta 2$ 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). α D β 2 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 α L β 2 to different ICAMs has been observed previously (Buckley et al., 1997; Al-Shamkhani and Law, 1998). However, the relationship between α L β 2 affinity states and its conformations is unclear, because structural data of full length integrin was lacking at that time. The resolution of α V β 3 crystal structure (Xiong et al., 2001) allows us to understand how α L β 2 affinity states are linked to its conformation. Hence, we hypothesize that the movement of β 2 hybrid domain is involved in the α L β 2 transition between different activation states. This section of the study made use of an activation reporter mAb MEM148 to demonstrate that α L β 2 hybrid domain movement is involved in ICAM-1 and ICAM-3 binding.

1.8.2 Differential activation of α L β 2 and α M β 2 I-domain

Nine integrin α subunits contain an I-domain which is involved in ligand binding. Many single-residue mutations were introduced into the α L 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 α L β 2 and α M β 2 in terms of ligand binding (Huth et al., 2000; Xiong et al., 2000). However, it was also reported that systematic

truncation of integrin $\beta 2$ leads to differential activation of $\alpha L\beta 2$ and $\alpha M\beta 2$ 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 $\alpha L\beta 2$ and $\alpha M\beta 2$ 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 $\beta 2$ 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 $\alpha M\beta 2$.

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 α L)	From Prof. LAW SK (School of Biological Sciences, NTU)
CD11b (integrin α M)	From Prof. LAW SK (School of Biological Sciences, NTU)
CD11c (integrin α X)	From Prof. LAW SK (School of Biological Sciences, NTU)
CD18 (integrin β 2)	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- α L	From Prof. McMichael (John Radcliffe Hospital, Oxford, UK)
LPM19c	anti- α M	From Prof. LAW SK (School of Biological Sciences, NTU)
KB43	anti- α X	From K. Pulford (LRF Diagnostic Unit, Oxford, UK)
MHM23	anti- β 2 heterodimer dependent	From Prof. McMichael (John Radcliffe Hospital, Oxford, UK)
1B4	anti- β 2 heterodimer dependent	From Prof. LAW SK (School of Biological Sciences, NTU)
MEM48	anti- β 2 activating mAb	From Prof. Horejsi (Prague, Czech Republic)
H52	anti- β 2	From Prof. LAW SK (School of Biological Sciences, NTU)
7E4	anti- β 2	From Beckman Instruments
MEM148	anti- β 2	From Prof. Horejsi (Prague, Czech Republic)
KIM127	anti- β 2	From Dr. Robinson (UCB, CellTech, UK)
KIM185	anti- β 2 activating mAb	From Dr. Robinson (UCB, CellTech, UK)
KIM202	anti- β 2	From Dr. Robinson (UCB, CellTech, UK)

2.1.6 *Ligands for cell binding analysis*

iC3b	From Dr. A W Dodds (MRC Immunochemistry 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 ₂ O, 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 ₂ O
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 OD₂₆₀ of 1. The OD₂₈₀ was also read and the ratio of OD₂₆₀/OD₂₈₀ was calculated to estimate the purity of the DNA solutions. An OD₂₆₀/OD₂₈₀ 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 transilluminator 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 QIAquick 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 volume. 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 DH5 α 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 OD₅₅₀ 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 CaCl₂ and combined into a single bottle. Cells were resuspended again in 250 ml ice cold 0.1 M CaCl₂ and centrifuged at 8000 rpm for 8 min at 4°C. Finally the pellet was resuspended in 43 ml ice cold 0.1 M CaCl₂ in ddH₂O with 7 ml sterile glycerol. Competent cells were distributed into convenient aliquots (0.2 ml) in cold microfuge tubes. Cells were stored 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 μ l 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 μ l 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 T_m of the two primers, and finally an extension at 72°C for an appropriate time (normally 1 min per kb). T_m 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 volume 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% CO₂ 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 5x10⁶ 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 cm² 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% CO₂ 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 25 μ M chloroquine was added to replace the old media one hour prior to the transfection. 10 μ g DNA was added to ddH₂O (1095 μ l total) in a 15 ml sterile tube, followed by adding in 155 μ l 2M CaCl₂. 1250 μ l of 2xHBS (8.0g NaCl, 0.37g KCl, 201mg Na₂HPO₄•7H₂O, 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 μ l of growth media without antibiotics to achieve a 70-90% confluency at the time of transfection. On the next day, 1 μ l Lipofectamine™ 2000 and 0.8 μ g DNA were added to two different tubes which contained 50 μ l 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 10⁶ 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-HCl 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 sephrose (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 minigel 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 electrophoresis 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-HCl, 95 mM Glycine, 10 % (v/v) 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 transferred 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 (2x10⁴ 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 $\alpha\text{L}\beta 2$ -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 $\mu\text{g/ml}$) were used. For blocking studies, function-blocking αL -specific mAb MHM24 (10 $\mu\text{g/ml}$) 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 redTM-X succinimidyl ester labeled zymosan

Zymosan particles (Sigma) were labeled with rhodamine redTM-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 redTM-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 $\alpha\text{L}\beta 2$ -specific MHM24; $\alpha\text{M}\beta 2$ -specific LPM19c; $\alpha\text{X}\beta 2$ -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 $\alpha\text{M}\beta 2$, Hck, Lck, and CD4, the surface antigen antibodies used were mAbs LPM19c (anti $\alpha\text{M}\beta 2$) 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 α M β 2 mutants having β 2 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 $\beta 2$ cDNA in J8.1E (Douglass et al., 1998) was used as a template for construction of the $\beta 2$ in mammalian expression vector pcDNA3.0 (Invitrogen) (Figure 2.1). KpnI and SpeI were used to digest $\beta 2$ 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 α L or α X cytoplasmic tail respectively. Restriction enzymes SspBI and XbaI were used for digestion. Because wild type α L cDNA in pcDNA3.0 has two SspBI cut sites, the one at the position of 1652 in α L cDNA was removed by point mutation before digestion with SspBI and XbaI (Figure 2.3). Thereafter, cytoplasmic tails of α L and α X were digested with SspBI and XbaI and ligated into wild type α M 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. α L I331G FL or α L I331G I-domain
 F 5' CTGCAGAAGAAGGGCTATGTCATTGTC 3'
 R 5' GACAATGACATAGCCCTTCTTCTGCAG 3'
2. α M I332G FL or α M I332G I-domain
 F 5' CTTGCGGAGAAAGGGCTTTGCGATCGAAG 3'
 R 5' CTTGATCGCAAAGCCCTTCTCCCGAAG 3'
3. β 2 D709R
 F 5' CTGATCCACCTGAGCCGCCTCCGGGAGTACAGG 3'
 R 5' CCTGTACTCCCGGAGGCGGCTCAGGTGGATCAG 3'
4. β 2 N741*
 F 5' CCACGACGGTCATGTAGCCCAAGTTTGCTGAG 3'
 R 5' CTCAGCAAACCTGGGCTACATGACCGTCGTGG 5'
5. β 2 N727*
 F 5' AAGTCCCAGTGGAAC TAGGATAATCCCCTTTTC 3'
 R 5' GAAAAGGGGATTATCCTAGTCCACTGGGACTT 5'
6. β 2 K702*
 F 5' GCTGGTCATCTGGTAGGCTCTGATCCAC 3'
 R 5' GTGGATCAGAGCCTACCAGATGACCAGC 5'
7. β 2 S734*
 F 5' CCCCTTTTCAAGTGAGCCACCACGACGGTC 3'
 R 5' GACCGTCGTGGTGGCTCACTTGAAAAGGGG 3'

