CONTROL OF β_1 INTEGRIN FUNCTION:

LOCALIZATION AND CHARACTERIZATION OF REGULATORY EPITOPES ON HUMAN β_1 INTEGRIN CHAIN

By: Heyu Ni

Supervisor: John A. Wilkins

A Thesis Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my supervisor Dr. John A. Wilkins. I thank you for your extreme appreciation of my data and my behavior in your laboratory. I thank you for your scrutiny and discussions on my hypotheses in order to make them closer to nature.

I would like to thank the members of my dissertation committee, Drs. Alec Sehon, Arnold Froese, Leigh C. Murphy, and Martin E. Hemler, for their patience, insightful contributions and genuine enthusiasm.

I will always be grateful to many friends in Dr. Wilkins laboratory, especially to Yanglong Mou, Luo Ma, Chantal Binda, and Di Wang for their cooperation and friendship. I am also thankful to all the technicians and our secretary, Heather Gray for their help during my Ph.D. program.

Finally, I would like to thank my parents, my brothers and sisters who gave me endless love. To my wife Ju Fu, and my daughter Xun Fu, I say thank you for your understanding, love, and encouragement. To my son Taylor Heyu Ni, I thank you for joining us in the end of my Ph.D. program and bringing me a lot of fun and joy.

ABSTRACT OF THESIS

The $ß_1$ integrins are heterodimeric cell surface receptors that mediate adhesion between cells or with components of the extracellular matrix. There are several different conformations of ß1 integrins on cell surface (i.e. inactive and active or plus ligand occupied conformations). Only integrins in the active state are capable of binding their ligands. However, it is not clear how the activities of these molecules are regulated at the structural level.

Previous investigators in this laboratory have generated regulatory monoclonal antibodies to the human integrin $ß_1$ chain. Based on their effects on cell adherence to fibronectin, these antibodies can be divided into three groups, i.e. stimulatory antibodies such as N29, B44, and B3B11, neutral antibodies such as JB1 and 6F4, and inhibitory antibodies such as JB1A and 3S3. In this study, I found N29 epitope expression highly correlated with ß1 integrin functional status, it seems that expression of this epitope is a prerequisite for cell adhesion. In contrast, B44 epitope is a cation ligand induced binding site (CLIBS), whereas JB1A and 3S3 epitopes are quite stable in different functional states of $ß_1$ integrins. It was therefore questioned whether the localization of the epitopes of these mAbs could provide information about ligand binding sites or the regions involved in the control of integrin conformational transition. To map these regulatory epitopes, several techniques were employed 1) Phage peptide display libraries. 2) PCR amplification and integrin peptide expression in *E.coli*. 3) Western blot analysis of interspecies $ß_1$ chimeric proteins and 4) Peptide ELISA.

Using these approaches, three distinct stimulatory epitopes (B44, N29, and B3B11) and two different types of inhibitory epitopes (JB1A and 3S3) were localized. These results

suggested regulatory roles for the conserved -NH2 terminal cysteine rich domain, and regions containing residues 82-87, 355-425 and 636-705. Furthermore the potential contributions of disulfide bonds and O-linked carbohydrate in integrin integrity/functionality were also suggested. The data allowed us to develop a model of some the changes associated with the acquisition of integrin ligand binding competency and occupancy.

LIST OF ABBREVIATONS

aa	amino acid
ADP	adenosine diphosphate
Ag	antigen
BCIP	5-bromo-4—chloro-3-indolyl phosphate
Biotin-BMCC	1-biotinamido-4-[4'-(maleimidomethyl)-cyclohexane-carboxamido] butane
bp	base pair
BSA	bovine serum albumin
CCBD	Central Cell-binding Domain
cDNA	complementary deoxyribonucleic acid
Da	dalton
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
E. coli	Escherichia coli
EDTA	ethylene diamine tetra-acetic acid
FACS	Flow cytometry analysis
FBS	Fetal Bovine Serum
FN	fibronectin
g	gram
ICAM	Intercellular Adhesion Molecule
Ig	immunoglobulin
IgSF	immunoglobulin superfamily

kDa	kiloDalton
LFA	Lymphocyte Function Associated Antigen
LIBS	Ligand-Induced Binding Site
LN	Laminin
MAdCAM	Mucosal Addresion Cell Adhesion Molecule
NBT	nitroblue tetrazolium chloride
NCM	nitrocellulose membrane
NP-40	nonidet-p40
PEG	polyethylene glycol
PBS	phosphate buffered saline
PMA	Phorbol Myristyl Acetate
PMSF	Phenylmethylsulfonyl Fluoride
RGD, R-G-D	Arginine-Glycine-Aspartate residues
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrlamide gel electrophoresis
TBST	Tris-Buffered Saline, 0.5% Tween-20 v/v
TCR	T cell receptor
TFMS	trifluoromethanesulfonic acid
Tris-Cl	tris hydrochloride
Tween-20	Polyoxyethylaminomethane
VCAM	Vascular Cell Adhesion Molecule
VN	Vitronectin

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CHAPTER 1: INTRODUCTION

1.1 Cell adhesion is the basis of the existence of metazoans.

The assembly of individual cells into a multicellular architecture is a prerequisite for the existence of all metazoans. This process is mediated by the adhesion of cell-cell and cell-extracellular matrix proteins. Adhesion molecules not only play the role of sticking one cell to its neighboring cells, but also through their ligand selection and transmembrane signaling, guide cell arrangement, migration, proliferation, differentiation and programmed death (1, 2). Thus, adhesion molecules provide one of the prerequisites for the emergence of primitive multicellular organisms and maintenance of the very diverse, highly sophisticated tissue architecture of animals, including humans.

Many of the families of adhesion molecules are clearly very ancient. The cadherin, integrin and immunoglobulin superfamilies exist in both insects and mammals indicating that these molecules must predate the divergence of the deuterostome and protostome lineages. The same is true for their ligands such as collagens and laminins (3). In fact, the extracellular matrix glycoproteins, collagen and laminin, appear to predate the most primitive metazoan, *Cnidaria* (4). The recent amino acid sequence data of $ß_1$-class integrin from the Cnidaria (coral *Acropora millepora*) and another primitive metazoan, the Porifera (marine sponge *Ophlitaspongia tenuis*) suggest that $ß_1$integrin may have changed relatively little over the past 500 million years (5). Although the precise time when adhesion molecules appeared in this world is obscure, the crucial role of adhesion molecules in the earliest stages of metazoan evolution is certain.

Adhesion molecules are required for the existence of mammals. Adhesion molecules mediate mammalian egg fertilization. Mouse egg $ß_1$ integrin has been defined as a receptor of mouse sperm and this kind of sperm-egg adhesion may be a ubiquitous mechanism for all mammalian egg fertilization, including human (6,7). Adhesion molecules are also crucial for embryogenesis and implantation. Gene knockouts of many adhesion molecules or their ligands result in embryonic lethality (8).

Adhesion molecules exist in all human nucleated cells. Alterations in their adhesive properties, not surprisingly, are observed in many human diseases such as cancer (invasion and metastasis), thrombosis, inflammatory diseases (infections and autoimmune diseases) and problems arising from ischaemia/reperfusion injury (heart attacks, stroke, organ transplantation, frostbite) (9).

1.2 Adhesion molecules in immune system

Several families of adhesion molecules have been characterized molecularly, they are cadherins, members of the immunoglobulin superfamily, selectins, integrins, leucine-rich glycoproteins (LRG), mucins, CD36 family and CD44 (10). In the immune system, the integrin, selectin and immunoglobulin superfamilies (IgSF) play important roles in lymphocyte development, migration and activation, including the antigen specific receptors of T and B lymphocytes.

Adhesion molecules are required in lymphocyte development. Lymphocytes, especially T and B lymphocytes, are the central elements of the immune system. These lymphocytes originate from so-called hemangioblasts, which are common precursors for all blood and endothelial cells. Hemangioblasts differentiate at both intraembryonic and

extraembryonic sites, i.e., aorta-gonad-mesonephros (AGM) region and yolk sac tissue. At around embryonic day 9, when blood circulation begins, precursor cells from both regions enter the blood and settle in the fetal liver at around day 10. Integrin β_1–null precursor cells are present in AGM, yolk sac and the fetal blood but never in the fetal liver (11). Thus, fetal liver can not provide precursor cells to fetal thymus, fetal spleen and bone marrow to form central immune organs and major peripheral immune organs (12). During fetal life, T cell development is α_4 integrin independent, but after birth further production of T cells becomes α_4 dependent (13). Precursors for both T and B cells require α_4 integrins for normal development within bone marrow. In α_4 –null chimeric mice, B cell differentiation is blocked at an earlier stage than that observed in RAG-1 - or RAG-2 –deficient mice, i.e., before the pro-B cell stage, and T cell differentiation is blocked at the point before they can exit the bone marrow and migrate to the thymus (13). In the thymus, integrins also play important roles in thymocyte maturation (14, 15, 16, 17). Overall, integrins are required for development of immune system.

Besides the integrin family, members of the immunoglobulin superfamily such as the antigen receptors of T cells and B cells and MHC molecules play pivotal roles in T cell and B cell differentiation and maturation. The selectin family, which includes P-, E- and L-selectins, plays an important role in leukocyte migration but their roles in lymphocyte development and maturation are currently unclear. It seems that gene knockouts of adhesion molecules from this family do not block lymphocyte development during fetal life (18,19).

Adhesion molecules are crucial for lymphocyte migration and homing.

Lymphocytes as well as other leukocytes migrate extensively throughout the body to mediate immune surveillance and responsiveness for foreign antigens such as infectious pathogens and mutant self-antigens of malignant cells. The route of lymphocyte circulation starts from the blood, through the tissue, into the lymph, and returns to the blood. To patrol the body effectively, these cells must circulate in the blood and lymph as non-adherent cells and migrate through the tissues as adherent cells. Adhesion molecules dynamically control these physiological processes.

Leukocyte extravasation can be divided into four steps (*Figure 1.21*), i.e., (1) tethering and rolling, (2) triggering, (3) arrest and strong adhesion, (4) transendothelial migration.

Tethering and rolling are predominantly mediated by selectins. Adhesion molecules of this family have rapid association and dissociation rate constants for their ligands, thus, mediate tethering of a flowing cell in the span of a millisecond and subsequently, rolling (20). Blocking selectin function with either monoclonal antibodies or their antagonists and ablating selectins in gene knockout mice severely impairs neutrophil and lymphocyte tethering and rolling (18, 21, 22, 23, and 24). Furthermore, clinical evidence from two so-called leukocyte adhesion deficiency II (LAD-II) patients indicates that lack the ligands of selectins display strikingly depressed leukocyte emigration into inflammatory sites (25). These studies indicate the exclusive province of selectins in the earliest step of leukocyte extravasation. Although, there is some evidence to suggest that α_4 integrin is also capable of mediating tethering and rolling (26, 27).

After tethering and rolling, leukocytes are triggered by chemoattractants such as chemokines e.g. MCP-1 and IL-8 or by cell surface interactions. The roles of chemokines and their receptors of serpentine G-protein-linked proteins in this step have been highlighted recently (20, 28). These signals rapidly activate integrin family members such as $ß_2$ (e.g. $\alpha_L ß_2$) and α_4 (e.g. $\alpha_4 ß_1$ and $\alpha_4 ß_7$) integrins and subsequently induce tight adhesion between leukocyte and endothelial cells. The integrin ligands on the endothelium surface, which mediate this strong adhesion, are immunoglobulin superfamily members such as VCAM-1, ICAM-1, ICAM-2 and MAdCAM-1 (20, 28). Mutation of $ß_2$ integrin in patients results in leukocyte adhesion deficiency I syndrome (LAD-I), which is phenotypically similar to LAD-II in its effects on leukocyte extravasation. The α_4 integrin null T lymphocytes cannot migrate to Peyer's patches (13). Therefore, both integrins and molecules of immunoglobulin superfamily are required for leukocyte tight adhesion to endothelial cells.

Transendothelial migration requires both deactivation of integrins and cell migration between two endothelia. The mechanisms that control this process are not known.

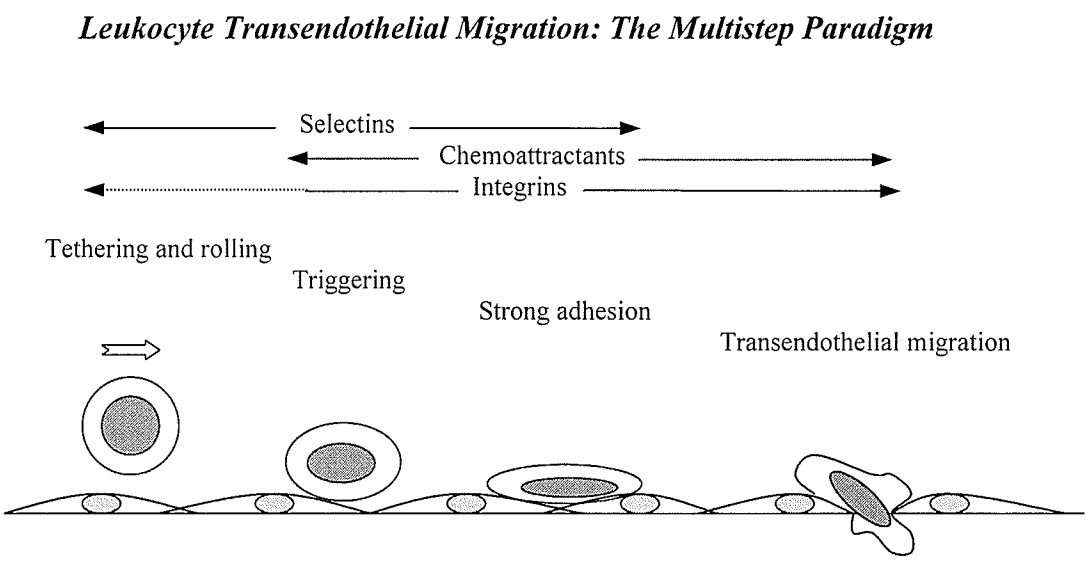


Figure 1.21: Leukocyte transendothelial migration: 4 steps are involved in this process, i.e. 1) tethering and rolling, 2) triggering, 3) strong adhesion, and 4) transendothelial migration.

Lymphocyte homing has been studied since the 1950s (29). The most important conclusion from these earlier studies is that lymphocyte homing is not random. Different classes of lymphocytes migrate preferentially through various lymphoid and non-lymphoid tissues. For example, when collected from lymph draining gut or skin, lymphocytes from adult animals (containing memory and effector cells) but not newborns (containing naive cells) show a 2-fold or higher preference to recirculate to the same type of organ from which they came (30). This property is of significance for several reasons. First, it is an economical way to enhance the efficiency of the immune system by targeting immune surveillance to tissues most similar to those where the cells initially encountered the antigen. Second, it may reduce the opportunities for autoimmune cross-reactions with tissue components from unrelated tissues because this property decreases the chance of a lymphocyte staying in these unrelated tissues.

Our understanding of the molecular mechanisms involved in lymphocyte homing has advanced over the last 10 years. Several pairs of adhesion molecules which control this process mediate interactions between lymphocytes and endothelial cells. ***Figure 1.22*** shows the molecules that contribute to this process in the first three steps (20) (*Permission of reprints granted by Dr. Springer and Cell press*). These lymphocyte surface molecules may be induced by the particular environment in which the specific antigen was first encountered.