8. α MLP1110*

F 5' CCGAATGGAATCCCTTGAGAAAGACTCTGAGCAGCTG 3'
R 5' CAGCTGCTCAGAGTCTTCTCAAGGGATTCCATTTCGG 3'

9. α MXN1132*

F 5' CAAATTGCCCCAGAAACTGAACACAGACCCCCAG 3'
R 5' GCTGGGGGTCTGTGTTCAAGTTTCTGGGGCAATTTG 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 $\beta 2$ cDNA in J8.1E (Douglass et al., 1998) was used as a template for construction of the $\beta 2$ in mammalian expression vector pcDNA3.0. KpnI/SpeI was used to digest $\beta 2$ 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 α L and α M I- domain.

FIG 2.3 Schematic illustration of plasmid construction of α ML and α MX chimera. The α ML and α MX cytoplasmic tail chimeras were generated by exchanging the α M cytoplasmic tail with α L or α X cytoplasmic tail respectively. Restriction enzymes SspBI and XbaI were used for digestion. There are two SspBI sites on α L cDNA, the SspBI* site at the position 1652 was removed by point mutation before digestion.

Chapter Three: Different activation states of α L β 2 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 α Ib β 3 headpiece with that of unliganded-closed α V β 3 revealed a $\sim 10\text{\AA}$ 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 α 5 β 1 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 α Ib β 3, α V β 3, and α 5 β 1 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 α IIb β 3 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 α 4 β 1 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 α 4 β 1 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 α L β 2, it has been reported that α L β 2 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 α L β 2 I-domain (Binnerts et al., 1994; Binnerts et al., 1999). However, based on crystal structure of an engineered high affinity α L 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 α L I-domain. Ligand ICAM-1 exhibits higher affinity for purified α L β 2 from T cells than ICAM-3 (de Fougerolles et al., 1992). Prior exposure of α L β 2 to ICAM-1 increased

its binding to ICAM-3 (Buckley et al., 1997). Soluble ICAM (sICAM)-3 binds to $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ adhesion to ICAM-3 while only one activating mAb was required for $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ affinity states are required to bind to different ICAM ligands. Many inferences on $\alpha\text{L}\beta 2$ 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 $\beta 2$ subunit or Mg^{2+} /EGTA-activated $\alpha\text{L}\beta 2$ but not resting $\alpha\text{L}\beta 2$ (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 $\alpha\text{L}\beta 2$ 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 $\beta 2$ hybrid domain. It is not exposed in resting integrin $\alpha\text{L}\beta 2$ 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 $\beta 2$ human/mouse "knock-out" mutants generated by colleague Dr. Tng (Tng et al., 2004). These human integrin $\beta 2$ mutants have their residues replaced by corresponding mouse $\beta 2$ residues at positions where they differ in the mid-region (Figure 3.1B). COS-7 cells, which can express $\beta 2$ integrin in the absence of the α subunit (Tan et al., 2001) were transfected with the $\beta 2$ 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 Leu⁵³⁴, Phe⁵³⁶, and His⁵⁴³ of human integrin $\beta 2$ 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 $\beta 2_{Hu}$ (H370S/R371I) or $\beta 2_{Hu}$ (N372G/Q373K) as compared with $\beta 2_{Hu}$ (wt). The $\beta 2_{Hu}$ (P374S) variant showed significant (>90%) abrogation of MEM148 epitope expression.

Next, an integrin $\beta 2$ human/mouse chimera ($\beta 2_{Hu/MoA}$) (Figure 3.1A) in which Met²²-Asn⁵⁶² of human integrin $\beta 2$ is replaced by the corresponding region from mouse integrin $\beta 2$ (Tng et al., 2004) was used to generate two "knock-in" mutants. The $\beta 2_{Hu/MoA}$ (S374P) has the Ser³⁷⁴ of the mouse mid-region substituted by the corresponding human Pro residue. The $\beta 2_{Hu/MoA}$ (A368V-S374P) has the segment Ala³⁶⁸-Ser³⁷⁴ of the mouse mid-region replaced by the corresponding human segment Val³⁶⁸-Pro³⁷⁴. In this case, mAb KIM185, which maps to integrin $\beta 2$ 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

FIG 3.1. (A) Linear organization of the human integrin β_2 subunit. The human integrin β_2 subunit is shown with the domains and regions indicated. The mouse fragment in human/mouse chimera β_2 Hu/MoA is shaded in black. TM, transmembrane domain. (B) Sequence alignment of human and mouse integrin β_2 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 Cys⁴²⁵ involved in the long range disulfide bond indicated by an asterisk (*).

FIG 3.2. Epitope expression of mAb MEM148 on β_2 knock-out and knock-in mutants. (A) β_2 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 β_2 which is constitutively exposed) for each mutant. The mAb MEM48 maps to the C-terminal region of human β_2 subunit and was used as a reference antibody in this case. (B) β_2 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 β_2 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 $\beta_{2\text{Hu/MoA}}$. Epitope expression was shown based on expression index (EI).EI was calculated by % percentage cells gated positive multiply by geo-mean fluorescence

$\beta 2_{\text{Hu/MoA}}$. The expression of MEM148 epitope on $\beta 2_{\text{Hu/MoA}}$ (S374P) was low as compared with $\beta 2_{\text{Hu}}$ (wt) (Fig. 3.2B). However, transfectant bearing $\beta 2_{\text{Hu/MoA}}$ (A368V-S374P) fully restored the epitope of MEM148. Thus, it suggests that although human integrin $\beta 2$ Pro³⁷⁴ is a critical residue, other residues, His³⁷⁰, Arg³⁷¹, Asn³⁷², Gln³⁷³ are also required for the effective presentation of MEM148 epitope.

3.2.2 Model of $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ was generated by MODELLER (Tng et al., 2004) using $\alpha\text{V}\beta 3$ 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, $\beta 2$ PSI, I-EGFs, and β -tail domains were not included. The critical residue Pro³⁷⁴ of the mAb MEM148 epitope resides on the surface of the hybrid domain facing the αL subunit. In contrast, the epitope of mAb 7E4 (Val³⁸⁵) (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 $\alpha\text{L}\beta 2$; because if resting $\alpha\text{L}\beta 2$ is in a severely bent conformation as proposed for $\alpha\text{V}\beta 3$ (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 $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$. (A) The model of $\alpha\text{L}\beta 2$ was generated by the MODELLER using the $\alpha\text{V}\beta 3$ 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 $\beta 2$ subunit are shown. The critical determinants Pro³⁷⁴ (yellow) for mAb MEM148 and Val³⁸⁵ (red) for mAb 7E4 (Tng et al., 2004) are indicated. (B) Surface representation with different rotation of the model. Pro³⁷⁴ (yellow) is located in the interface between the αL and $\beta 2$ headpiece and between the $\alpha\text{L}\beta 2$ headpiece and tailpiece interface. Val³⁸⁵ (red) is exposed. (C) Diagram illustrating the masking of Pro³⁷⁴ (*) in resting $\alpha\text{L}\beta 2$ 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 α L β 2 from one affinity state to another is reversible*

The mAb MEM148 does not bind to α L β 2 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 α L β 2 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 β 2 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 α L-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 α L β 2 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. α L-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 α L-specific function-blocking mAb MHM24. Together, our data suggest that reversion of Mg^{2+} /EGTA-activated α L β 2 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 α L β 2·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 α L β 2 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 $\beta 2$ 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 1×10^6 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 $\beta 2$, 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 $\alpha L\beta 2$ when cells were treated with Mg^{2+} /EGTA but not with PMA. No corresponding protein bands were detected using irrelevant mAb, but $\alpha L\beta 2$ bands were detected under all conditions using the heterodimer dependent $\beta 2$ -specific mAb MHM23. Truncated $\beta 2$ (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 $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$, although it was reported to promote $\alpha\text{L}\beta 2$ ligand binding (Tng et al., 2004; Rothlein et al., 1986). It is possible that PMA promotes an active $\alpha\text{L}\beta 2$ conformation that is different from that triggered by $\text{Mg}^{2+}/\text{EGTA}$ for example. Alternatively, PMA increases the lateral mobility of $\alpha\text{L}\beta 2$ on plasma membrane, allowing receptor clustering, which effects ligand binding (Kucik et al., 1996).

FIG 3.5. Effect of PMA on MEM148 staining to $\alpha\text{L}\beta 2$. MOLT-4 cells were surface-biotinylated. Labeled cells were treated with $\text{Mg}^{2+}/\text{EGTA}$ 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. $\text{Mg}^{2+}/\text{EGTA}$ (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 $\alpha\text{L}\beta 2$ to immobilized ICAM-1 and ICAM-3 requires different affinity states

To understand the molecular basis of $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ by either Mg^{2+} /EGTA or one of the activating mAbs may convert $\alpha\text{L}\beta 2$ into an intermediate affinity state. Promotion of this population of $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ 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 $\alpha L\beta 2$ and ICAM-3. Noteworthy, the combination of Mg^{2+} /EGTA with mAb MEM148 only had a marginal effect on $\alpha L\beta 2$ /ICAM-3 binding as compared with other mAbs. This could not be due to insufficient ICAM-3 ligand coated on the well (1 ng/ μ l) 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 $\beta 2$ hybrid domain is permissible on Mg^{2+} /EGTA-activated but not resting $\alpha L\beta 2$. The ICAM-3 binding data suggest that Mg^{2+} /EGTA-activated $\alpha L\beta 2$ adopts a conformation in which the epitope of MEM148 becomes exposed. The quaternary conformation adopted by Mg^{2+} /EGTA-activated $\alpha L\beta 2$ may be similar to one of the intermediate affinity states proposed for $\beta 3$ integrins (Xiao et al., 2004).

3.2.5 Binding of $\alpha L\beta 2$ to soluble ICAM-1 and ICAM-3

If resting $\alpha L\beta 2$ assumes a severely bent conformation like $\alpha V\beta 3$, 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 α L-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/ μ l) 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 $\alpha L\beta 2$ -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 $\alpha L\beta 2$ 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 multiply by geo-mean fluorescence. Binding was specific because the addition of α L-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 $\alpha\text{L}\beta 2$ may be analogous to the integrin $\alpha\text{IIb}\beta 3$ 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 $\beta 2$ I-like domain of integrin $\alpha\text{L}\beta 2$ allosterically regulates ligand binding of the αL I-domain through binding of the $\beta 2$ metal ion-dependent adhesion site (MIDAS) to Glu³¹⁰, found in the last helix of the αL I-domain. This was shown by replacing αL Glu³¹⁰ with Cys and either $\beta 2$ MIDAS Ala²¹⁰ or Tyr¹¹⁵ to Cys, allowing a disulfide connection between the two domains (Yang et al., 2004). Downward displacement of this helix by $\beta 2$ 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 $\alpha\text{L}\beta 2$ hybrid domain maps the epitope of mAb 7E4 to Val³⁸⁵ and demonstrates the importance of the hybrid domain for the transfer of activation signal from $\beta 2$ membrane proximal region to the αL I-domain (Tng et al., 2004). Preventing the movement of integrin $\alpha 5\beta 1$ hybrid domain away from its $\beta 1$ I-like domain by mAb SG/19 maintains $\alpha 5\beta 1$ 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 Pro³⁷⁴ and residues His³⁷⁰-Gln³⁷³ of the integrin $\beta 2$ hybrid domain. Distinct from the exposed epitope Val³⁸⁵ recognized by mAb 7E4, these residues are hidden in the $\alpha\text{L}\beta 2$ model using the bent $\alpha\text{V}\beta 3$ 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 $\beta 1$ hybrid domain that are partly masked by integrin $\alpha 5$ β -propeller (Mould et al., 2003). Accessibility of mAb MEM148 to these epitope(s) would, therefore, be sterically unfavorable when $\alpha L\beta 2$ adopts a bent and closed headpiece conformation. It accounts for the lack of mAb MEM148 reactivity to unactivated $\alpha L\beta 2$ observed previously (Tan et al., 2001; Drbal et al., 2001). However, when exposed to Mg^{2+} /EGTA, $\alpha L\beta 2$ can undergo a conformational change possibly via unbending, thereby exposing the epitope of MEM148.