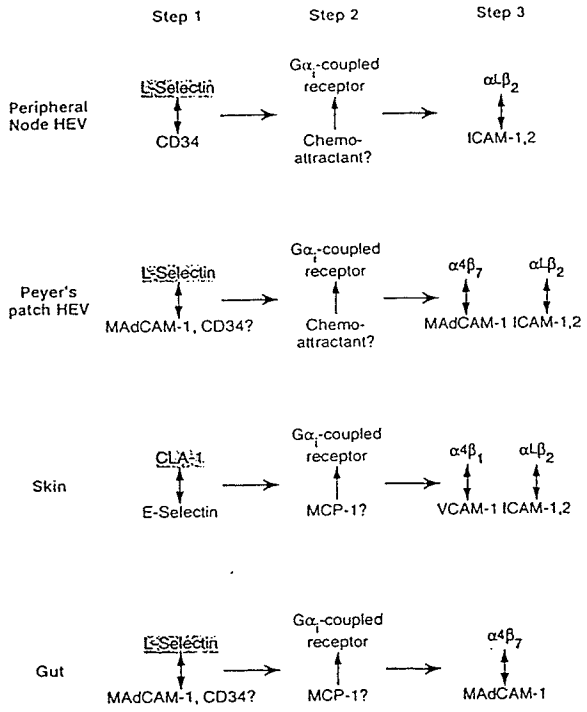


Figure 1.22. The molecules that mediated first three steps of lymphocyte homing (Springer,1994). For each organ, the interacting molecules are shown on the top for lymphocytes and on the botton for endothelia.

Adhesion molecules are important in lymphocyte activation and communication.

Lymphocyte activation usually requires multiple signals to reach their activation threshold. In T and B lymphocytes, the primary signals are provided by the antigen receptor recognizing a specific antigen, the secondary signals are provided by the interaction of an array of adhesion or costimulatory receptors with their respective ligand molecules. Without secondary signals, the first signal can sometimes induce cell unresponsiveness or anergy. Besides the first signal from the antigen receptors, the $ß_1$, $ß_2$ and $ß_3$ integrin are clearly involved in secondary signals (31) and the costimulatory role of L-selectin has been recently noted (32). Thus, all three major families of adhesion molecules (i.e. integrin, selectin and IgSF) participate in T cell and B cell activation by transmitting signals from either extracellular matrix or counter receptors on other cells.

The interactions of lymphocytes with other cell types are critical for immune function. The three most important cell-cell interactions are: 1) T cell/antigen presented cells (APC) interaction, by which a naive T cell is activated by the MHC antigen peptide complex on APC surface. 2) T cell/B cell interaction, by which B cell triggering from T helper cells results in the differentiation of the former into antibody forming plasma cells. 3) T cell/ target cell interaction, by which a cytotoxic T lymphocyte (CTL) is able to destroy virus infected cells and tumor cells. It is now clear that interaction between antigen receptor and MHC antigen peptide is not enough to mediate this adhesion. Other adhesion molecules such as integrin LFA-1 and members of IgSF e.g. LFA-2, LFA-3, ICAM-1, ICAM-2, ICAM-3, play major roles in these adhesive interactions (33, 34). It is notable that interactions mediated by different molecules are highly cooperative. Initial weak adhesion provided from other adhesion molecules may provide an opportunity for

better TCR/MHC antigen peptide recognition. Subsequent signals from TCR can then dramatically upregulate cell adhesion by other molecules within minutes (35).

In summary, adhesion molecules are required in lymphocyte development, maturation, migration, homing, activation and lymphocyte communication. The immune system could not have existed without these molecules.

1.3 Functions and Structures of Integrin superfamily

Integrin functions:

Integrin was named by Dr. Richard O. Hynes in 1986 to denote its role as an integral membrane complex involved in the transmembrane association between the extracelluar matrix and the cytoskeleton (36). Since then, at least 22 integrins have been found or named. These versatile cell surface receptors not only mediate cell adhesion to extracellular matrix and plasma proteins (such as fibronectin, fibrinogen, von Willebrand factor, collagens, vitronectin, laminins, thrombospondin, coagulation factor X and complement iC3b) but also mediate cell interactions with their counter receptors (such as VCAM-1, MAdCAM-1, ICAM-1, -2, -3) (37). Integrins also mediate interactions between host cells and the proteins of some pathogens (such as adenovirus penton base, HIV Tat protein, *Leishmania* gp63 etc.). ***Table 1.31*** shows mammalian

Table 1.31. Mammalian Integrins and Their Extracellular Ligands (Sugimori *et al.*, 1997).

Integrin	Extracellular ligands
$\alpha_1\beta_1$ (CD49a/CD29)	Laminin, collagens I, IV, VI
$\alpha_2\beta_1$ (CD49b/CD29)	Collagnens I-IV, Laminin, vitronectin, Tenascin (cytotactin); $\alpha_3\beta_1$; pathogens (*Echovirus 1*). Motif: DGEA
$\alpha_3\beta_1$ (CD49c/CD29)	Epiligrin (kalinin), fibronectin, laminin, collagen I, entactin, $\alpha_2\beta_1$, $\alpha_3\beta_1$; pathogens (Invasin from *Yersinia spp.*). Motif: RGD
$\alpha_4\beta_1$ (CD49d/CD29)	VCAM-1 (CD106), Fibronectin, α_4 subunit[a], pathogens (Invasin from *Yersinia spp.*). Motif: LDV[b] (consensus: L/I-D/E-S/T/V-P/S) (e.g. IDSP, LDV)
$\alpha_5\beta_1$ (CD49e/CD29)	Fibronectin, denatured collagen, L1 adhesion molecule[c]; pathogens (Invasin from *Yersinia spp.*). Motif: RGD
$\alpha_6\beta_1$ (CD49f/CD29)	Epiligrin, laminin; pathogens (Invasin from *Yersinia spp.*)
$\alpha_7\beta_1$ (CD49g/CD29)	Laminin
$\alpha_8\beta_1$ (CD49h/CD29)	Fibronectin, tenascin, Vitronectin[d]
$\alpha_9\beta_1$ (CD49i/CD29)	Tenascin
$\alpha_V\beta_1$ (CD51/CD29)	Fibronectin, vitronectin; Motif: RGD
$\alpha_L\beta_2$ (CD11a/CD18)	ICAM-1, -2, -3 (CD54, 102, 50); Landsteiner-Wiener (LW) blood group glycroprotein[e]; pathogens (*H. Capsulatum*). Motif: LDV (e.g. GIETP, ALETSL, QIDSPL)[f]
$\alpha_M\beta_2$ (CD11b/CD18)	ICAM-1, -2, -3 (CD54, 102, 50), iC3b, fibrinogen, factor X, lipopolysaccarides (LPS), carbohydrates[g], heparin[h], Haptoglobin[i], CD23[j], kininogen[k], denatured proteins; Landsteiner-Wiener (LW) blood group glycoprotein[e]; pathogens (neutrophil inhibitory factor from *Ancylostoma C.*[l], filamentous hemagglutinin from *Bordetella pertussis*), gp63 from *Leishmania D.*, *H. Capsulatum*, WI-1 antigen from *Blastomyces dermatididis*[m]
$\alpha_X\beta_2$ (CD11c/CD18)	iC3b, fibrinogen, CD23[j], LPS[n], pathogens (*H. Capsulatum*)
$\alpha_D\beta_2$ (CD11d/CD18)	ICAM-3 (CD50)[o]
$\alpha_{IIb}\beta_3$ (CD41/CCD61)	Fibronectin, fibrinogen, von Willebrand factor (vWF), vitronectin, thrombospondin, collagens, denatured collagen, PECAM-1 (CD31)[p]; pathogens (Disintegrins, *Bortelia burgdorferi*). Motifs: RGD, KQAGDV
$\alpha_V\beta_3$ (CD51/CD61)	Fibronectin, fibrinogen, vitronectin, vWF, thrombospondin, osteopontin, tenascin, denatured collagen; L1 adhesion molecule[q], gelatinase A[r]; pathogens (HIV Tat protein, disintegrins, penton base protein of adenovirus type 2[s]). Motif: RGD
$\alpha_6\beta_4$ (CD49f/CD104)	Laminin
$\alpha_V\beta_5$	Fibronectin, vitronectin; pathogens (HIV Tat protein, penton base protein of adenovirus type 2[s]). Motif: RGD
$\alpha_V\beta_6$	Fibronectin, tenascin. Motif: RGD
$\alpha_4\beta_7$	MadCAM-1, CD106, fibronectin, Motif: (from MadCAM-1) LDV (GLDTSL)[t]
$\alpha_E\beta_7$ (CD103)	E-cadherin
$\alpha_V\beta_8$	Vitronectin

integrins and their extracellular ligands (38) (*Permission of reprints granted by Dr. Arnaout and Kidney International publisher, Blackwell Science, Inc*). Despite the diversity of their ligands, integrins generally recognize short sequences in their ligands, which contain a key acidic residue that is essential for receptor binding (e.g. RGD in fibronectin and other ligand proteins, QIDSPL in VCAM-1 etc.) (38). It should be noted that there is some controversy regarding the intermolecular interactions between $\alpha_2\beta_1/\alpha_3\beta_1$, $\alpha_3\beta_1/\alpha_3\beta_1$, and $\alpha_4\beta_1/\alpha_4$ listed in Table 1.31. The evidence for $\alpha_2\beta_1$ binding DGEA motif has also been questioned. These issues derive from the fact that others have not been able to duplicate these results.

Integrins are capable of transmitting bidirectional signals across the cell membrane. Upon binding to their ligands, integrins deliver signals into the cell through their intracellular region (Outside-in signaling) which can influence cell differentiation, migration, proliferation or apoptosis (1, 39, 40). On the another hand, triggering of other receptors on the cell surface, through intracellular signal pathways and integrin cytoplasmic domain interactions, can change integrin extracellular domain conformation (Inside-out signaling) which influences ligand binding (1, 33, 39, 41, 42, 43). Extracellular matrix proteins can also influence cell function by integrin outside-in signaling. Conversely integrin activation regulated by inside-out signaling may be essential for the assembly of extracellular matrix (44).

Integrins are expressed on all nucleated cells, although the expression patterns and levels are variable among different cell types. Thus integrins appear to be important in a wide variety of tissues and may play roles in renal, cardiovascular, gastrointestinal, hepatic, lung, skin, bone and joint, neurological, infectious and malignant diseases (45).

Recently, the genes encoding the $ß_2$ Integrin and its ligand have been identified as a new class of obesity genes (46) suggesting that $ß_2$ integrins may play an indirect role in the regulation of some aspects of hormone levels and energy expenditure.

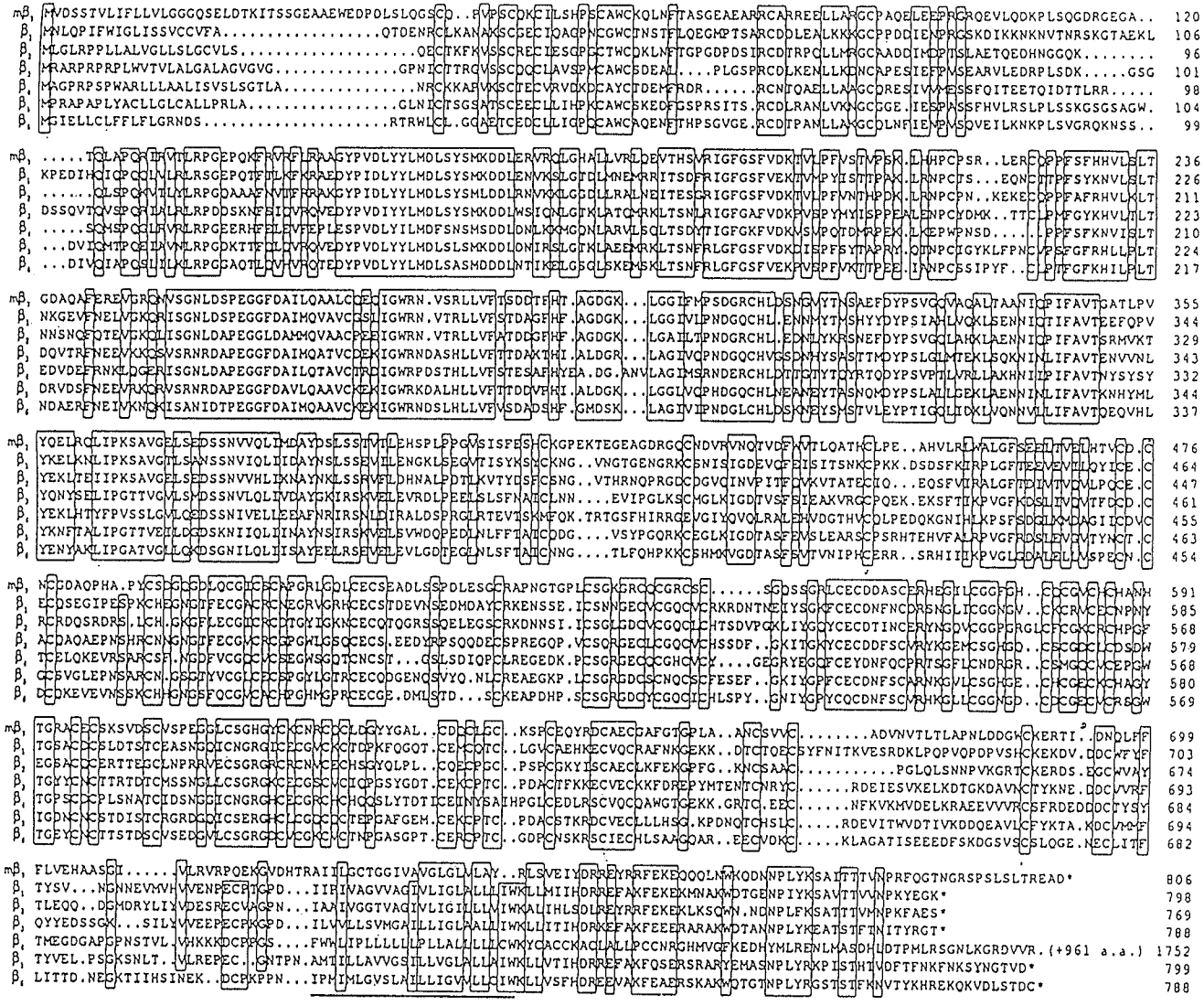
Integrin structure:

Integrins are αβ heterodimeric receptors. A pool of 16 α and eight β subunits can form at least 22 integrin heterodimers with many different ligand specificities (see *Table 1.31*). Each of the α subunits contains about 1000 amino acids, whereas each of ß subunits contains about 750 amino acids except $ß_4$ that contains a very long cytoplasmic tail (over 1000 amino acids, full length of human $ß_4$ is 1752 amino acids). Amino acid sequences of human ß subunits are shown in ***Table 1.32*** (47). Both α and ß subunits consist of a short C-terminal cytoplasmic tail (except $ß_4$ chain), a single transmembrane region and a large extracellular domain (see ***Figure 1.31***) (*Permission of reprints granted by Dr. Arnaout and Kidney International publisher, Blackwell Science, Inc*).

Comparison of amino acid sequences indicates α subunits can be divided into two groups, i.e. subunits with I-domains and subunits with cleavage sites. The homologies between α subunits vary from about 20% to 60% and the homologies within the same group are significantly higher. Among ß subunits, the overall amino acid homology is about 45-50% (about 80% in certain areas) with complete conservation of 56 cysteines in most of ß subunits ($ß_4$, $ß_7$, and $ß_8$ contain more cysteines). Interestingly, 82-90% amino acid identity is observed between human, mouse, chicken, and frog $ß_1$ integrins, and 45% identity between human $ß_1$ and a $ß_1$–like protein from *Drosophila* (48). These data

suggest that there may be considerable similarity in the structure and function of the different integrins.

Table 1.32 (Hu et al, 1992)



The extracellular domain of α subunits contains seven tandem repeats, each comprised of 60-70 amino acids, three or four of which have EF-hand-like divalent cation binding sites. A ß-propeller model, in which seven four-stranded ß-sheets are arranged in a torus around a pseudosymmetry axis, has been proposed recently as a structural model of these seven repeats (49) (***Figure 1.32***) (*Permission of reprints granted by Dr. Springer and PNAS publisher*). In seven α subunits (α_1, α_2, α_E, α_L, α_M, α_X, α_D), an extra A-type domain about 200 residues (called A- or I-domain) is inserted between the second and third repeats. These I-domains contain three EF-like motifs and adopt a Rossman-fold structure (***Figure 1.33***) (50) (*Permission of reprints granted by Dr. Arnaout and Cell press*). The large extracellular domains of the ß subunits contain a putative I-like domain in its N-terminal region (50) and a characteristic C-terminal cysteine-rich motif repeated four times. Electron micrographs of several integrin heterodimers reveal a mushroom-like head comprised of the two N-terminal halves of the α and ß subunits and two flexible tails representing the C terminal portions of the subunits (***Figure 1.34***) (1) (*Permission of reprints granted by Dr. Hynes and Cell press*).