Crystal structures of the integrins $\alpha I\beta 3$ and $\alpha V\beta 3$ with ligand mimetics, electron microscopy images of soluble integrin $\alpha V\beta 3$ with cyclo-RGDfV peptide in the presence of Mn^{2+} or Ca^{2+} , and that of integrin $\alpha 5\beta 1$ 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 $\alpha L\beta 2$ binding to ICAM-1 and ICAM-3 exhibit different affinities (de Fougères 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 $\alpha L\beta 2$ /ICAM-1 binding as compared with Mg^{2+} /EGTA alone. The observation could be attributed to the disruption of the extensive contacts between the $\alpha L\beta 2$ headpiece-tailpiece interface when

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

From our $\alpha L\beta 2$ /ICAM-3 binding data, two activating agents are required to promote binding. Collectively, this implies that the conformation adopted by $\alpha L\beta 2$ in the presence of Mg^{2+} /EGTA, mAb KIM185, or MEM48 represents an affinity state(s) capable of ICAM-1 but not ICAM-3 binding. The $\alpha L\beta 2$ 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 $\alpha L\beta 2$ /ICAM-1 binding and promote $\alpha L\beta 2$ /ICAM-3 binding. Indeed, a previous study showed that the mAb 7E4 binds to $\alpha L\beta 2$ hybrid domain and prevents $\alpha L\beta 2$ activation by mAb KIM185 but not Mg^{2+} /EGTA (Tng et al., 2004). The Mg /EGTA-activated $\alpha L\beta 2$ conformer has its hybrid domain re-oriented, as evidenced by its reactivity with MEM148 described herein. However, such re-positioning as a result of Mg^{2+} /EGTA activation still did not favor $\alpha L\beta 2$ binding of ICAM-3. We also found that the combination of Mg^{2+} /EGTA with mAb MEM148 did not promote effective $\alpha L\beta 2$ /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 $\alpha L\beta 2$, 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 $\alpha L\beta 2$ by binding to its re-positioned hybrid domain.

It was hypothesized that other intermediates may exist during $\alpha V\beta 3$ 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 $\alpha L\beta 2$ adopts a conformation akin to one of these intermediates. This may explain the reactivity of mAb MEM148 to Mg^{2+} /EGTA-activated $\alpha L\beta 2$. The Mg^{2+} /EGTA-activated $\alpha L\beta 2$ "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 $\alpha L\beta 2$, which was beforehand activated by Mg^{2+} /EGTA. EDTA does not directly affect these mAbs because staining could be detected for single integrin $\beta 2$ 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 $\beta 2$ hybrid domain and I-EGF2 movement during $\alpha L\beta 2$ 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 (Leu¹⁶¹-Cys/Phe²⁹⁹-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 $\beta 6$ - $\alpha 7$ 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 $\alpha 7$ helix (Jin et al., 2004), which is allosterically regulated by the $\beta 2$ I-like domain. It was demonstrated that Mg^{2+} /EGTA-treated $\alpha L\beta 2$ could be further activated to bind ICAM-1 by the addition of activating mAb KIM185 or MEM48. Mg^{2+} /EGTA treatment would have converted $\alpha L\beta 2$

into an intermediate affinity state. The addition of these mAbs would disrupt the interface between the α L and β 2 leg pieces with eventual propagation of conformational change to the hybrid domain and β 2 I-like domain. This allows further displacement of the I-domain α 7 helix transforming the α L β 2 from an intermediate to a high affinity state for ICAM-1 binding.

Why does binding to ICAM-1 and ICAM-3 require different α L β 2 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 α L β 2 also share similarity; Glu³⁴ and Gln⁷³ in IgSF domain 1 (D1) of ICAM-1 (Staunton et al., 1990) and the corresponding residues Glu³⁷ and Gln⁷⁵ in D1 of ICAM-3 (Klickstein et al., 1996; Sadhu et al., 1994; Holness et al., 1995). However, Lys³⁹, Met⁶⁴, Tyr⁶⁶, and Asn⁶⁸ of ICAM-1 are important for binding to α L I-domain (Shimaoka et al., 2003), but Asn²³, Ser²⁵, and Phe⁵⁴ 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 α L I-domain have also been determined. Residues surrounding the α L 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 α L 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 α L I-domain, whereas its N

terminus is located at its bottom. Recent crystal data of an engineered high affinity α L I-domain in complex with ICAM-3 D1 suggest that both ICAM-1 and ICAM-3 share a common docking mode with α L 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 α L β 2). However, the exact nature and differences of the interactions between intact ICAMs and α L β 2 require further investigations.

In conclusion, this study using β 2 hybrid domain-specific reporter mAb MEM148 suggests the requirement of different affinity states for integrin α L β 2 binding to ICAM-1 and ICAM-3. A proposed model explaining these observations would be the displacement of the β 2 hybrid domain in Mg^{2+} /EGTA-treated α L β 2, which renders α L β 2 capable of ICAM-1 binding. However, this movement did not generate an α L β 2 conformer that could bind to ICAM-3 effectively, which requires additional activation signal. It will be interesting in future studies to test the proposed model of α L β 2 activation by structural analyses of intact α L β 2 with ICAMs under different conditions.

Chapter Four: Differential activation of α L β 2 and α M β 2 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 β 2, 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 α L (Qu et al., 1995), α M (Lee et al., 1995a; Lee et al., 1995b), and α X (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 α M 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 α L 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 α M 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 α M β 2 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 α M β 2. 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 α X integrin subunit (Vorup-Jensen et al., 2003).

It was reported previously that systematic truncation of integrin β 2 leads to differential activation of α L β 2 and α M β 2 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 α L, α M or the β 2 subunits must account for the differences observed in α L β 2 and α M β 2 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 α L and α M, as well as the isolated I-domain of α L and α M. The ligand binding activities of these mutants were analyzed.

FIG 4.1. Sequence alignment of integrin α subunit I-domains. (A) Alignment of $\alpha 7$ helices of all known human integrin α I-domains. Amino acid numbers (aa no.) of the first residues in each $\alpha 7$ helix are indicated in italics (in all cases, the initiating methionine is numbered 1). The invariant isoleucine in the $\alpha 7$ helices (Ile³³¹ in the αL subunit and Ile³³² in the αM subunit) are shown in bold. (B) Alignment of the I-domains of integrin αL and αM subunits. The Ile³³¹ in αL and Ile³³² 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 α M 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 α M or α M(I332G) in combination with wild-type β 2. Cell surface expression of the wild-type and mutant α M β 2 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 α M subunit yielded α M β 2 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 α L subunit has minimal effect on α L β 2 binding to ligand*

The experiment with the mutation in α L β 2 was repeated. 293T cells were transfected with full-length wild-type α L or α L(I331G) in combination with wild-type β 2. Cell surface expression of wild-type α L β 2 was comparable to the α L(I331G) β 2 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 α L-specific mAb MHM24.

FIG 4.2. α M I332G variant is confirmed to be constitutively active in binding to α M ligand. (A) Cell surface expression of wild-type (wt) α M β 2 and α M(I332G) β 2 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 α M subunit, heterodimer, and the β 2 subunit respectively (solid lines). (B) Full-length α M β 2 wt and α M(I332G) β 2 mutant binding to BSA. Binding of α M(I332G) β 2 was 2-fold higher than that of wild-type α M β 2 in the absence of any activating antibodies. Binding of the wild-type α M β 2 was increased in the presence of mAbs KIM185 or MEM48 but α M(I332G) β 2 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) $\alpha L\beta 2$ and $\alpha L(I331G)\beta 2$ 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 $\beta 2$ subunit, respectively (solid lines). (B) Full-length $\alpha L\beta 2$ wt and $\alpha L(I331G)\beta 2$ 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 α L I-domain wild-type and mutant I331G show constitutive ligand binding activity

These differences in the activity of α M β 2 and α L β 2 isoleucine mutants prompted us to investigate further the activation of the isolated α L I-domain. α L I-domain cDNA coding for residues Glu¹⁴⁹ to Asp³⁴¹ (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, α L 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 α L 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 Mg²⁺/EGTA (Figure 4.4B). Similar binding profiles were observed for pDL-I331G. In contrast, intact full-length α L β 2 could bind to ICAM-1 only in the presence of Mg²⁺/EGTA. Furthermore, addition of Mg²⁺/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 α L I-domain and mutant α L 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 α L I-domain. (B) Isolated α L I-domain binding to ligand ICAM-1. pDisplay α L 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 α L β 2 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/ μ l (50 μ l is added in each well). In the absence of Mg^{2+} /EGTA activation, wild-type $\alpha L\beta 2$ 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 $\alpha L\beta 2$ 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 α M I-domain and mutant I332G show similar binding activity to BSA as compared to full length α M β 2

We furthered our study on binding property of isolated α M I-domain with or without isoleucine to glycine mutation. The cloning of isolated α M I-domain was similar to the generation of isolated α L I-domain but α M cDNA was used instead. In addition, α M I-domain having the I332G substitution was also constructed. These constructs will be referred to as pDL-wt(α M) and pDL-I332G(α M). 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 α M I-domain (Figure 4.6A). pDL-wt(α M) was found to have a similar binding profile to BSA as compared to full length α M β 2. A similar binding profile was also observed for pDL-I332G(α M) (Figure 4.6B).

A

B

FIG 4.6. Isolated wild-type αM I-domain and mutant αM I332G show similar binding activity to BSA as compared to full length $\alpha M\beta 2$ 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 $\alpha M\beta 2$. 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 α M 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 α 7 helix which undergoes a 10 Å downward shift in the ligand-occupied conformation. This movement of the α 7 helix has a potential physiologic regulatory role in integrin ligand binding. Analyses of other integrin I-domains, namely those of α 2 (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 α M showed that the side chain of Ile³³² packs into a hydrophobic pocket between the α 7 helix and the opposing β -sheet in the closed conformation. Here Ile³³² coordinates with Ile¹⁵¹, Leu¹⁸⁰, Ile²⁵², and Tyr²⁸³. It was proposed that packing of this residue into the isoleucine socket, or SILEN, might constrain the α M I-domain in the closed conformation (Figure 4.7) (Xiong et al., 2000). This implies that activation of α M β 2 involves the displacement of the isoleucine from the socket. Substitution of Ile³³² to a glycine (I332G variant) in the I-domain of α M was reported to result in a constitutively active α M β 2 (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 α M Ile³³² is a key residue in α M I-domain regulation. Docking of Ile³³² into SILEN will therefore determine in part the activity of the α M I-domain.

FIG 4.7. Ile³³² 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 (Ile¹⁵¹, Leu¹⁸⁰, Ile²⁵², Tyr²⁸³, 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 α M 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 α X I-domain. An α X I-domain-I333G variant was shown to have 200-fold higher affinity for iC3b as compared to wild-type α X I-domain (Vorup-Jensen et al., 2003). In a separate study, alanine scanning mutagenesis of the α L I-domain yielded many α L β 2 variants that showed higher ligand-binding capacity than the wild-type (Huth et al., 2000). Substitution of α L Ile³³¹, which corresponds to the invariant isoleucine in the integrin I-domains, to alanine generated an α L β 2 variant that was approximately 3-fold more adhesive to ICAM-1 as compared to wild-type.

Herein, we report that mutation of α L Ile³³¹ did not render the resultant α L β 2 variant constitutively active. When expressed on 293T, it showed an ICAM-1 binding profile indistinguishable from that of wild-type α L β 2. 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 α L β 2 with the Ile³³¹ 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 α L β 2 and α L(I331G) β 2 showed no difference in their ligand binding properties, we reasoned that Ile³³¹ is not the critical residue contributing to allosteric regulation of α L β 2 affinity.

Of interest, these data revealed that isolated wild-type α L 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 α_L I-domain using the pDisplay vector was shown not to adhere to ICAM-1 coated surfaces (Lu et al., 2001d). In our hands, α_L 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 α_M , constitutive binding to BSA can only be observed in full length $\alpha_M(I332G)\beta_2$, but not in isolated α_M 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., α_M , but may not serve as a prototypic model for the regulation of other I-domains. Such differential regulation of β_2 integrins activities was reported in our previous study on the functional regulation of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ via β_2 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 α_L I-domain exists in a default active conformation and that the α_L I-domain is prevented from binding ligand in the intact receptor unless activated. It is possible that the β_2 subunit plays a role in constraining the α_L I-domain in an inactive conformation in intact $\alpha_L\beta_2$. 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 α M β 2 but not α L β 2 or α X β 2