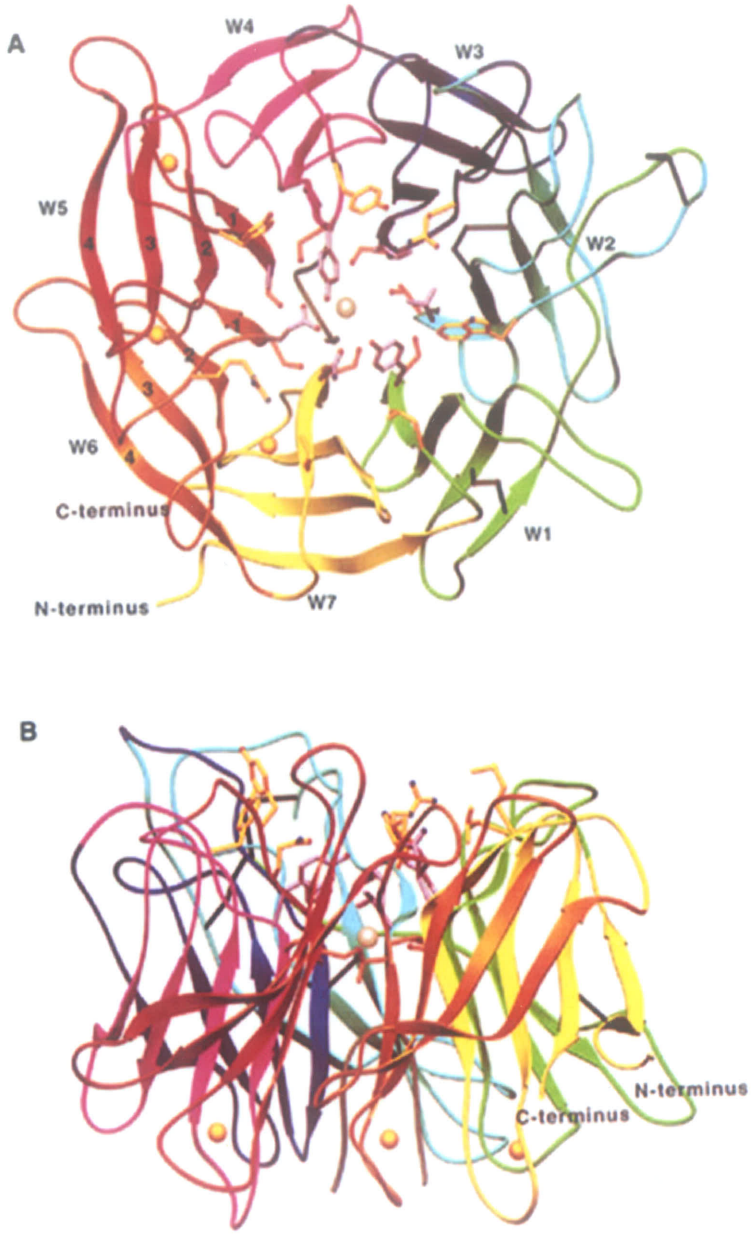


Figure 1.32. Ribbon diagrams of the model for the integrin α 4-subunit β–propeller domain. Views are from the top (A) and side (B). Each W is shown in a different color. A hypothetical polypeptide finger in the central cavity is gray. Cysteins in disulfides are black. Side chains in β-strand 1 in positions 0, b, and 2 are shown in gold, lavender, and rose, respectively; their oxygens and nitrogens are red and blue, respectively. Ca^{2+} ions and a hypothetical Mg^{2+} ion are gold and silver spheres, respectively. (reprint from[46]with permission from *PNAS*)

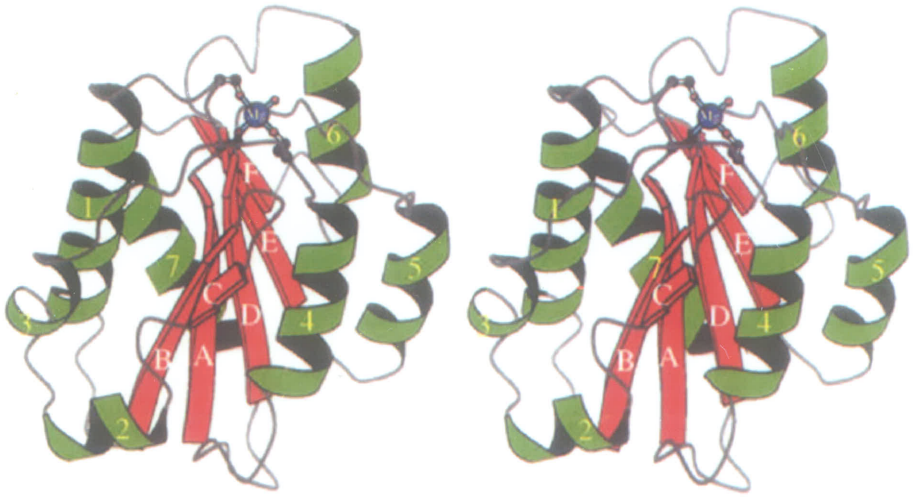


Fig 1.33a. Schematic Stereo Diagram of the I Domain Structure (Lee et al, 1995). The Mg2+ ion is shown on the top of Rossman-fold structure.

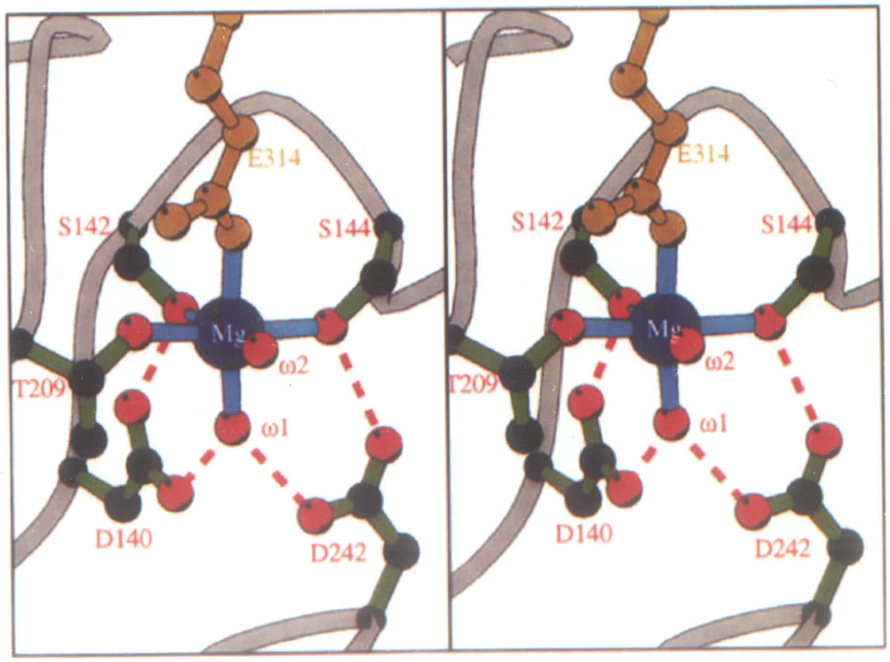


Figure 1.33b. Stereo Diagram of the Metal Ion-Dependent Adhesion Site (MIDAS) (Lee et al, 1995). The MIDAS motif, a DxSxS sequence (residues 140-144, where x represent any amino acid) and a threonine (T209) and aspartate (D242) from other parts of the chain are shown. The hydrogen bonds are shown by dashed lines.

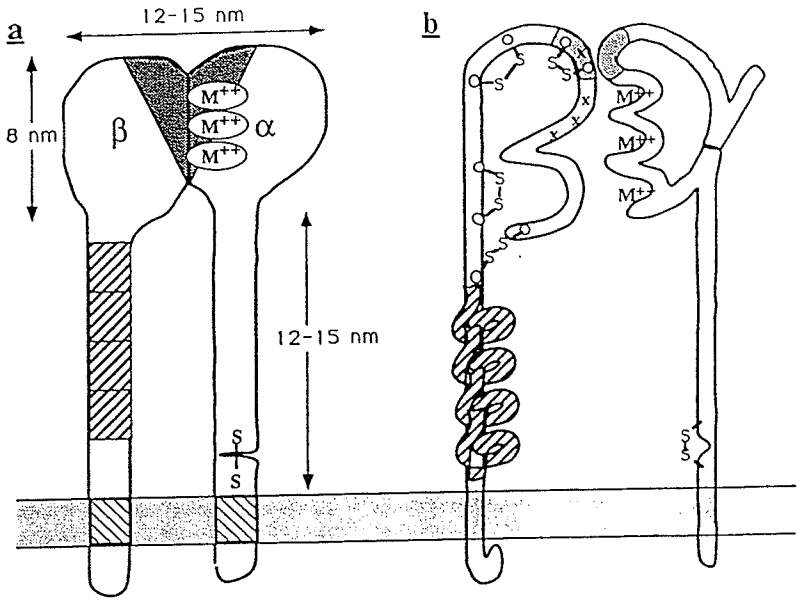


Figure 1.34. Structural Features of Integrin Receptors (Hynes, 1992)

(a) shows the overall shape, as deduced from electron microscopy, as well as the putative locations of the cystine-rich repeats of the β subunit (crosshatched) and metal-binding sites in the α subunit (M^{++}). The shaded area represents the ligand-binding region that is known to be made up from both subunits based on cross-linking and binding data.

(b) schematizes the arrangement of the polypeptide chains with the cystine repeats internally folded and the head region of the β subunit containing internal disulfide loops, some but not all of which are shown. A disulfide bond from the middle of the β subunit to a point close to the membrane has been proposed (Calvete et al., 1991) but is omitted here for clarity. Xs indicate positions of mutations (of human β_2 or β_3 subunits) known to affect ligand binding or αβ dimerization. The positions of alternatively spliced segments in Drosophila subunits are shaded.

Ligand binding sites and divalent cation binding sites:

The I- and ß- propeller domains in the α subunit and the I-like domain in the ß subunit have been identified as the ligand binding sites (51). Thus, α chains have either one ($\alpha_{3\text{-}9}$, α_{IIb}, α_v) or two (α_1, α_2, α_E, α_L, α_M, α_X, α_D) ligand binding domains, and ß chains have one ligand binding domain. These domains are located in the N-terminal half of α and ß subunits, in agreement with the morphologic studies that indicated mushroom-like head were the ligand binding area.

The I domain structure was proposed in 1995 (50, 52). This domain has hydrophobic ß-sheets in the middle and hydrophilic α-helices on both sides of the ß-sheets (a " Rossman" fold). This domain has a Mg^{2+}/Mn^{2+} coordination site at its surface (see ***Figure 1.33***). The residues in α_L and α_M critical for ligand binding, have been shown to be involved in the coordination of a divalent cation. Thus, both ligand and divalent cations bind to the same region of the top of I domain. These critical residues DXSXS (residue 140, 142, 144), T (residue 209) and D (residue 242) that form the divalent cation binding site in α_M are also present in all ß chains (50,51). The DXSXS is in the *conserved region 1* and the second D is in *conserved region 2* of the ß chain which further supports previous observations that these two conserved regions in ß chain are ligand and divalent cation binding sites (53, 54). The *conserved region 1* (i.e. residue 120-139 of $ß_1$ chain) and the *conserved region 2* (i.e. residue 221-241 of $ß_1$ chain) are defined here to describe the two most highly conserved regions among integrin ß chains.

The ß-propeller domain structure was proposed in 1997 (49). This domain contains seven four-stranded ß-sheets (called "W") arranged in a torus around a pseudosymmetry

axis (see ***Figure 1.32***). Integrin ligands and a putative Mg^{2+} ion are predicted to bind to the upper face of the ß-propeller. The Ca^{2+} binding motifs in the integrin α subunit are on the lower face of the ß-propeller. This part of the ß-propeller domain may be involved in interactions with the ß subunit and in conformational changes that regulate ligand binding (49). This model has been supported by several experimental observations (51,55).

Disulfide bonds in integrin family:

Disulfide bonds play important roles in maintaining protein structure. Both integrin α and ß subunits contain multiple cysteines. In the model of platelet glycoprotein IIb/IIIa ($\alpha_{IIb}ß_3$), the disulfide bridges in the α_{IIb} subunit are all sequential, but in the $ß_3$ chain they are much more complicated (***Figure 1.35***) (56) (*Permission of reprints granted by Dr. Calvete and the publisher of Thrombosis and Haemostasis*). In the $ß_3$ chain three long-range disulfide bonds which join the N-terminus and C-terminus of extracellular side to the proteinase-resistant core (Cys^{5}- Cys^{435} and Cys^{614}- Cys^{687}) and the I-like domain to the extracellular side of the C-terminus (Cys^{406}- Cys^{655}) (57). As proposed by Juan J. Calvete, the cysteine-pairing pattern in GPIIIa may be extended to all ß subunits of the integrin family. However, this has not been experimentally confirmed for other integrins. It is notable that both the I-like domains in ß chains and the ß-propeller domains in α chains contain disulfide bonds although there is no disulfide bond in I-domain. These disulfide bonds could be targets of reducing agents and may be involved in thiol exchange mechanisms that control integrin function.

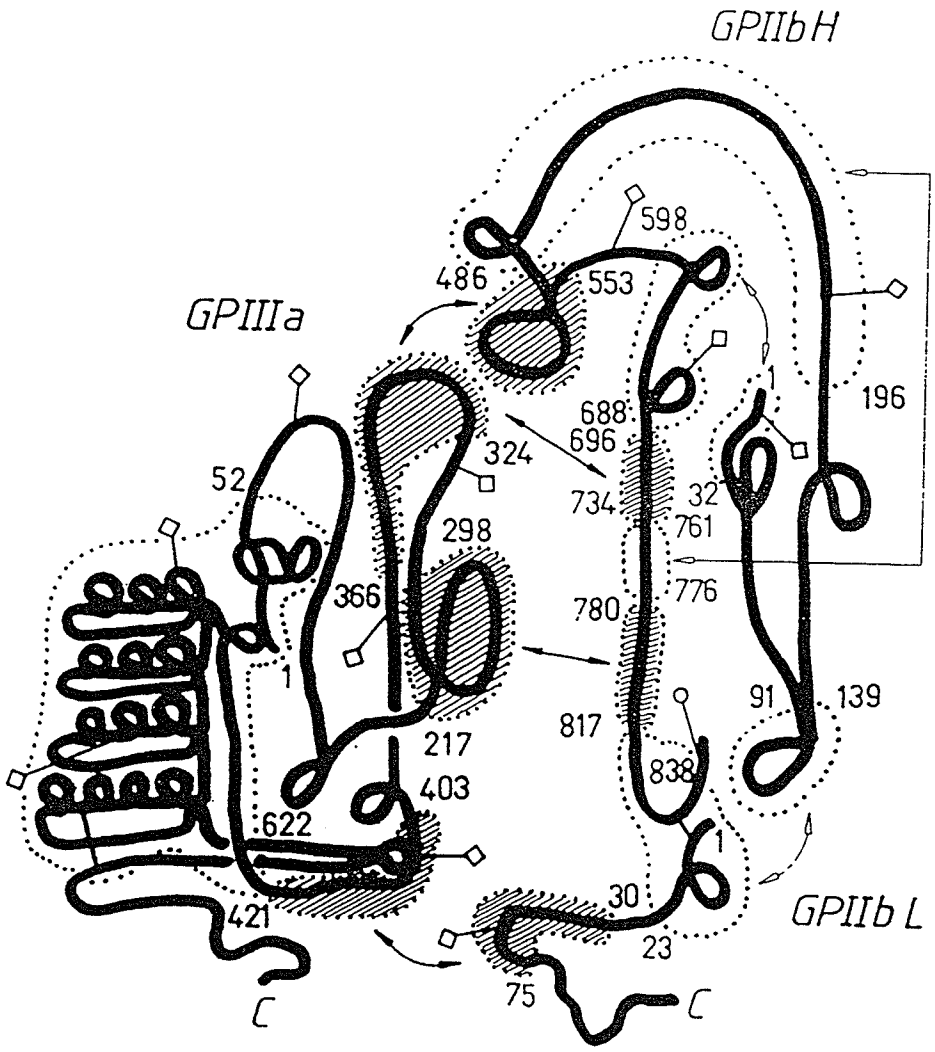


Figure 1.35. A model for isolated platelet glycoprotein IIb/IIIa (αIIβ/β3) based on biochemical data (Calvete, 1994). The protein domains participating in the intra- and intersubunit surface of the heterodimer are shown as dotted areas and shadowed regions, respectively. In α chain the disulfide bridges (loops) are all sequencial; the intrincate disulfide bond pattern of β chain is indicated. The diamonds connected to the polypeptide chain represent N-glycosylation points; the open circle at the C-terminus of α chain shows the position of O-glycosylated Ser^{847}, C, C-terminus.

Glycosylation sites in integrin family:

It has been suggested that carbohydrate moieties play important roles in glycoproteins including stabilization of specific protein conformations, protection against proteolytic degradation and modulation of biological activity. Integrins are glycoproteins that contain multiple N-linked glycosylation sites. The fibronectin receptor ($\alpha_5\beta_1$), for instance, contains 14 and 13 potential N-linked glycosylation sites on α and β subunits, respectively (58). These oligosaccharides are important for maintenance of integrin/ligand interaction (59) and α and β subunit association (60). There is little information about the presence and the role(s) of O-glycosylation site(s) in the integrins. One O-glycosylation site has been localized to Ser^{847} in αIIb subunit (61). Inhibition of O-linked glycosylation has also been shown to impact on VLA4- and VLA5-dependent HL60 cell adhesion (62). Therefore, it appears that there may be a role for the latter type of glycosylation in integrin function.

1.4 Modulation of Integrin functions:

As discussed above, integrin functions are tightly regulated in different physiological and pathological processes. The hierarchy of control includes 1) regulation of integrin expression on the cell surface, (i.e. quantitative regulation) and 2) regulation of integrin functional states (i.e. qualitative regulation). See table 1.41.

1.41 Regulation of integrin expression: A variety of agents and factors have been found to affect the expression of integrins and their function. These include cytokines/ chemokines, hormones, infectious agents, some pharmacological agents and mechanical factors. The mechanisms involved in this type of regulation include (1) regulation of protein levels by transcriptional or posttranscriptional mechanisms, (2) alternative splicing of mRNA, (3) processing of αß heterodimers and transportation of preexisting intracellular stores, and (4) mechanisms involved in integrin internalization and shedding (1, 63, 64, 65, 66).

Table 1.41. Regulation of Integrin Function

Expression Level (quantitative)

Biosynthesis
Alternative splicing
Receptor processing
Transport/ mobilization of intracellular stores
Internalization & shedding

Functional States (qualitative)

Clustering

Affinity Regulation (Conformational change):
Extracellular Domain Stimuli: DTT, Mn^{2+}, mAbs, and ligands
Plasma Membrane Lipid Composition
Intracellular Domain Stimuli: inside-out signaling
Other associated proteins

1.42 Regulation of integrin functional states: There are two ways to quickly change integrin function without new protein synthesis. They are integrin conformational changes and integrin clustering (37).

1) Integrin conformational changes

Many biological processes require promptly changing integrin conformation. Platelet aggregation and adhesion at the site of injured vessels, for example, are critical for preventing fatal bleeding. This process requires immediate transition of $ß_3$ integrins from the inactive form to an active conformation after platelet exposure to the subendothelial matrix (67). Leukocyte extravasation, as described in the previous discussion, is another example. Cell surface integrins need to change their conformation from an inactive form to an active one for firm leukocyte adhesion to the endothelium, then return to the inactive form for leukocyte transendothelial migration to the inflammatory site.

To monitor integrin conformational changes, several *in vitro* methods have been established since the late 1980s, such as: **a**) Detection of changes in the affinity of integrin for to radioactive isotopes or fluorescence labeled soluble ligands or biotinylated soluble ligands. This method has been widely used to distinguish integrin conformational change from cell adhesion resulting from integrin clustering or cell spreading (41). **b**) Detection of functional status associated neo-epitopes with monoclonal antibodies (see section 1.5). **c**) Fluorescence resonance energy transfer studies with a pair of monoclonal antibodies, which recognize different epitopes on the integrin heterodimers (e.g. one to α_{IIb}, another to $ß_3$), these two antibodies respectively conjugated with either a donor

fluorescein (FITC) or an acceptor tetramethylrhodamine (TR) chromophore. This method can test spatial alteration between two epitopes (68). **d**) Detecting alteration of integrin hydrodynamic parameters (69) etc. Among these methods, the first two have been most frequently used in evaluating integrin conformational changes.

Regulating integrin conformation by triggering inside-out signalling:

Signaling via integrins takes two forms: regulation of the affinity and conformation of the receptor from inside the cell (inside-out signaling), and triggering of intracellular events by ligand occupation of the receptors (outside-in signaling). *In vitro* experiments have clearly demonstrated that platelets did not bind GPIIbIIIa ($\alpha_{IIb}ß_3$) ligands such as fibrinogen without activation. These results are consistent with the observation that platelets do not spontaneously aggregate under physiological conditions *in vivo*. After activation with agonists, such as adenosine diphosphate (ADP) or thrombin, the affinity of $\alpha_{IIb}ß_3$ for soluble fibrinogen dramatically increases, which leads to platelet aggregation (70). Fluorescent energy transfer studies on $\alpha_{IIb}ß_3$ have also demonstrated that these agonists induced a change in the spatial separation or orientation of extracellular domains within α_{IIb} and $ß_3$ and that this conformational change occurred before ligand binding (68). In the case of leukocyte integrin activation, *in vitro* experiments demonstrated that triggering of human monocytes with ADP activated $\alpha_M ß_2$ dependent binding of soluble ligand, factor X (71). Triggering of the T cell receptor of the lymphoid cell line, HUT-78, by OKT3 antibody (anti-CD3) induced $\alpha_4ß_1$ and $\alpha_5ß_1$ binding of soluble fibronectin (41). Triggering T lymphocytes with phorbol esters (PMA) resulted in a 4-fold affinity increase of a subpopulation of $\alpha_L ß_2$ for soluble recombinant

ICAM-1 (72). Thus, conformational changes induced by natural agonists *in vivo* are likely a general property of all members of integrin family.

Regulating integrin conformation by divalent cations:

Divalent cations are required to maintain integrin structure. As previously discussed, each integrin heterodimer contains multiple divalent cation binding sites. I domains contain a Mg^{2+} binding site, ß -propeller domains contain a Mg^{2+} / Mn^{2+} binding sites on the upper and three Ca^{2+} sites on the lower face, I-like domains contain a Mg^{2+} binding site. Thus integrins contain either 5 (without I domains) or 6 (with I domains) divalent cation binding sites. Although only three divalent cations (i.e. Ca^{2+}, Mg^{2+}, and Mn^{2+}) were intensively studied, other divalent cations may also play roles in integrin structure (73, 74).

Studies in the middle 1980s demonstrated that the exposure of platelets to Ca^{2+} chelators resulted in the dissociation of the $\alpha_{IIb}ß_3$ complex into monomeric subunits and the loss of the aggregation response of platelets (75). Subsequently, divalent cations have been demonstrated to be indispensable factors for the functionality of all integrins. Although the precise relationship between these cations and integrin function is still unclear, linkage of integrins (cation binding sites or ligand binding sites) and ligand (acidic residue such as "D" in RGD sequence) through divalent cations might be a general mechanism of initiating ligand binding. Following the bridging of ligands, the cations may be eventually displaced from integrin receptor (76).

Divalent cation induced high affinity conformations of integrins have been demonstrated in several cases, for example, Mn^{2+} can induce 15-20 fold higher affinity of

VLA-4 ($\alpha_4\beta_1$) to soluble recombinant VCAM-1/IgG Fc fusion protein on normal peripheral blood T lymphocytes (77). High concentrations (e.g. 2mM) of Mg^{2+} can induce higher affinity of LFA-1 ($\alpha_L\beta_2$) for soluble ICAM-1/IgG Fc fusion protein on T cell surface (78). Furthermore, Mn^{2+} and Mg^{2+} can induce purified $\alpha_5\beta_1$ binding of the CCBD fibronectin fragment (79), suggesting divalent cations may directly change the conformation of integrins.

In many cases, Ca^{2+} appears to interfere with the activities of Mg^{2+} and Mn^{2+}. For example, Ca^{2+}inhibited Mg^{2+} activated $\alpha_2\beta_1$ anchored in liposomes from binding to collagen (80). Ca^{2+} also inhibited $Mn^{2+}$and Mg^{2+} activated LFA-1 ($\alpha_L\beta_2$) binding to ICAM-1 by T cells (81). Using purified $\alpha_5\beta_1$, Mould *et al* demonstrated that Ca^{2+} is a noncompetitive inhibitor of Mn^{2+} activated ligand binding, but Ca^{2+} can be an inhibitor or stimulator of Mg^{2+} activated ligand binding depending on the concentrations of Mg^{2+} and Ca^{2+}. When the concentration of Mg^{2+} is low, low levels of Ca^{2+} can bind to a distinct high affinity site, which increases the affinity of Mg^{2+} for its ligand-competent site. However, Ca^{2+} can compete directly with Mg^{2+} for binding to the Mg^{2+} ligand-competent site when Ca^{2+} concentration is high. This study suggested that $\alpha_5\beta_1$ exists in several distinct divalent cation-binding sites, each of which has different specificity for Mn^{2+}, Mg^{2+} and Ca^{2+} (79). It is still not known today which of the cation sites are occupied in any of these situations. Several questions are still unanswered, i.e. whether a certain type of cation can bind different cation binding sites or they have their own specificities; how many cation binding sites must be occupied by cations in order to support ligand binding; why does Mn^{2+} confer a much higher ligand binding affinity on many integrins than Mg^{2+} and Ca^{2+}.

Regulating integrin conformation by the reducing agent, Dithiothreitol (DTT):

As discussed previously, both α and ß chains of integrin contain multiple highly conserved cysteines that are predicted to be involved in intrachain disulphide bonds. Protein disulphide isomerase (PDI), an enzyme involved in the exchange of protein disulfide has been described on the surface of different cells (e.g. lymphocytes) and can be released from activated platelet (82, 83). Recently, several extracellular matrix proteins (fibronectin, von Willebrand Factor) have been shown to display PDI like activity (84, 85). Thus exchange or deassociation of disulphide bonds within integrin receptors might be involved in regulating integrin conformation and integrin functional status.

In 1974, MacIntyre *et al* demonstrated that dithiothreitol induced platelet aggregation, and that this aggregation was caused by reduction of disulphide bonds in platelet proteins rather than reduction of fibrinogen (86). In 1984, Zucker *et al* showed that DTT induced specific ^{125}I-fibrinogen binding to platelets and this binding resulted from reduction of fibrinogen receptor, $\alpha_{IIb}ß_3$ (87). Since then, DTT activation of $ß_1$, $ß_2$, and $ß_3$ integrins has been reported (88, 89, 90). Because DTT was found to enhance the binding of fibronectin to purified $\alpha_5ß_1$, it seems that DTT can directly change integrin conformation. In other words, alteration of disulfide bonds within the integrin heterodimer is able to influence integrin ligand binding and is a probable mechanism *in vivo.*

Regulating integrin conformation by other factors:

In addition to physiological agonists, divalent cations, and reducing agents, integrin conformation can be regulated by cell membrane lipid constitution (91); integrin associated proteins (92, 93, 94) (some of them may need more evidences), and regulatory monoclonal antibodies (see section 1.5). These molecules may regulate integrin conformation through their unique interactions with the integrin extracellular domain, the transmembrane domain or the intracellular domain. However, similar to those previously described factors, their roles in control of integrin structure and function are not fully understood.

We are still far from understanding integrin conformational transitions *in vivo*, where divalent cation concentrations may be lower than those used *in vitro* and where DTT does obviously not exist. On the another hand, multiple stimuli that exist *in vivo*, including shear stress in blood (95), may synergistically induce integrin conformational changes, which have yet to be demonstrated *in vitro*. Thus, better systems to examine integrin conformation *in vivo* may be required for the future investigation of this point.

2). Regulating integrin function by integrin clustering:

The regulation of integrin diffusion/clustering, through integrin cytoplasmic tail interactions with the cytoskeleton, is another important mechanism in controlling cell adhesion (37). $ß_2$ integrin (LFA-1), for instance, distributes homogeneously on resting lymphocytes. These cells bind poorly to ICAM-1, which is a ligand of LFA-1. This nonadhesive state is actively maintained by the lymphocyte cytoskeleton. Disruption of

actin cytoskeleton of T and B lymphocytes allows lateral movement and activation of LFA-1, resulting in ligand binding and "outside-in" signaling, that subsequently stimulates actin polymerization and LFA-1 clustering which stabilizes strong cell adhesion (96, 97). LFA-1 is clustered on the cell surface of interleukin-2/phytohemagglutinin-activated T lymphocytes. These cells bind strongly to ICAM-1. Disruption of the actin cytoskeleton of these cells with cytochalasin D results in a decreased LFA-1-mediated adhesion to ICAM-1. Thus, the cytoskeleton maintains both the nonadhesive state (homogeneous integrin distribution) and the strong adhesive state (integrin clustering). However, the intracellular molecular mechanism that controls integrin distribution on the cell surface remains to be further elucidated.

Although integrin conformational change and clustering are considered as two separated events, they may happen concurrently on the cell surface and synergistically facilitate ligand interaction (98). It is also important to keep in mind that varied integrin functional states may exist in the same cell (e.g. different affinity $\alpha_4\beta_1$ pool on leukocyte surface) (99). In addition, integrins in different functional parts of the same cell may be also different (e.g. focal-adhesion and macroaggregate formation on fibroblasts) (66). All of these are sophisticatedly controlled by the cell and its environment although we still don't know the detailed mechanisms.

1.5 Structure and function analysis of integrins with regulatory antibodies:

Structure and function analyses of integrins with regulatory antibodies have improved our knowledge in several aspects. These include 1) Detection of integrin

conformational changes. 2) Identification of ligand binding sites. 3) Identification of regulatory sites.

1) Detection of integrin conformational changes

As discussed in the last section, integrin conformations can be regulated at three levels: i.e. integrin extracellular domain, lipid constitution of cell plasma membrane (91) and integrin intracellular domain. Divalent cations (100), reducing agents such as DTT (87, 88), ligands (101) and some regulatory monoclonal antibodies (1,100) can directly act on the extracellular domain. PMA and other physiological signals generated by the triggering of cell surface receptors can affect the integrin intracellular domain by intracellular signal cascades (35, 102, 41, 42, 43, 103). In addition, some integrin-associated proteins, which regulate integrin functions, may do so through unique interactions with the integrin extracellular domain, intracellular domain or transmembrane domain (92, 93, 94). However, the structural bases of integrin conformational changes are still unclear. How different cells maintain different integrin conformational states (e.g. partially active forms on Jurkat cells *vs* latent forms on peripheral blood lymphocytes) is an enigma.

Detecting integrin-ligand affinity is a reliable criterion to test integrin conformation. However, this method may be not able detect some integrin conformation changes below its threshold (i.e. partial active form) or integrin conformation change for binding other associated protein(s). For example, phorbol ester (PMA) increases the expression of an epitope recognized by mAb 15/7 in β_1 integrins under conditions where an increase in soluble ligand binding was not observed (37). Furthermore, this method can not provide any information about structure basis of integrin conformational transition. Although X-ray

crystallography and electron microscopy have provided some information about integrin structure (see previous discussion), they are not applicable to monitor integrin conformational transition.