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 β 2 integrins (α L β 2 CD11aCD18; α M β 2 CD11bCD18; α X β 2 CD11cCD18; α D β 2 CD11dCD18). Increasing evidence indicate that the α cytoplasmic tail determines integrin functional specialization. Integrin α 2 β 1 (VLA-2) showed differential cell-migration on collagen and laminin when α 2 cytoplasmic tail was replaced by that from integrin α 5 or α 4 (Chan et al., 1992). Integrin α 4 β 1 (VLA-4) exhibited differential adhesion properties to ligand VCAM-1 under shear flow analyses when α 4 tail was exchanged with that from integrin α 2 or α 5 (Kassner et al., 1993). The difference in α cytoplasmic tails of α 4 β 1 and α 5 β 1 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 α 5 (Alahari et al., 2000), paxillin with α 4 (Liu et al., 1999), calcium integrin binding protein (CIB) with α IIb, CD45 with α L (Geng et al., 2005), and T-cell protein tyrosine phosphatase (TCPTP) with α 1 (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 $\beta 2$ integrin-mediated adhesion and respiratory burst in response to tumor necrosis factor (Lowell et al., 1996). Hck was reported to co-localize with $\alpha M\beta 2$ around phagosome in $\alpha M\beta 2$ transfectants (Astarie-Dequeker et al., 2002). In this part of study, we adopted a similar approach using $\alpha M\beta 2$ transfectants to examine the effect of $\alpha M\beta 2$ tail modifications on Hck recruitment. We showed that: (i) the co-capping of Hck to $\alpha M\beta 2$ 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 $\beta 2$ tails and (iii) we identified a region within $\beta 2$ tail that is required for $\alpha M\beta 2$ and Hck co-capping. Together, these data suggest a primary role of the integrin α tail in regulating $\beta 2$ tail interaction with cytosolic factors.

5.2 Results

5.2.1 *Hck co-capped with α M β 2 but not α L β 2 or α X β 2*

We examined Hck co-capping with the β 2 integrins in human monocytic THP-1 cells. In our hands, suspension THP-1 cells express lower basal level of α X β 2 as compared to α L β 2 and α M β 2 (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 β 2 integrins. Specific β 2 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 α M β 2 but not α L β 2 or α X β 2 (Figure 5.1B). To verify Hck and α M β 2 co-capping specificity, CHO-K1 transfectants expressing α M β 2 and Hck or another SFK Lck, which is not known to interact with α M β 2, were examined.

Importantly, Hck but not Lck co-capped with cross-linked α M β 2 (Figure 5.2A). However, Lck co-capped efficiently with its known interactor CD4 (Kim et al., 2003). Because the α M β 2-specific function-blocking mAb LPM19c was used in the cross-linking of α M β 2, we extended the analyses using the heterodimer-specific function-blocking mAb IB4 (Wright et al., 1983), the β 2-specific activating mAb KIM185 (Andrew et al., 1993), and the β 2-specific mAb KIM202 that is not known to activate or abrogate α M β 2 function (unpublished data from our lab) (Figure 5.2B). In all cases, Hck co-capping with α M β 2 was detected. It is therefore apparent that the association of Hck with α M β 2 is

constitutive. Of note, the SFK c-Src was shown to bind to platelet integrin $\alpha\text{IIb}\beta 3$ constitutively (Arias-Salgado et al., 2003).

FIG 5.1. Specific co-localization of Hck with integrin $\alpha\text{M}\beta 2$. (A) FACS analysis of PMA-treated THP-1 cells using integrin $\alpha\text{L}\beta 2$ (MHM24), $\alpha\text{M}\beta 2$ (LPM19c) and $\alpha\text{X}\beta 2$ (KB43) specific mAb (shaded histograms). Background staining with irrelevant mAb (open histograms). (B) Co-localization of Hck with $\alpha\text{M}\beta 2$ but not $\alpha\text{L}\beta 2$ or $\alpha\text{X}\beta 2$ 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 $\alpha\text{M}\beta 2$ in CHO-K1 transfectants. Lck co-capped with CD4. Representative images of three separate experiments were shown (B) Co-capping of Hck with $\alpha\text{M}\beta 2$ in CHO-K1 transfectants. $\alpha\text{M}\beta 2$ was cross-linked with function-blocking heterodimer-specific mAb IB4, $\beta 2$ -specific activating mAb KIM185, or $\beta 2$ -specific mAb KIM202 that is non-function-blocking nor activating. Representative images are shown. Bar, 10 μm .

5.2.2 *Substitution of α M tail with α L or α X tail abrogated Hck recruitment to α M β 2-mediated phagosome*

The length and composition of the α L, α M and α X tails differ (Figure 5.3A). To test whether these variations could account for Hck and α M β 2 co-capping specificity, we generated tail variants of α M. The α M tail (Lys¹¹¹⁴-Gln¹¹³⁷) was replaced with α L (Lys¹⁰⁸⁷-Asp¹¹⁴⁵) or α X (Lys¹¹¹⁰-Lys¹¹⁴⁴) tail to generate α ML and α MX variants respectively (Figure 5.3B). CHO-K1 transfectants of α ML β 2 and α MX β 2 showed comparable expression levels to α M β 2 as determined by FACS (Figure 5.4A). The binding properties of these variants to α M β 2 ligand iC3b, a serum complement opsonin, were also comparable to α M β 2 transfectants (Figure 5.4B). Further, α M β 2, α ML β 2 and α MX β 2 transfectants can phagocytose OZ (Figure 5.5). Hck accumulation around OZ-containing phagosomes was reported in α M β 2 CHO-K1 transfectants (Astarie-Dequeker et al., 2002). To extend our study in the context of a biological process, co-localization of Hck with α ML β 2 and α MX β 2 during phagocytosis was examined. CHO-K1 transfected with Hck and α M β 2, α ML β 2 and α MX β 2 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 α M β 2 transfectants but poorly in α ML β 2 or α MX β 2 transfectants.

FIG 5.3. Schematic representation of integrin and mutants cytoplasmic tails. (A) The α L, α M, α X and β 2 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 α ML β 2 or α MX β 2 chimeras are illustrated by a black box followed by the α L or α X tail sequence respectively. The α MLP1110* and α MXN1133* are similarly illustrated but with corresponding deletions of the α cytoplasmic tails. (C) The β 2 cytoplasmic tail that has its Asp (salt-bridge forming residue) substituted by Arg (black box) is shown. The other β 2 cytoplasmic tail truncation mutants are illustrated.

FIG 5.4. Effect of integrin α cytoplasmic tails on α M β 2 binding to iC3b. (A) Cell surface expression of α M β 2, α ML β 2, and α MX β 2 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 β 2 specific activating mAb KIM185 was included. Specificity of adhesion was demonstrated by its abrogation in the presence of integrin α M β 2 function-blocking mAb LPM19c.

FIG 5.5. Opsonized zymosan (OZ) phagocytosed by $\alpha M\beta 2$, $\alpha ML\beta 2$, and $\alpha MX\beta 2$ 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 $\alpha M\beta 2$ 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 α M β 2. (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 μ m. Representative images of three separate experiments were shown (B) % transfectants with Hck clustering around phagocytosed-OZ by counting an entire field. α M β 2 value was taken as 100%.

5.2.3 Tail truncation or salt-bridge disruption restored α ML β 2 or α MX β 2 recruitment of Hck.

Nuclear magnetic resonance studies reveal hydrophobic and electrostatic contacts between integrin α IIb and β 3 tails (Vinogradova et al., 2000; Vinogradova et al., 2002). The proximity of α L and β 2 tails is also evidenced by fluorescence resonance energy transfer (FRET) study (Kim et al., 2003). Hence, C-terminal truncated variants α MLP1110* and α MXN1133* were generated such that the length of their α L and α X cytoplasmic elements are comparable to that of the α M tail (Figure 5.3B). Significant Hck accumulation at the periphery of the phagocytosed-OZ could be detected in α MLP1110* β 2 and α MXN1133* β 2 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, β 2D709R variant having its Asp⁷⁰⁹ substituted with Arg was generated (Fig. 5.3C). Marked accumulation of Hck around phagocytosed-OZ was detected in α ML β 2D709R and α MX β 2D709R transfectants (Figure 5.7).

The binding properties of α M β 2, α ML β 2, or α MX β 2 transfectants may be similar at high coating-concentration (7.5 μ g/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 $\alpha\text{M}\beta 2$, $\alpha\text{ML}\beta 2$, and $\alpha\text{MX}\beta 2$. To resolve this concern, we examined the binding of $\alpha\text{M}\beta 2$, $\alpha\text{ML}\beta 2$, and $\alpha\text{MX}\beta 2$, $\alpha\text{M}\beta 2\text{D709R}$, $\alpha\text{ML}\beta 2\text{D709R}$, $\alpha\text{MX}\beta 2\text{D709R}$, $\alpha\text{MLP1110}^* \beta 2$, and $\alpha\text{MXN1133}^* \beta 2$ transfectants on varying concentrations of iC3b (Figure 5.8). All variants had similar cell surface expression profiles as compared to that of $\alpha\text{M}\beta 2$ (Figure 5.8A). Further, comparable iC3b-binding profiles were observed for $\alpha\text{M}\beta 2$, $\alpha\text{ML}\beta 2$, $\alpha\text{MX}\beta 2$, $\alpha\text{MLP1110}^* \beta 2$ and $\alpha\text{MXN1133}^* \beta 2$ transfectants even at low concentration (0.3 $\mu\text{g/mL}$) of iC3b without or with activating mAb KIM185. The $\alpha\text{M}\beta 2\text{D709R}$, $\alpha\text{ML}\beta 2\text{D709R}$, $\alpha\text{MX}\beta 2\text{D709R}$ transfectants showed higher level of iC3b-binding as compared to $\alpha\text{M}\beta 2$ 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 $\alpha\text{ML}\beta 2$, and $\alpha\text{MX}\beta 2$ transfectants as compared to transfectants expressing $\alpha\text{M}\beta 2$ 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. $\alpha M\beta 2D709R$, $\alpha ML\beta 2D709R$, $\alpha MX\beta 2D709R$, $\alpha MLP1110^* \beta 2$, and $\alpha MXN1133^* \beta 2$ 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 $\alpha\text{M}\beta_2$ variants (continued). (B) CHO-K1 transfectants were allowed to adhere wells coated with different concentrations (0.3, 0.8, 2.5, and 7.5 $\mu\text{g/mL}$) of iC3b in the presence or absence of β_2 -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 $\mu\text{g/mL}$) was effectively abrogated in the presence of $\alpha\text{M}\beta_2$ function-blocking mAb LPM19c.

5.2.4 $\beta 2$ tail sequence Asp⁷²⁸-Ser⁷³⁴ is required for Hck recruitment

Hck was reported to interact with $\beta 2$ tail in pull-down analyses using platelet lysate and purified recombinant $\beta 2$ tail protein (Arias-Salgado et al., 2003). However, we were unable to co-immunoprecipitate Hck with $\alpha M\beta 2$ from lysates of THP-1 or transfectants although $\alpha M\beta 2$ was effectively precipitated. What accounts for this discrepancy is unclear. It is possible that co-capping of $\alpha M\beta 2$ and Hck may involve an adaptor protein that is susceptible to disruption under conditions of immunoprecipitation.

Notwithstanding, we generated truncated $\beta 2$ tail variants ($\beta 2M740^*$, $\beta 2S734^*$, $\beta 2N727^*$, and $\beta 2K702^*$) with sequential deletion from their C-termini (Figure 5.3C) and examined their effects on the co-capping of $\alpha M\beta 2$ and Hck in transfectants (Figure 5.9). $\alpha M\beta 2$, $\alpha M\beta 2M740^*$, and $\alpha M\beta 2S734^*$ co-capped with Hck. This was markedly diminished in $\alpha M\beta 2N727^*$, and $\alpha M\beta 2K702^*$. These data suggest that the $\beta 2$ tail sequence Asp⁷²⁸-Ser⁷³⁴ is required for Hck recruitment to $\alpha M\beta 2$ in CHO-K1 transfectants.

FIG 5.9. Defining the region in integrin $\beta 2$ 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 $\alpha\text{M}\beta 2$, $\alpha\text{M}\beta 2\text{M740}^*$, $\alpha\text{M}\beta 2\text{S734}^*$ but not with $\alpha\text{M}\beta 2\text{N727}^*$ and $\alpha\text{M}\beta 2\text{K702}^*$ 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 $\beta 2$ 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 phagocytose OZ (Welch et al., 1997). There are also evidences suggesting a role of activated Hck in regulating $\alpha M\beta 2$ 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 $\alpha M\beta 2$ but not $\alpha L\beta 2$ or $\alpha X\beta 2$. What is the underlying mechanism? Specific binding of paxillin to the $\alpha 4$ tail affects focal adhesion kinase (FAK) phosphorylation and recruitment by integrin $\alpha 4\beta 1$ (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 $\beta 2$ tail. Indeed, we observed that a reduction in length of the αL or αX tail in $\alpha ML\beta 2$ or $\alpha MX\beta 2$ 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 $\beta 2$ tail. The highly-charged cytoplasmic C-terminal half of αIIb is reported to regulate $\alpha IIb\beta 3$ 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 $\alpha ML\beta 2D709R$ and $\alpha MX\beta 2D709R$ suggest that the close proximity and possibly interaction of the αL or αX with the $\beta 2$ tail may prevent Hck recruitment to the $\beta 2$ tail. Structural determination of these cytoplasmic tails, which is currently lacking for the $\beta 2$ integrins, will be required to define these molecular mechanisms. Although we could not show direct interaction between Hck and the $\beta 2$ tail as reported (Arias-Salgado et al., 2003), our data suggest that the region Asp⁷²⁸-Ser⁷³⁴ of the $\beta 2$ tail is required for effective Hck and $\alpha M\beta 2$ 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 $\beta 3$ 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 $\alpha M\beta 2$, Hck, and talin warrants future investigations.