Detecting integrin conformation with monoclonal antibodies (mAb), which specifically recognize the active form of integrin, is an another simple and reliable method. In 1985, Coller demonstrated that mAb 7E3, which can block fibrinogen binding, bound slowly to native platelets but much more rapidly to ADP and thrombin activated platelets. As a control, another blocking antibody 10E5 bound nearly equally rapidly to both native and activated platelets (104). Almost at the same time, Shattil et al found that mAb PAC-1, another murine blocking antibody, bound to ADP activated platelets, but not to unstimulated platelets (105). Thus, 7E3 and PAC-1 are able to recognize the active conformation of GPIIbIIIa ($\alpha_{IIb}ß_3$). These two antibodies were classified as ligand-mimetic antibodies (37). Similar to the case for $ß_3$ integrin, several ligand-mimetic antibodies such as CBRM1/5, and 7A10 to $ß_2$ integrin were also identified. However, this type of antibody has not yet been found in $ß_1$ integrins (37).

Different from ligand-mimetic antibody, another group of antibodies recognizes ligand occupied integrins. Because they bind ligand induced binding sites (LIBS), these antibodies are named as anti-LIBS. The first anti-LIBS was described by Mark H. Ginsberg and colleagues in 1988. They found that mAb PMI-1 did not bind native platelets and ADP activated gel filtration-washed platelets (without fibrinogen), but bound to ADP activated platelet in plasma (with fibrinogen) and ADP activated gel filtration-washed platelets if exogenous fibrinogen was added. They further demonstrated that the peptide ligand GRGDSP, induced PMI-1 epitope expression, and this result could be reproduced in

purified $\alpha_{IIb}ß_3$ (106). Thus ligand occupancy generated a new conformation which can be detected by monoclonal antibody. This concept was quickly spread to other members of integrin family. At least a dozen anti-LIBS have been found in $ß_1$, $ß_2$, and $ß_3$ integrin since the end of 1980s (37). More recently, it was realized that most LIBS sites are also regulated by divalent cation, therefore these sites can be also named as CLIBS, i.e. cation and ligand influenced (induced) binding sites (107).

In addition to ligand-mimetic and anti-CLIBS antibodies, other antibodies also exist, which can be used to detect integrin conformational changes. For example, mAb N29 described in this study can detect active $ß_1$integrin on Jurkat and K562 cells, it is also able to detect DTT, Mn^{2+}, and probably anti-CD3 activated $ß_1$integrin, however this antibody is not a ligand-mimetic antibody and no evidence suggests it is an anti-CLIBS. We should keep in mind that anti-CLIBS, in some cases, can bind not only ligand binding conformation but also integrins treated with other reagents such as SDS or EDTA (37). We also demonstrated that anti-CLIBS B44 could bind PMA activated, ligand-independent conformation of human $ß_1$ integrin. Thus different antibody may give different insights into integrin structure/function relationships if we can find theirs binding sites (epitopes) on integrins.

2) Identification of ligand binding sites with monoclonal antibodies

Several methods have been successfully used to identify ligand-binding sites in the integrin ß chain. A Cross-linking study with Arg-Gly-Asp (RGD) peptide, a sequence recognized by many integrins, demonstrated that residues 109-171 and 66-203 of the $ß_3$ chain were RGD binding sites in $\alpha_{IIb}ß_3$ and $\alpha_v ß_3$ respectively (108, 109). These results

were supported later by a natural mutation in a Glanzmann's Thrombasthenia patient, whose $ß_3$ chain residue119D was mutated to Y, and an artificial mutation of homologous residue 130D to Y of the $ß_1$ chain. Both mutations resulted in the loss of ligand binding potential (110, 111). Panning phage peptide display library with the RGD peptide further demonstrated that KDDLW (residues 125-129) in the first conserved region (i.e. residue 109-128 of $ß_3$ chain) is the ligand-binding site (54). Several studies with cross-linking agents, and mutational analysis also suggested that the second conserved region (i.e. residues 212-233 of $ß_3$ and 221-242 of $ß_1$) in ß chain is also a ligand binding site (53, 112). A cross-linking study with another peptide KQAGGV from C-terminal (chain of fibrinogen demonstrated that a residues 294-314 of $ß_3$ chain in $\alpha_{IIb}ß_3$ was another ligand-binding site (108). To avoid confusion, it is worthwhile to keep in mind that most of these ligand-binding sites exist around the top of I-like domain, i.e. divalent cation and ligand binding site (see previous discussion).

Localization of ligand blocking antibody (inhibitory antibody) epitopes, especially the epitopes of ligand mimetic antibodies, was thought to be useful for identifying ligand-binding sites. That ligand mimetic antibody AC7 recognized the first conserved region of $ß_3$ chain, and several inhibitory antibodies recognized residue 207-218, which is immediately before the second conservative region of $ß_1$ chain, supported this idea (113, 111). However, the information from this research orientation is limited because only a few antibodies are available and most of their epitopes are difficult to identify.

3) Identification of regulatory sites of integrins

Antibodies to integrin can be divided into three groups, depending on effects on integrin function (i.e. adhesion): stimulatory antibodies, inhibitory antibodies and neutral antibodies. The anti-LIBSs belong to the stimulatory antibodies because they stabilize integrin in their active forms. The ligand mimetic antibodies belong to inhibitory antibodies since they block ligand binding. However, stimulatory antibodies are not necessarily anti-LIBS and inhibitory antibodies are not necessarily ligand mimetic, for example, N29 strongly stimulates cell adhesion but ligand binding does not influence the expression of this epitope (114). Similarly JB1A inhibited cell adhesion, but its epitope is located far from putative ligand binding sites (115). Thus regulatory roles of different antibodies and their epitopes in integrins may be very complicated, identifying their individual epitopes may contribute to understanding integrin conformational transition.

Three anti-LIBS binding sites on $ß_3$ and $ß_1$ chain have been identified in different laboratories before and during this study (37). The epitopes of anti- $ß_3$ monoclonal antibodies AP-5 and D3GP3 were localized in residues 1-6 and 422-490, respectively; the epitopes of another group of anti-LIBSs (i.e. P41, LIBS-2, -3, and -6) are localized between residues 602-690 of the $ß_3$ chain. In comparison to $ß_3$ integrin, most of the information about $ß_1$ chain LIBS sites appeared only in the last a few years. The epitopes of 8A2 and 12G10 were localized in residues 207-218; The epitopes of 15/7, HUTS-4, -7, -21 and B44 (in this study) were localized in residues 354-425; and epitope of 9EG7 was localized in residues 495-602. Thus although significant homology between $ß_3$ and $ß_1$ chains, exists three separate CLIBS sites on the $ß_1$ chain do not overlap with three different CLIBS sites on the $ß_3$ chain (37).

Several inhibitory epitopes were localized within I-like domain, which may compete directly with binding of ligands to integrin (see above). However, some inhibitory antibody may bind other sites. For example, although the epitope of inhibitory monoclonal antibody 13 (mAb 13) is attenuated by ligand occupancy, the concentration of ligand required for half-maximal inhibition of antibody binding is independent of antibody concentration (116). Thus the ligand may acts as an allosteric inhibitor of mAb 13 binding, i.e. mAb13 does not compete directly with ligand for integrin binding, in other words, mAb13 epitope may be not a ligand-binding site. My results also indicated ligand occupancy could enhance B44 epitope (CLIBS) exposure, but did not affect inhibitory epitopes such as JB1A and 3S3 (114). Hence, inhibition by many inhibitory antibodies may be through an allosteric mechanism rather through competition with ligand binding. Unfortunately, the precise epitopes of many inhibitory antibodies are not known. In addition, many epitopes localized with chimeric integrins or mutations can not distinguish between whether a certain region is just "involved" in epitope formation from whether a certain region itself is the epitope, this is a potential problem associated discontinue epitope mapping.

It is interesting that a cluster of stimulatory and inhibitory antibodies recognizes the same region of residues 207-218 of the β_1 chain (111). This region is located between two conserved regions and is immediately before the second conserved region. This region is on one of the outer alpha helices of the Rossman fold formed by I-like domain, (see figure 1.33a). The fact that both stimulatory and inhibitory antibodies recognize this region suggests that this region is highly dynamic and may exist in different conformational states, reflecting inactive, active, or ligand-occupied conforms of the β_1 integrin.

In summary, studies on integrin structure and function with monoclonal antibodies have demonstrated that monoclonal antibodies are powerful probes to monitor integrin activation states; mapping regulatory antibody epitopes has provided some insights into the structure basis of integrin conformational transition. Some antibodies (such as 7E3) have been demonstrated to be useful in clinical approaches to manipulate integrin function in patients (117). In addition, our results suggest that monitoring different epitope expressions might help to design and evaluate new integrin related drugs (e.g. Bacitracin), which may be used for immune-regulation, inflammation control and tumor therapy.

1.6 Objectives of the study

Integrins can be expressed in at least two or three different functional states on the cell surface. The conformational transitions from one state to another which control cell adhesion and deadhesion are of important physiological significance. However, the molecular and structural bases of integrin conformational change are currently unclear.

We have attempted to begin to address this question using three groups of monoclonal antibodies (mAb) against human $ß_1$ integrin, which were produced in our laboratory. The groups are: (1) stimulatory mAbs such as N29, B44 and B3B11, which enhance $ß_1$ integrin binding to their ligands. (2) Inhibitory mAbs such as JB1A and 3S3, which block $ß_1$ integrin dependent cell binding to their ligands. (3) Neutral mAbs such as JB1 and 6F4, which do not affect adhesion. It was questioned whether the localization of the epitopes which these mAb recognize could provide information about ligand binding

sites or the regions involved in the control of the transition from the inactive to the active state.

Specific aims:

1. **To map regulatory epitopes using several molecular biological and immunological methods.**

2. **To monitor the expression of these epitopes in different functional states of $ß_1$ integrins.**

3. **To examine the role of disulfide bonds in $ß_1$ integrin structure and function.**

CHAPTER 2: MATERIALS AND METHODS

Materials:

Unless otherwise indicated all chemicals were purchased from Sigma Chemicals, St. Louis, MO. Media and fetal bovine serum were obtained from Gibco Life Sciences, Burlington, ON. Fibronectin was obtained either from Gibco Life Sciences or Chemicon Intl., Temecula, CA. Biotin-BMCC was purchased from PIERCE, Rockford, IL . O-Sialoglycoprotein Endopeptidase was obtained from Cedarlane Laboratories Ltd, Hornby, ON. Custom synthesized peptides were purchased from Chiron Mimotopes Peptide Systems, Emeryville, CA and from Research Genetics, Huntsville, AL. Oligonucleotides were synthesized with Bio-Rad oligonucleotide synthesis system, Department of Medical Microbiology, Univ. of Manitoba and purified according to manufacturer's instruction. Phagemid pBS (+) was obtained from Stratagene, La Jolla, CA. Expression vector pET-14b and competent *Escherichia coli* BLR(DE3)plyss strain were purchased from Novagen, La Jolla, CA. DNA restriction endonucleases were purchased from Gibco BRL. The 15-mer and 26-mer random phage peptide display libraries were provided by Chiron Corporation, Emeryville, CA.

Monoclonal antibodies B44, 13B9, N29, B3B11, JB1B, 21C8, JB1, 6F4, JB1A, C30B, D11B, 3S3 were generated from this laboratory as described before (118-122, 114, 115). Stimulatory monoclonal antibodies A1A5 and TS2/16 were provided by Dr. M. Hemler, Dana-Farber Cancer Institute, Harvard University. These two antibodies recognize the region of residue 207-218 (114). Stimulatory monoclonal antibody HUTS-21 was provided by Dr. Sanchez-Madrid, Universidad Complutense, Madrid, Spain. Stimulatory

monoclonal antibody 15/7 was provided by Dr. T. Yednock, Athena Corp, LA, CA. These two antibodies recognize the LIBS (ligand-induced binding site) of residue 355-425 (114). Inhibitory antibody AIIB2 was provided by Dr. C. Damsky, UCSF, CA. This antibody recognizes an epitope dependent on residues 207-218 of the ß1 chain(114). Some properties of antibodies are shown in ***Table 2.1***.

The human cell lines Jurkat (T Leukemia), IM9 (B cell) and K562 (erythroleukemia) were obtained from ATCC, Rockville, MD. Cell lysates of Chinese Hamster Ovary cells (CHO) transfected with human/chicken and human/mouse chimeric $ß_1$ integrins were provided by Dr. Y. Takada, Scripts Institute, La Jolla, CA.

Table 2.1: **Summary of Antibody Properties and Competitive Blocking Studies**

	S 1			S 2		S 3	I 1			I 2
	JB1B	B3B11	21C8	B44	13B9	N29	C30B	D11B	JB1A	3S3
JB1B	+	+	+	-	-	-	-	-	-	-
B3B11	+	+	+	-	-	-	-	-	-	-
21C8	+	+	+	-	-	-	-	-	-	-
B44	-	-	-	+	+	-	-	-	-	-
13B9	-	-	-	+	+	+	-	-	-	-
N29	-	-	-	-	-	+	-	-	-	-
C30B	-	-	-	-	-	-	+	+	+	-
D11B	-	-	-	-	-	-	+	+	+	-
JB1A	-	-	-	-	-	-	+	+	+	-
3S3	-	-	-	-	-	-	-	-	-	+

S = Stimulatory Monoclonal Antibodies, I = Inhibitory Monoclonal Antibodies
+, > 80% and -, <10% inhibition of binding of labeled antibody by 20ug/ml of competitor antibody

Methods:

Cell adhesion Assay: The assay was performed as previously described (115). Non-tissue culture treated microtiter wells were coated with purified plasma fibronectin (5μg/ml) in bicarbonate buffer at 4°C overnight. The wells were washed and blocked with 1% BSA in RPMI-1640. Jurkat cells were washed twice with RPMI-1640 and incubated with the stimulatory agents at the indicated concentrations at room temperature for 30 minutes. The cells were then added to fibronectin or BSA coated wells (2×10^5 cells/ well) and incubated for 60 minutes at 37°C. The non-adherent cells were removed by centrifugation of the inverted plates for 5 minutes at 70×g and the supernatants were removed. The adherent cells were fixed and stained for 60 minutes with 0.5% crystal violet in a 30% solution of methanol in water. The plates were washed with water to remove unbound dye. The residual dye was solubilized in methanol and the absorbance at 550 nm was determined.

In the case of examining the effects of different divalent cations on cell adhesion, cells were washed twice with Puck's saline (KCl 0.4g, NaCl 8.0g, $NaHCO_3$ 0.35g, D(+) Glucose 1g, and Phenol Red 0.005g were dissolved in H_2O to 1 liter) and incubated with different divalent cations in Puck's saline at the indicated concentrations at room temperature for 30 minutes. The cells were then added to fibronectin or BSA coated wells and adhesion assay was performed as described above. To examine the effect of reducing agent on cell adhesion, cells were incubated with the indicated concentration of DTT in RPMI –1640 at room temperature for 30 minutes, cells were then washed twice to remove the DTT prior to their addition to wells.

Flow cytometry analysis:

Cells were preincubated with the indicated stimulant at room temperature and then incubated with the indicated antibody (5μg/ml) for 30 min at 37°C. The cells were washed twice with PBS buffer and incubated for 60 min at 4 °C with a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (Chemicon, CA). All assays included cells incubated with the second antibody alone as a control for nonspecific binding. Fluorescence analysis was performed with a BD FACScaliber. To examine the expression of regulatory epitopes in the different divalent cations and DTT, cells were washed and suspended in the same condition as cell adhesion assay. In some cases, FACS protocol had to be modified, in these cases, the individual methods were described in Chapter 3.

Peptide ELISA and peptide competitive ELISA:

Peptides were suspended at 10μg/ml in 0.1% dimethyl formamide in water and allowed to dry overnight, 0.5μg/well, in Nunc Maxisorb plates. The plates were washed three times with 0.5% Tween 20 in TBS (0.15M NaCl, 50mM Tris-HCl, pH 7.5) and blocked for 2 hours at room temperature with 2% BSA in TBS. The indicated antibodies (5μg/ml) were added to the wells, and the binding was quantitated using an alkaline phosphatase conjugated anti-mouse IgG and substrate. In the case of biotinylated peptides, avidin was first suspended at 5μg/ml in water and allowed to dry overnight, 0.5μg/well, in Nunc Maxisorb plates. After washing and blocking, biotinylated peptides, 100pM, were added to each well and ELISA was performed as described above.