Chapter Six: Summary

The focus of this study is the $\beta 2$ integrins. The $\beta 2$ 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 $\beta 2$ integrin activation states for ligand binding and the role of its cytoplasmic domain in integrin functions.

$\alpha L\beta 2$ integrin has been the therapeutic target in certain clinical trials (Leonardi et al., 2005). In psoriasis, an anti- $\alpha L\beta 2$ antibody (Efalizumab), which can prevent $\alpha L\beta 2$ 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 $\alpha L\beta 2$, not only those active $\alpha L\beta 2$ but also resting ones, the treatment may be improved if an antibody which is specific for only active $\alpha L\beta 2$ is used. Therefore, the understanding of the integrin activation states is very important. A few observations lend support to the different activation states for $\alpha L\beta 2$. COS-7 cells expressing wild type $\alpha L\beta 2$ 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 $\alpha L\beta 2$ 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 $\alpha 5 \beta 1$ 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 Pro³⁷⁴ and residues His³⁷⁰-Gln³⁷³ of the integrin $\beta 2$ hybrid domain. Unlike the exposed epitope Val³⁸⁵ recognised by mAb 7E4 (Tng et al., 2004), the epitope for MEM148 is hidden in the $\alpha L \beta 2$ model, when using the bent $\alpha V \beta 3$ as template (Figure 3.3). Therefore MEM148 could be used as a reporter mAb to detect $\beta 2$ integrin which are activated. First, we performed a series of FACS analyses to show the $\alpha L \beta 2$ integrin can shuttle between MEM148-staining (active) conformation and non MEM148-staining (inactive) conformation. Next, we tested the ligand-binding ability of $\alpha L \beta 2$ to ICAM-1 and ICAM-3. Interestingly, we found that MEM148 itself cannot promote binding of $\alpha L \beta 2$ to ICAM-1 but rather can stabilize the $\alpha L \beta 2$ 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 $\alpha L \beta 2$

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 $\alpha\text{L}\beta 2/\text{ICAM-1}$ and $\alpha\text{L}\beta 2/\text{ICAM-3}$ interaction may also cause the difference in $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ in the process of migration. By contrast, ICAM-3 is the major $\alpha\text{L}\beta 2$ 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 $\alpha\text{L}\beta 2$ binding to ICAM-3 is essential to prevent cell-cell firm contact in resting T cells. While in the initial step of immune response, $\alpha\text{L}\beta 2$ becomes active and the $\alpha\text{L}\beta 2/\text{ICAM-3}$ adhesive event may facilitate interaction of T cells with antigen-presenting B cells for antigen presentation (de Fougères and Springer, 1992; Fawcett et al., 1992; Vazeux et al., 1992). Although the affinity requirements of $\alpha\text{L}\beta 2$ binding to ICAM-1 and ICAM-3 are different, $\alpha\text{L}\beta 2/\text{ICAM-1}$ and $\alpha\text{L}\beta 2/\text{ICAM-3}$ binding are interrelated as in, (i) $\alpha\text{L}\beta 2/\text{ICAM-3}$ interaction will enhance $\alpha\text{L}\beta 2/\text{ICAM-1}$ binding; (ii) $\alpha\text{L}\beta 2/\text{ICAM-3}$ adhesion is critical for $\alpha\text{L}\beta 2/\text{ICAM-1}$ dependent T cell proliferation (Bleijns et al., 2000).

As MEM148 is a $\beta 2$ specific mAb and has been used to study different activation states of $\alpha\text{L}\beta 2$, we may also want to extend the study on other $\beta 2$ integrin family members, such as $\alpha\text{M}\beta 2$. Does $\alpha\text{M}\beta 2$ also have more than one active conformation? It may not be

true that $\alpha M\beta 2$ will behave exactly the same as $\alpha L\beta 2$, because several studies have revealed that activation mechanism of $\alpha L\beta 2$ and $\alpha M\beta 2$ may not be the same (Tan et al., 2000; Walters et al., 2005).

In chapter four, we mainly compared the I-domain of $\alpha L\beta 2$ and $\alpha M\beta 2$ 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 $\alpha L\beta 2$ and $\alpha M\beta 2$ 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 $\alpha M\beta 2$. 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 $\alpha L\beta 2$ to bind to ICAM-1, while the same mutation in $\alpha M\beta 2$ does lead to $\alpha M\beta 2$ 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 $\alpha L\beta 2$ /ICAM-1 and $\alpha M\beta 2$ /BSA binding were analyzed in this part of study. Since $\alpha L\beta 2$ and $\alpha M\beta 2$ 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 α L β 2 and α M β 2. Nevertheless, our data revealed that the proposed 'SILEN' regulation of I-domain is applicable to specific I-domain containing integrins, e.g., α M, but may not serve as a universal model for the regulation of other I-domains. Such differential regulation of β 2 integrins activities was reported in our previous study on the functional regulation of α L β 2 and α M β 2 via β 2 subunit truncation (Tan et al., 2000). In that report, deletions of β 2 transmembrane and cytoplasmic domain will lead to activation of α L β 2 but not α M β 2. This should not happen if α L β 2 and α M β 2 share a common regulation mechanism for their I-domains. It is proposed that β 2 subunit may have different roles in the function of α L β 2 and α M β 2. Differential activation of α L β 2 and α M β 2 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 β 2 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 $\alpha 2\beta 1$ (VLA-2) showed differential cell-migration on collagen and laminin when $\alpha 2$ cytoplasmic tail was replaced by that from integrin $\alpha 5$ or $\alpha 4$ (Chan et al., 1992). Integrin $\alpha 4\beta 1$ (VLA-4) exhibited differential adhesion properties to ligand VCAM-1 under shear flow analyses when $\alpha 4$ tail was exchanged with that from integrin $\alpha 2$ or $\alpha 5$ (Kassner et al., 1993). The difference in α cytoplasmic tails of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ may also account for the differential requirement of FAK mediated signaling pathway (Hsia et al., 2005). In a recent study, a dimerized $\beta 2$ 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 $\beta 2$ and SFKs. In the third part of the study, we found (i) the co-capping of Hck to $\alpha M\beta 2$ 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 $\beta 2$ tails; (iii) the identification of a region within $\beta 2$ tail that is required for $\alpha M\beta 2$ and Hck co-capping. Together, these data suggest a potential role of the integrin α tail in regulating $\beta 2$ tail interaction with cytosolic partners. Furthermore, there are several areas that need further investigation: (i) how does Hck and talin co-operatively affect $\alpha M\beta 2$ function since they seem to interact with $\beta 2$ 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.

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