Peptide competitive ELISA were performed by preincubating antibodies (150 ng/ml) with the indicated peptide concentration in either room temperature for 2 hours or 4°C overnight. The antibodies were then added to Nunc plates coated with affinity-purified human placental β_1 integrin. The color was developed with alkaline phosphatase-conjugated rabbit anti-mouse IgG and substrate as described above.

Expression of NH_2 terminal peptide of human $ß_1$ chain in *E.coli:*

To localize the N29 epitope, a fusion protein, which contains residues 1-57 of the $ß_1$chain, was expressed in the pET-14b expression vector (Novagen) in *E. coli*. Polymerase chain reaction (PCR) amplifications were performed with the $ß_1$chain primer pair GTGAATTCATATG**CAAACAGATGAAAATAGATG** (5' primer) /GAGGATCCATATGTCA**TGGAGGGCAACCCTTCTTTT** (3' primer) (DNA sequence of $ß_1$chain were bolded here) using the plasmid from clone B105, which contains the first 105 aa of the human integrin $ß_1$chain and which can be recognized by monoclonal antibody N29. The products were digested with restriction enzymes EcoRI and BamHI, ligated into pBS(+) phagemid to introduce an restriction enzyme NdeI digestion site (Stratagene, La Jolla, CA). The recombinant phagemid was expanded, purified, and digested with NdeI. The $ß_1$fragment was purified and ligated to the expression vector pET-14b. The resulting insert was predicted to code for residues Gln^1 through Pro^{57} of the mature $ß_1$chain. The corresponding fusion protein was expressed in the competent *E.coli* BLR(DE3)plyss strain, purified with Ni^{2+} columns, and visualized by 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel by Coomassie Blue staining or immunoblotting with the indicated antibodies.

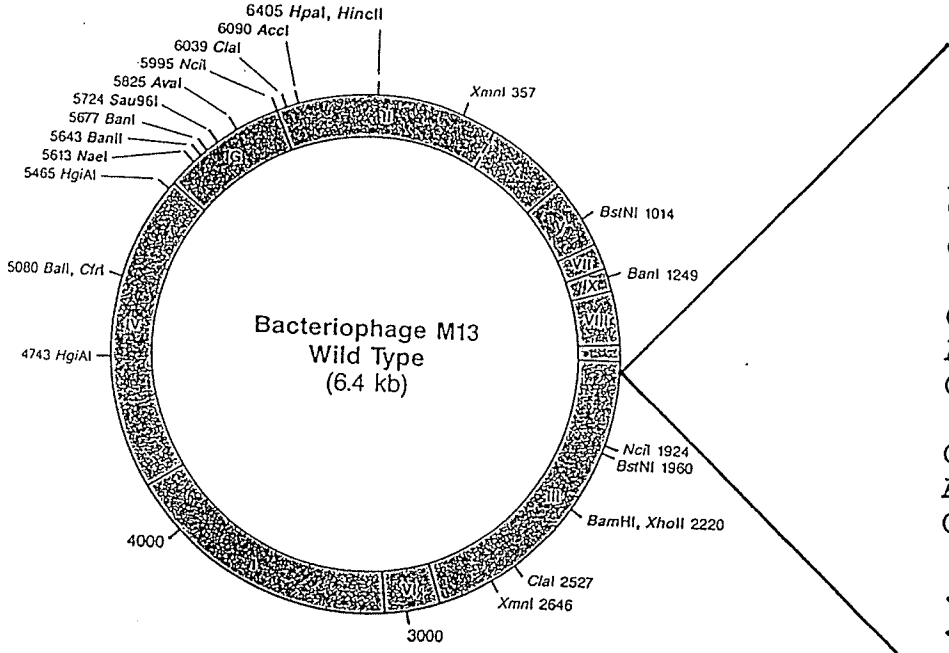
2.1 Localization of regulatory epitopes with random phage peptide display libraries:

The M13 phage based random peptide display library CMCC#3858 (15-mer) and 26-mer library was provided by Chiron Corporation. In these libraries, 45 or 78 base pairs of random synthetic oligonucleotides were inserted into gene III of phage M13 (***Figure 2.1***). Thus 15-mer or 26-mer peptides were displayed on the phage surfaces as a gene III fusion protein (123).

Phage expressing the appropriate gene III fusion protein can be recognized and isolated by the corresponding monoclonal antibody (***Figure 2.2***). The resulting phage can then be purified and analyzed (123).

Figure 2.1

RANDOM PEPTIDE DISPLAY LIBRARY IS MADE FROM BACTERIOPHAGE---M13

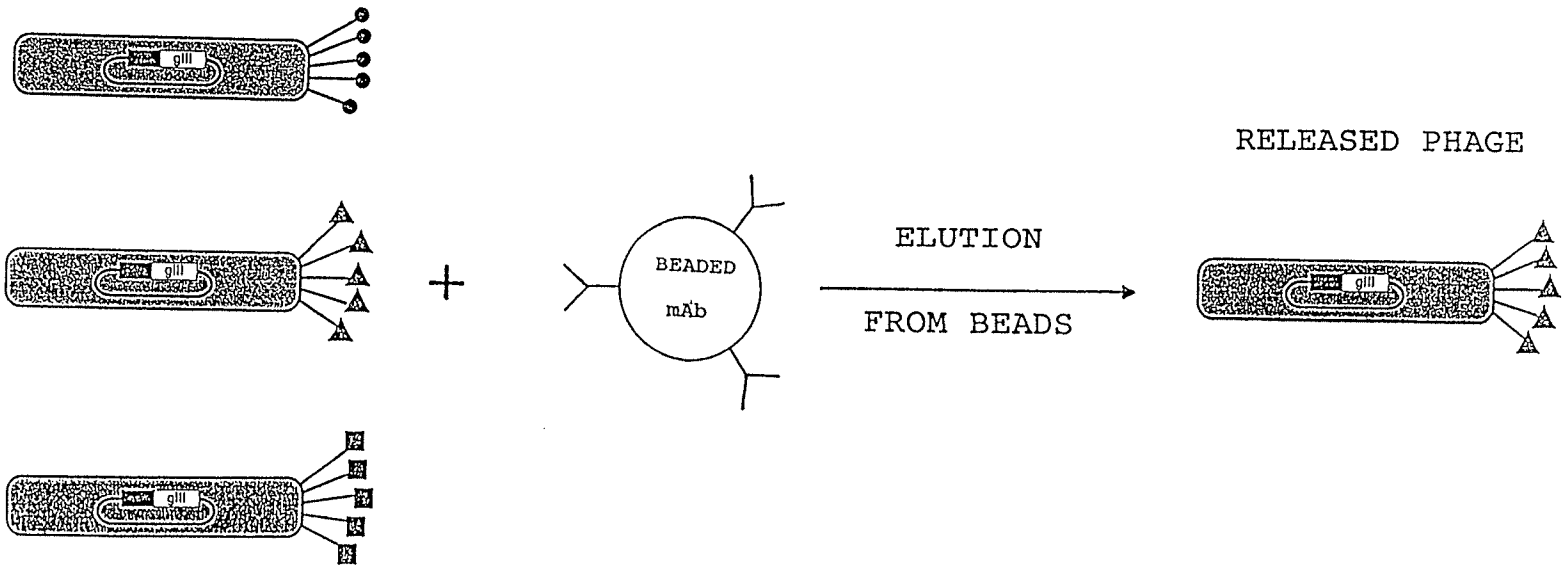


A	T	C	G
C	G	T	C
G	T	C	G
A	A	C	G
T	G	A	C
C	A	G	T
C	G	T	A
A	G	C	T
C	C	G	A
C	T	T	G
A	C	A	G
C	G	C	T
.	.	.	.
.	.	.	.
.	.	.	.
1	2	3	n

INSERT 45 bp
RANDOMLY
SYNTHESIZED
NUCLEOTIDES
TO GENE III

Figure 2.2

FOREIGN PEPTIDES DISPLAYED ON PHAGE SURFACES CAN BE SELECTED BY ANTIBODIES AND OTHER TARGET PROTEINS etc.



Phage selection: Clones were selected by incubating 10^{10} pfu phage in 1 ml of PBS with 1μg of biotinylated monoclonal antibodies (e.g. JB1A, B3B11 and 3S3) for 15 minutes with shaking. 20 μl of streptavidin conjugated magnetic beads (Dynal Inc., NY) were then added to the phage antibody mixture and incubated for an additional 30 minutes with shaking. The beads were magnetically collected and washed 8 times (1ml per wash) with PBS containing 0.1% BSA, 0.5% Tween 20. The phage were eluted from the beads in 100 μl of 0.1M glycine-HCl, pH 2.2, 0.1% BSA for 5 minutes. The beads were removed and the supernatant was neutralised with 9 μl of 2M Tris pH 8.0.

Phage titration: The eluted phages were titered with MV1190 *E.coli* cells. This step includes 1) Culture MV1190 in 10 ml LB medium at 37°C, (shake at 270 rpm) for about 6 hours to obtain fresh host cells. 2) Dilute eluted phage (10^2 to 10^{11} dilutions) in 200 μl volume with freshly prepared host cells in 96 well non-tissue treated culture microtiter plate (Flow Laboratories, Inc. Mclean, VA). 3) Mix 100 μl of diluted phage with another 100 μl of host cells and spot (20 μl/spot) this mixture on 1.5% LB medium agar plates (150x15mm, Fisher Scientific) and incubate at 37°C overnight. 4) Determine the titration of phage elution by counting the number of plagues on LB agar medium from this 20 μl mixture.

Phage amplification: For amplifying eluted phage, 200 μl of 6 hours cultured MV1190 cells were mixed with each individual 50 μl phage elute and incubated at 37°C for 15 minutes. This mixture was then added to 3ml melted (55°C) 0.7% agarose and

immediately poured on a 1.5% agar LB plate. The phage was amplified at 37°C overnight.

Phage purification: 5ml of sterilized TBS (0.15M NaCl, 50mM Tris-HCl, pH 7.5) were added to each plate and the plate was shaken gently for at least 4 hours to elute phage from LB plate. The TBS phage solution was collected and centrifuged at 13,000 rpm for 5 minutes to remove bacteria and debris. The supernatant was mixed with 0.15 volume PEG/NaCl and the phage was allowed to precipitate in 4°C for at least 4 hours. The phage were pelleted at 13,000 rpm and dissolved in fresh TBS for re-precipitation. Following a second precipitation the pellet was re-dissolved in 100 µl TBS for titration and for the second round of antibody selection.

Isolation of individual phage clones: After the 3rd round of antibody selection, individual plaques were isolated on LB agar plates. Individual plaques were picked with a Pasteur pipette and stored in 1ml TBS and shaken overnight at 4°C to elute phage from agarose and agar. 100µl supernatant of each plaque was used for amplification.

Phage ELISA: Individual clones were amplified and purified as above. For phage ELISA, PEG/NaCl re-precipitated phages were dissolved in 100 µl 0.15M NaCl and further precipitated with 11µl 1M acetic acid at room temperature for 10 minutes and on ice for another 10 minutes. After centrifugation, the phage pellet was dissolved in 500 µl TBS.

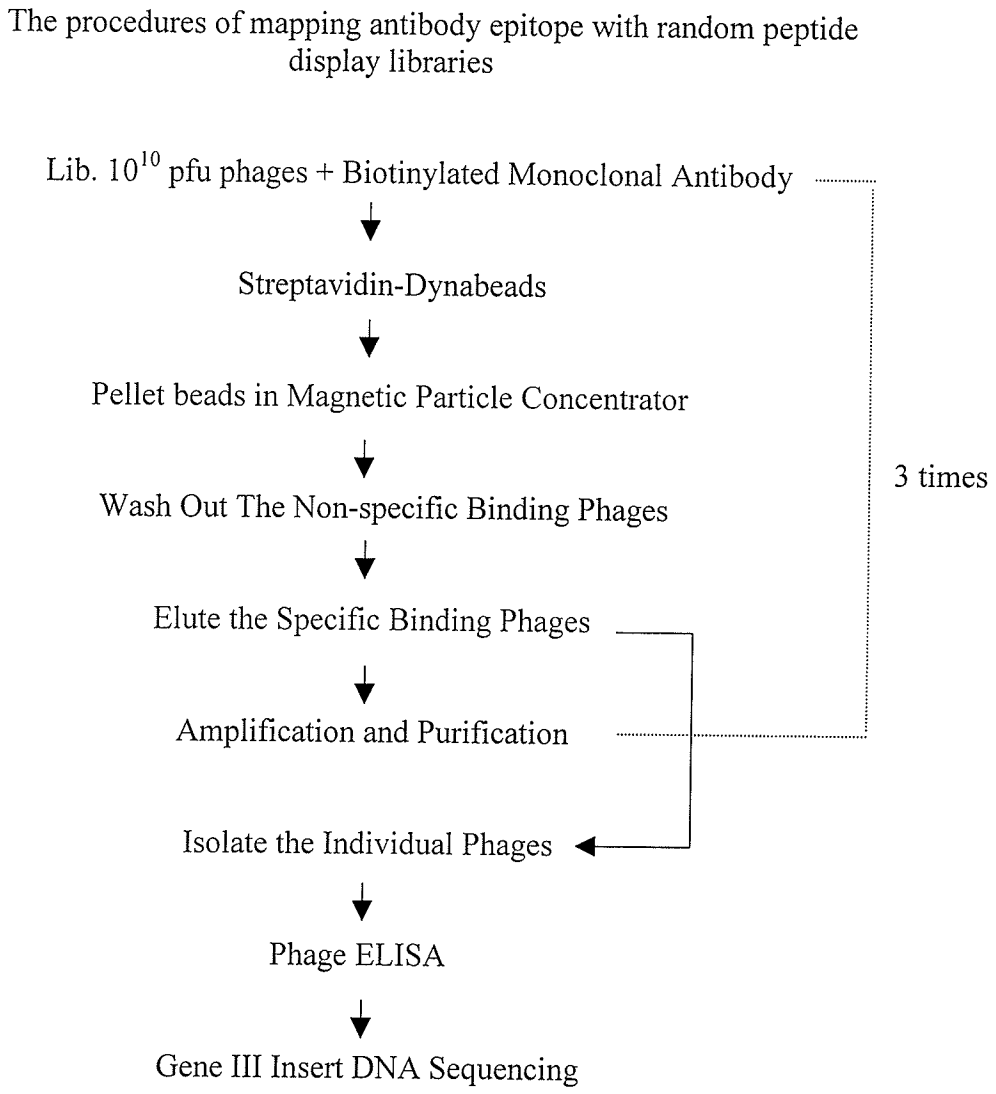
The phage was then coated in ELISA plate at 3x 10^{10} phage/100 μl/well in 4°C overnight. The excess phage was removed and the wells were washed and blocked with Blotto solution (5% non-fat milk, 0.02% NaN_3 TBS). After washing 4 times with TBST (0.5% Tween 20, TBS), 100 μl/well antibody (5μg/ml) was added and the reaction was monitored with 1:500 diluted anti-mouse IgG HRP conjugate.

Phage DNA isolation: 200 μl of purified phage (about 2x 10^{10}) were mixed with 100 μl Tris-HCl saturated phenol and vortexed twice. The aqueous phase was separated by centrifugation at 13,000rpm for 1 minute and transferred to 600 μl Alcohol/Na Acetate (absolute alcohaol/3M NaAc = 25:1). Phage DNA was precipitated in 4°C overnight. DNA pellet was washed once with 70% alcohol and air dried. This DNA sample was dissolved with 15 μl sterilized ddH_2O and analyzed by electrophoresis in 0.7% agarose gels and used for template of DNA sequencing.

Phage insert DNA sequencing: The protocol was based on instructions for the double strand DNA sequencing kit (BRL/Gibco). 6 steps were involved in this protocol, they are 1) End-labeling of primer with [γ-^{32}P] ATP, 2) Prereaction mixture, 3) Sequencing reactions, 4) Incubation in a thermal cycler, 5) Termination of PCR reaction, 6) Gel electrophoresis. The oligonucleotide sequence of gene III: ACA GAC AGC CCT CAT AGT TAG CG was used as primer (124). [γ-^{32}P] ATP and X-ray films were respectively purchased from Amershan and Kodak. X-ray films were developed in either a Kodak or Fuji automatic X-ray film developing system.

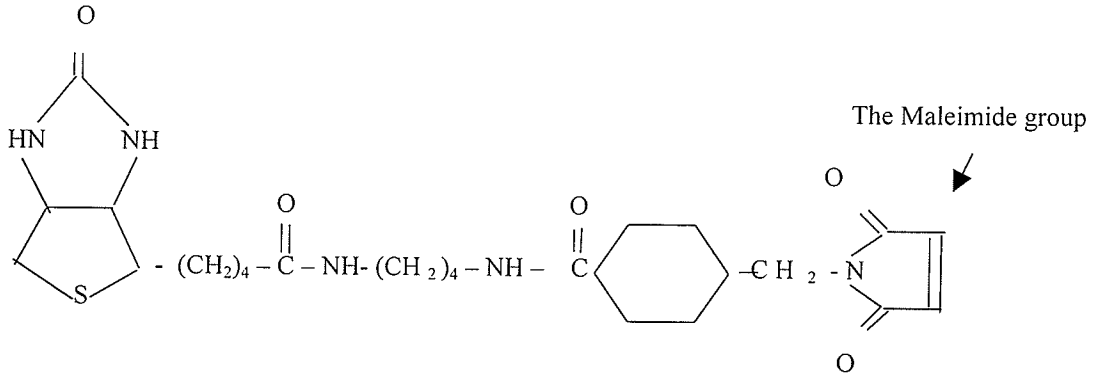
The procedures of mapping antibody epitopes with random peptide display libraries as shown on ***Figure 2.3***.

Figure 2.3:



2.2 Labeling free sulfhydryls of human $ß_1$ integrins with biotin-BMCC (1-biotinamido-4-[4'-(maleimidomethyl)-cyclohexane-carboxamido] butane) on cell surface.

The reducing agent dithiothreitol (DTT) induces platelet aggregation (87) and $ß_1$ integrin activation (114) suggesting a possible regulatory role for disulfide bonds in integrin function. To further investigate the role of disulfide bonds of $ß_1$ integrin, both adhesive (Jurkat) and non-adhesive (IM9) cells or the same cells following treatment with DTT, EDTA or Mn^{2+} were labeled with biotin-BMCC. BMCC contains a maleimide group that is highly specific for free sulfhydryls. This reagent is membrane impermeable. The chemical structure of biotin-BMCC is shown below.



Labeling cells with biotin-BMCC:

IM9 cells and Jurkat cells were washed twice with Hank's balanced buffer (Gibco Life Sciences) and suspended in Hank's buffer at 2 x 10^7/ml (IM9) and 1 x 10^7/ml (Jurkat). Each type of cell was aliquoted into 4 tubes (5ml/tube) for different treatment i.e.10mM EDTA, 2mM Mn^{2+}, 10mM DTT, and untreated control. After treatment at room

temperature for 30 minutes, biotin-BMCC dissolved in DMSO was directly added to each tube at a concentration 1 μM except in the case of DTT treated cells which were washed 3 times to remove excess DTT before biotin-BMCC labeling. The labeling reactions were kept in the dark at room temperature for 2 hours. The BMCC labeled cells were washed three times with PBS containing 10% DMSO to remove excess biotin-BMCC. The cells were then lysed with cell lysis buffer (10 mM PBS pH 7.4, 1% NP-40, and 1 mM PMSF) on ice for 30 minutes and –20°C for overnight.

Western blot analysis of biotin-BMCC labeled integrins:

The supernatant of 400 μl cell lysate/each treatment was immunoprecipitated with 50μl JB1A coupled Sepharose 4B beads at 4°C overnight. After 4 washes, the bead-bound materials were treated with SDS gel-loading buffer (50mM Tris.Cl pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and separated on an 8% SDS PAGE gel in Tris-glycine electrophoresis buffer (25mM Tris, 250 mM glycine pH 8.3, 0.1% SDS) and electrotransferred to nitrocellulose membranes in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol). The membrane was blocked with 1% BSA buffer A (200 mM NaCl, 100 mM Tris-Cl, pH 7.5) overnight and washed once with 0.05% Triton X-100 buffer A. The membrane was then incubated with 1:4000 diluted avidin-alkaline phosphatase in antibody diluted buffer (buffer A plus 0.1%BSA, 0.05% Triton X-100, 0.05% NaN_3) at room temperature for 1 hour. After washing 3 times with washing buffer (0.05% Triton X-100 buffer A) and once with buffer C (100 mM

NaCl, 49 mM $MgCl_2$, 100mM Tris-Cl, pH 9.5), the color was developed with NBT and BCIP.

Digestion of human β_1 integrin with O-sialoglycoprotein endopeptidase:

Human purified β1 integrin 10μl (about 20μg) was boiled 5 minutes and then digested with 20μl (2.4mg/ml) O-sialoglycoprotein endopeptidase at 37°C overnight. Protein fragments were separated by 10% SDS-PAGE under reducing condition and transferred to nitrocellulose membranes. The membranes were first blocked with 1% BSA buffer A (200 mM NaCl, 100 mM Tris-Cl, pH 7.5) at room temperature for 1 hours. The membranes were then incubated with biotinylated B44 or JB1A (1μg/ml) in antibody diluted buffer (buffer A plus 0.1%BSA, 0.05% Triton X-100, 0.05% NaN_3) for 2 hours, and subsequently were incubated with 1:2000 diluted avidin-alkaline phosphatase for 3 hours. After each step, the membranes were washed 3 times with TBST. The color was developed with NBT and BCIP as described above. Purified human β1 integrin treated with 20μl PBS was used as a negative control.

CHAPTER 3: RESULTS

3.1. GENERAL INTRODUCTION OF THIS STUDY

Integrins can be expressed in different functional states on the cell surface (i.e. inactive and active or ligand occupied conformations)(100). Although the physiological significance of conformational transitions from one state to another has been gradually understood, the structural bases for integrin conformational change are currently unclear.

Different techniques, such as electron microscopy and x- ray crystallography have been used to address this question. Electron microscopic images suggest that the α and β subunits interact with each other, and form a globular structure near their NH_2- termini. A model based on this profile suggests that these globular structures contain the ligand binding site (1); X-ray crystallography has provided a three-dimensional structure of the I domain of α_L (CD11a) and α_m (CD11b) chains (50, 52) and it has been suggested that all β subunits contain I domain-like structures (50). However, the crystal structures of the intact α or β chain and integrin heterodimer are not available at present. In fact, because of the inherent limitations of these techniques, it has not been possible to determine the conformations of integrin molecules in different functional states. Monitoring antibody epitope expression patterns has provided an alternate approach for examining the dynamics of $ß_3$ integrin conformational changes (125-129). In contrast, information about the $ß_1$ integrins was considerably less well developed when this project was initiated, despite the fact that members of the $ß_1$ group represents the largest subfamily of integrins.

Previous investigations in this laboratory have generated monoclonal antibodies against human integrin $ß_1$ chain. Depending on their effects on Jurkat cell adhesion to

fibronectin, these antibodies can be divided into three groups (i.e. stimulatory, neutral, and inhibitory) (114, 122). As shown in ***Figure 3.11***, antibodies B44, N29 and B3B11 enhance cell adhesion to fibronectin, while antibodies JB1A and 3S3 block this process. These results raise several important questions: How do these antibodies influence cell adhesion? Where are their binding sites i.e. " epitopes"? Does their epitope expression depend on the functional status of $ß_1$ integrins, or in another words, can these epitopes provide some insights into $ß_1$ integrin conformational transition at the structural level? And finally, can these epitopes provide some guidance in drug design in order to control cell adhesion and inflammation?

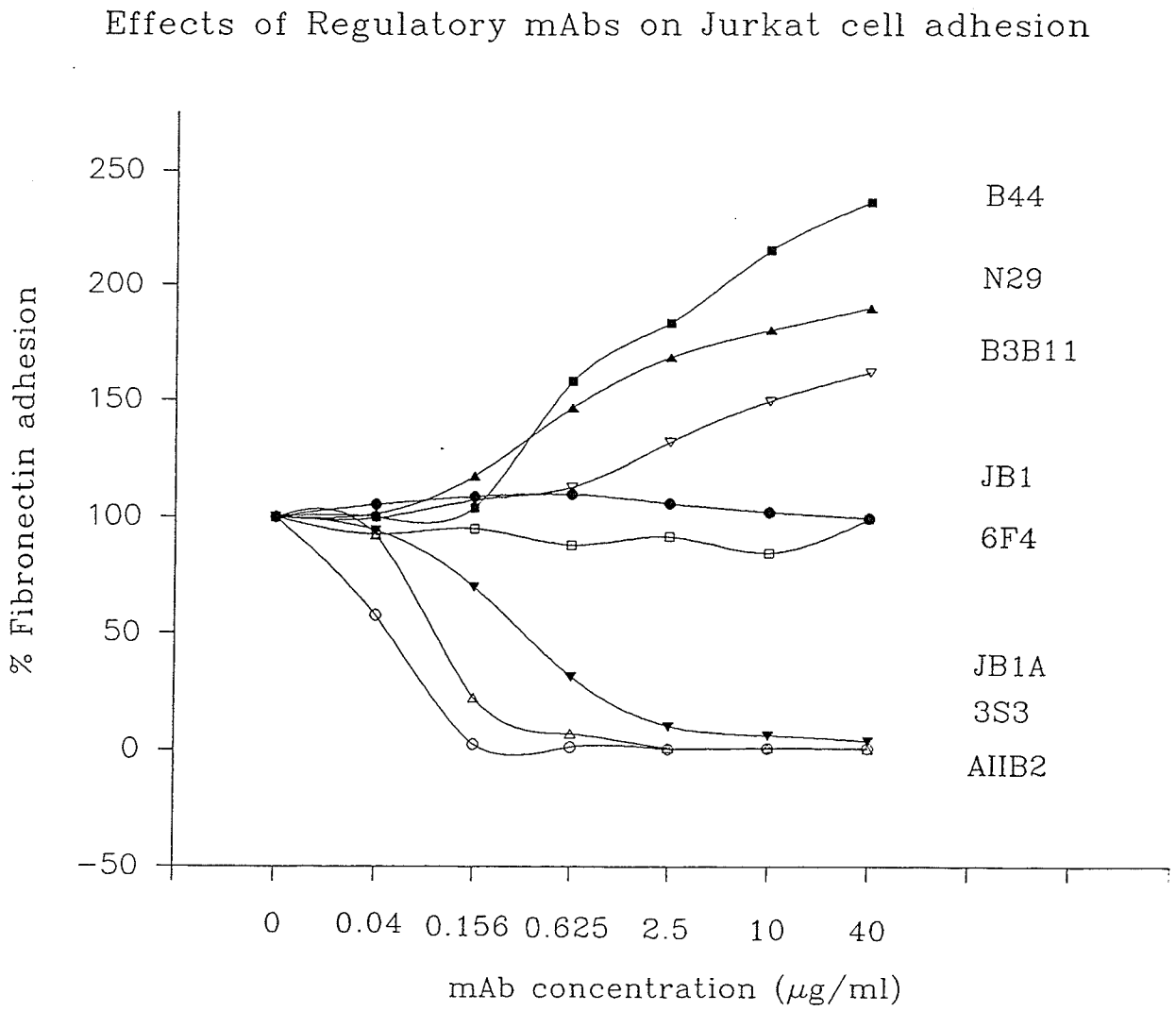


Figure 3.11. A comparison of the effects of antibodies to β_1 integrin on Jurkat adherence to fibronectin. Cells were pre-treated with the indicated antibodies for 30 minutes and assessed for their adherence to immobilized fibronectin (5μg/ml)(see method in chapter 2). The absorbance of untreated Jurkat cell adhesion is considered as 100%. The results are the mean of triplicate assays and they are representative of one of three experiments. The SEM was less than 10% of mean.

To map these regulatory epitopes, several molecular biological and immunological methods were employed in this study. 1) Screening random peptide phage display libraries; 2) PCR amplification and expression in *E.coli* of integrin derived peptide sequences 3) Western blot analysis of human /chicken and human /mouse $ß_1$chimeric proteins; 4) Peptide ELISA. In order to monitor the epitope expression in different functional states of $ß_1$ integrins, adherent, non-adherent cells and cells activated by different stimuli were used. The adherent cells included Jurkat cell (T cell) and K562 cell. The non-adherent cells included IM9 (B cell) and peripheral blood lymphocytes. The stimuli used in these studies included divalent cations, PMA, the reducing agent DTT, or anti-CD3 antibody that induces T cell adhesion by triggering T cell receptor mediated signaling events. Epitope expression levels were quantitated either by FACS or by antibody mediated cell capture assays.

Using these methods, three distinctive stimulatory epitopes (i.e. N29, B44, and B3B11) and two different types inhibitory epitopes (i.e. JB1A and 3S3) have been localized. The regulatory roles of -NH2 terminal cysteine rich domain (residue 15-55), residue 82-87, residue 355-425 and residue 636-705 and the possible contributions of disulfide bonds and O-linked carbohydrates to integrin functionality were suggested by these studies.

3.2. LOCALIZATION OF REGULATORY EPITOPES

3.21. Localization of N29 epitope

Several molecular techniques such as NovaTope libraries and phage peptide display libraries were used to map regulatory epitopes in this laboratory. These systems usually

are not able to localize carbohydrate or discontinuous epitopes because the host cells are prokaryotic bacteria, i.e. *E. coli*, in which protein folding and posttranscriptional carbohydrate modification can be different from eukaryotic human cells.

The N29 epitope was expressed on adhesive cells (such as Jurkat and K562) but its expression level on non-adhesive cells (such as IM9 cells and peripheral blood lymphocytes) was significantly lower (see following study in this section). Previous studies had demonstrated that the epitope was detected by western blot analysis under reducing conditions. This raised the possibility that this epitope is a cryptic, linear epitope in nonadhesive cells rather than an activation dependent discontinuous epitope. To address this question, cell lysates from Jurkat and IM9 were analyzed with JB1A and N29 in a western blot analysis. β_1 integrins chains of both Jurkat (adhesive) and IM9 (nonadhesive) express the N29 epitope under reducing conditions (***Figure 3.21a)***. Thus N29 epitope is a linear or continuous epitope on human integrin $\beta_1$chain which is apparently only accessible on the cell surface under certain conditions (i.e. active form of $\beta_1$integrin, see the following discussion). This result suggested that those molecular approaches used in the laboratory might be useful to identify this epitope if it is not a carbohydrate dependent epitope. That β_1 chain band of IM9 cells is wider than that of Jurkat cells may result from different carbohydrate post-translation modifications between this two types of cells.

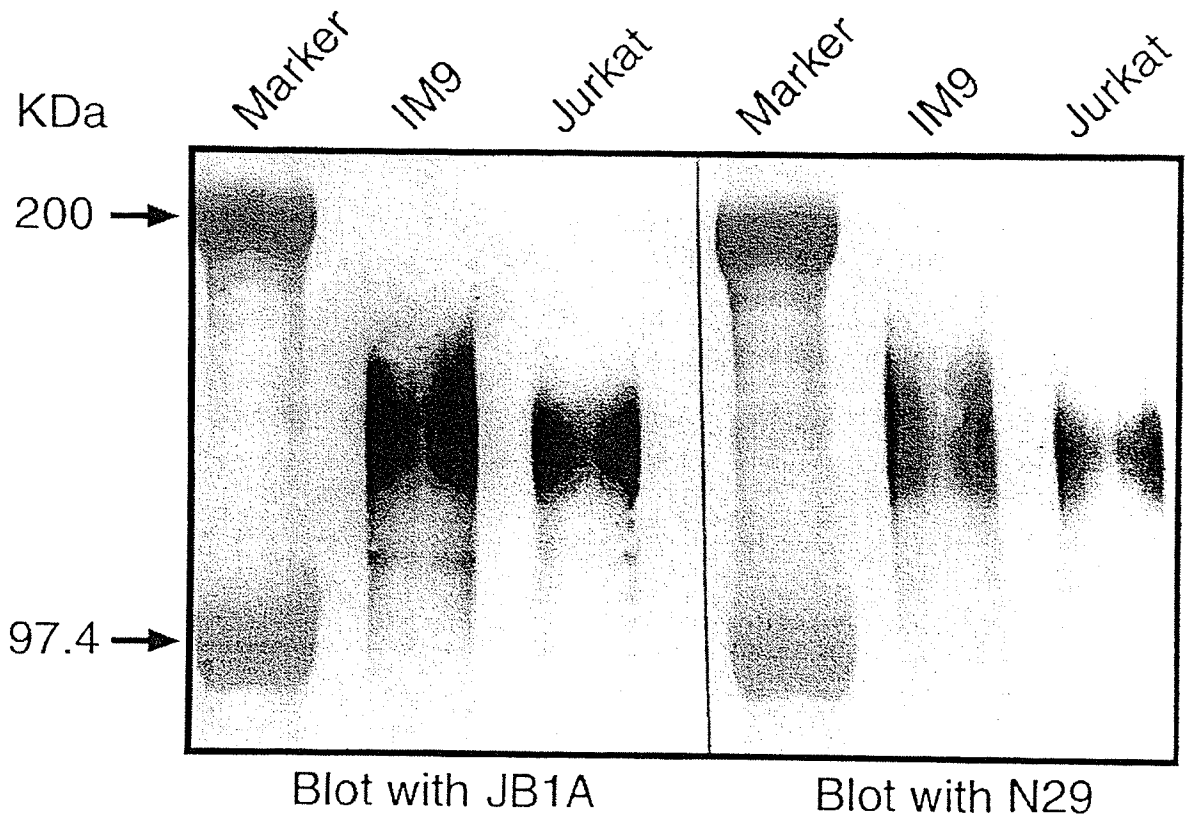


Figure 3.21a. Western blot analysis of the human integrin chain using N29 and JB1A monoclonal antibodies. 1x 10^8 cells/ml were lysed in PBS containing 1% NP-40, 1mM PMSF. 200µl supernatants of each cell lysates were immunoprecipitated with 50µl JB1A beads (containing 200µg JB1A). JB1A beads and integrin complexes were then mixed with SDS DTT loading buffer and heated at 100 °C for 5 minutes. Immunoprecipitated proteins were separated in 8% SDS PAGE gel and transferred to nitrocellulose membrane. JB1A and N29 epitopes were detected by individual monoclonal antibodies and rabbit antimouse IgG alkaline phosphatase conjugate. Color was developed with NBT and BCIP.

Localization of N29 epitope to residue 15-55 of integrin ß$_1$ chain

Screening a ß$_1$ epitope library identified a single N29 reactive clone, B105, (Anli Li data not shown). DNA sequencing of B105 indicated that this clone contained the first 105 residues of the ß$_1$chain plus an unknown source of 60 residues in its NH2- terminus. These 60 amino acids are not encoded from the immediate 5' insert plasmid. Since this clone was also recognized by JB1A which recognizes residue 82-87 of the ß$_1$chain (see section 3.22), this suggested that the recombinant protein was in the correct reading frame. Thus the N29 epitope was in either the first 105 aa of ß$_1$chain or the NH2-terminal 60 aa of B105 clone. Previous studies had determined that N29 did not recognize residues 55-105 (Caixia Shen data not shown). Therefore, it was likely that N29 epitope was located in the first 55 aa of ß$_1$chain.

To localize the N29 epitope, a fusion protein, which contained residues 1-57 of ß$_1$chain, was expressed in a pET-14b expression vector (Novagen) in competent *E.coli* BLR(DE3) plyss strain and purified with Ni^{2+} columns(see Materials and Methods in Chapter 2). The fusion protein was analyzed in 15% SDS-PAGE gel and western blot with N29 antibody. As indicated in ***Figure 3.21b***, N29 specifically recognized this fusion protein (indicated by the larger arrows). Thus, N29 epitope is located in the first 55 aa of integrin ß$_1$chain.

Figure. 3.21b

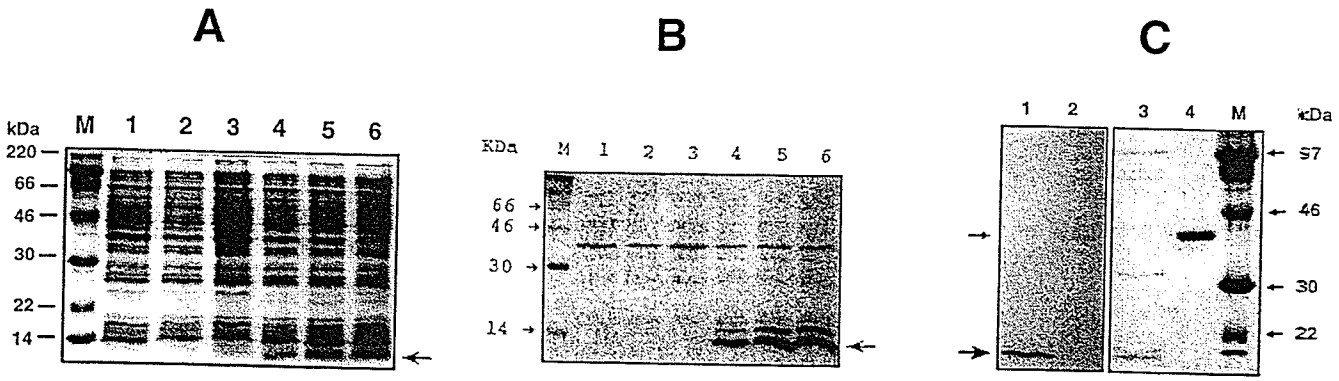


Figure 3.21b. The expression and reactivity of the $\beta_1$1-57 fusion protein. Panel A, Coomassie Blue-stained SDS-PAGE gel; Lane 1, no vector; lane 2, vector only; lane 3, vector plus insert uninduced; lane 4-6, isopropyl-1-thio-ß-Dgalactopyranoside-induced cells containing the $\beta_1$chain 1-57 insert at 1, 2, 3 hours post induction. The arrow indicates the location of the fusion protein. Panel B, a Western blot of a replicate of panel A stained with N29. Panel C, lanes 1 and 3 contain purified fusion protein, lanes 2 and 4 contain a gonococcal porin (1b) fusion protein produced in the same vector as a control. Lanes 1 and 2 were reacted with N29; lanes 3 and 4 were stained with Coomassie Blue.

Considering that the stimulatory antibody AP5 recognizes residues 1-6 of $ß_3$ chain (129), it was questioned if the N29 recognized the homologous region of the $ß_1$chain. An examination of a peptide containing the first 14 aa from $ß_1$ chain clearly demonstrated that N29 does not recognize this sequence (***Figure 3.21c***). Therefore, N29 epitope is located between residue 15-55, a cysteine rich domain of the human integrin $ß_1$chain. It may be noteworthy that this region is adjacent to the long-range disulfide bonds (Cys^{7}-Cys^{444}) and close to the epitope recognized by the inhibitory antibody JB1A (residues 82-87 see below).

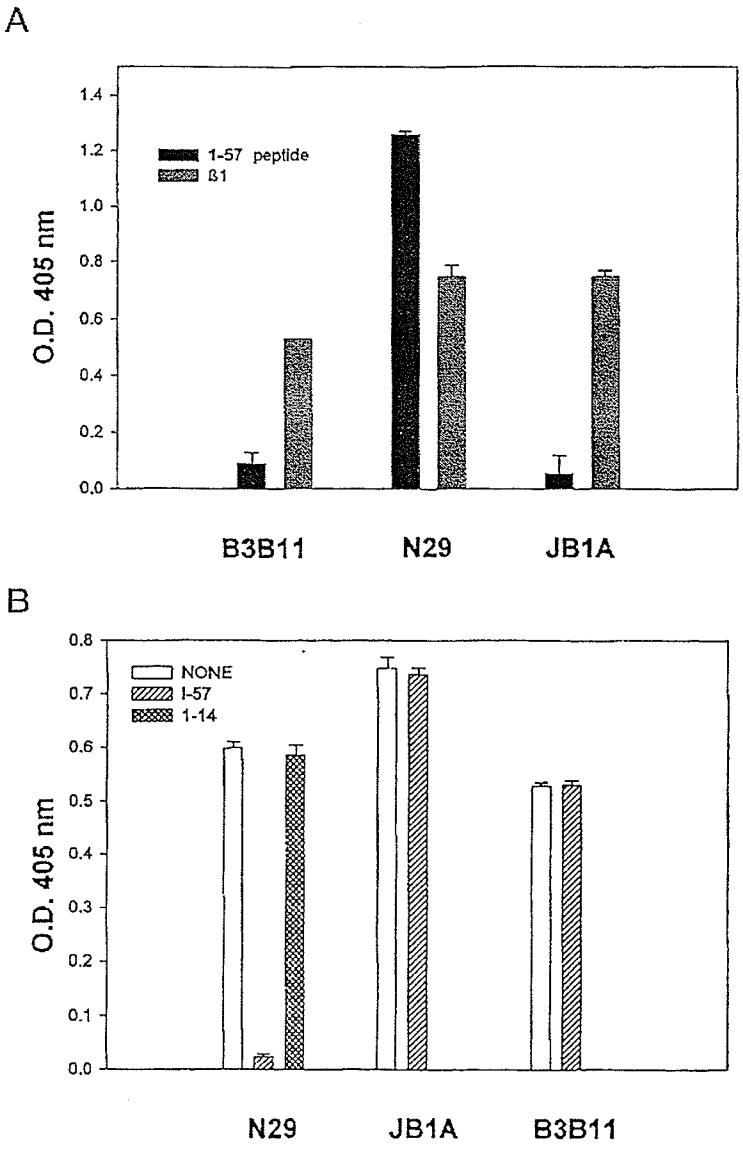


*Figure 3.21c. The specificity of antibody binding to the fusion protein 1-57. A. The binding of B3B11, JB1A, and N29 to immobilized fusion protein 1-57 or to purified native β_1 integrin were compared in an enzyme-linked immunosorbent assay. **B**, the capacity of fusion protein 1-57 to block the binding of the indicated antibodies to immobilized purified β_1 integrin. The effects of a synthetic peptide-containing residue 1-14 on N29 binding were also determined.*

3.22. Localization of epitopes of JB1A group antibodies

The studies in this laboratory have previously demonstrated that inhibitory antibodies JB1A, C30B, and D11B can each inhibit the binding of one another to the cell surface (Stupack DG, data not shown). However, none of these antibodies was able to block the cell binding of the inhibitory antibody 3S3. Thus, at least two different groups of inhibitory antibodies have been classified in this laboratory. Since the JB1A group of antibodies recognizes continuous epitopes, it was speculated that their epitopes might be close to each other.

Localization of epitopes of JB1A group antibodies to residue 82-87 of integrin $ß_1$ chain:

Screening of an epitope library (NovaTope library), localized the JB1A epitope between residues 61-93 of $ß_1$chain (Shen C, data not shown, please see the Figure 2 of the appendix of published paper II). Subsequent screening of a 15 mer random peptide phage display library determined that JB1A recognized a major motif (i.e. an aa sequence that was represented in most of phage clones) S/TxxKLK (potentially corresponding to $ß_1$ sequence TAEKLK, i.e. residue 82-87) and a minor motif TxxKLR (potentially corresponding to $ß_1$ sequence TPAKLR, i.e. residue 179-184) (Hunt N, data not shown). The major and minor motif were determined by the frequency of phage clones isolated by JB1A (see the Figure 3 of the appendix of published paper II).To further identify the JB1A epitope, synthetic peptides biotin-SGSGTAEKLK and biotin-SGSGTPAKLR were used, and examined by ELISA for their binding of the JB1A group of antibodies. ***Figure 3.22a*** shows that JB1A recognizes TAEKLK, but the affinity for TPAKLR is very low.

Surprisingly, C30B and D11B also recognized the former 6 aa. Peptide inhibitory ELISA (i.e. preincubation of antibodies with TAEKLK or control peptides) further confirmed that peptide TAEKLK blocked this group of antibodies but did not affect the binding of other inhibitory antibodies such as 3S3, AIIB2 binding native $\beta_1$integrin (***Figure 3.22b &c***). Thus, the JB1A group of antibodies recognizes the same residue TAEKLK (residue 82-87) which were close to the stimulatory N29 epitope.

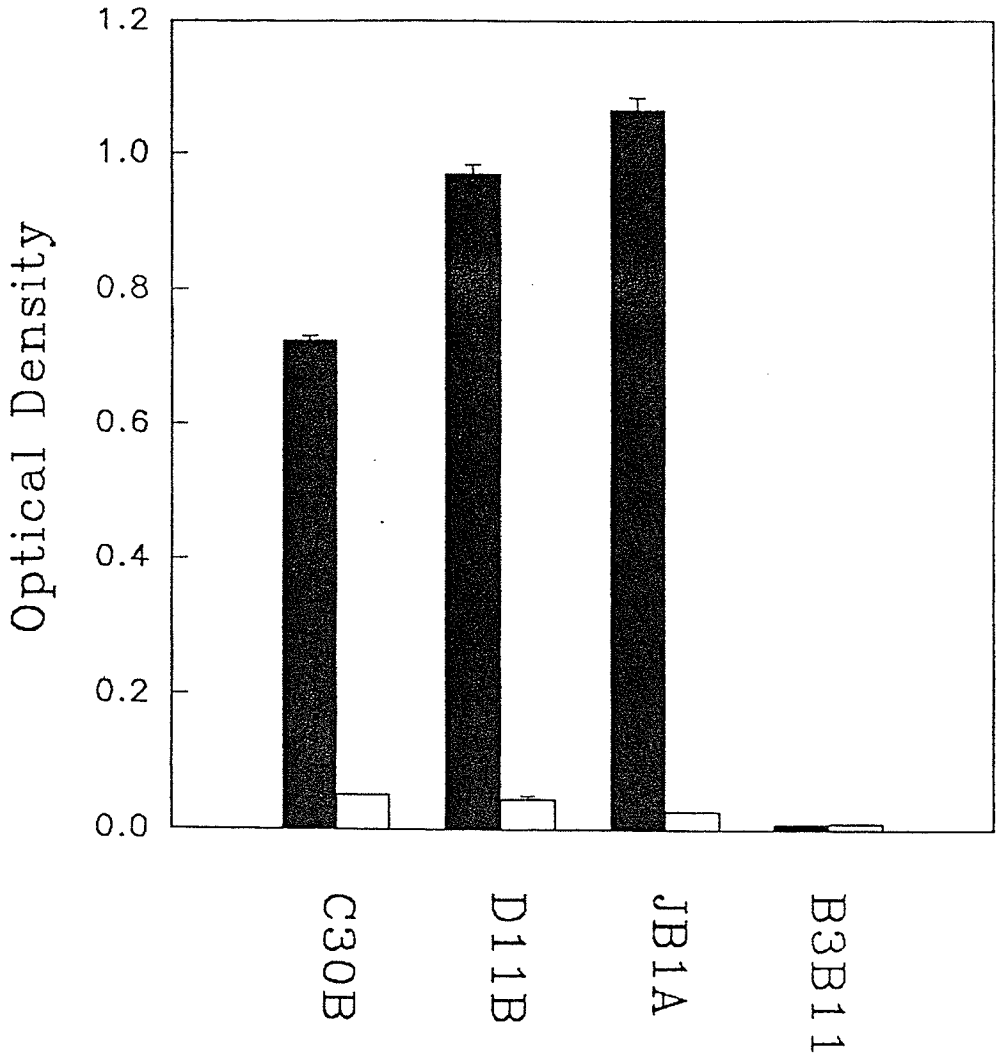


Fig.3.22a The binding of anti-β_1 monoclonal antibodies to peptides containing the predicted JB1A epitopes. The binding of JB1A, C30B, D11B, and B3B11 to the peptides, TAEKLK (82-87) (solid bar), and TPAKLR (179-184) (open bar) identified respectively by the major and minor consensus sequences in the random peptide phage library isolates. Antibody B3B11 has previously been shown to bind to a peptide corresponding to residues 657-670 .

Figure 3.22b

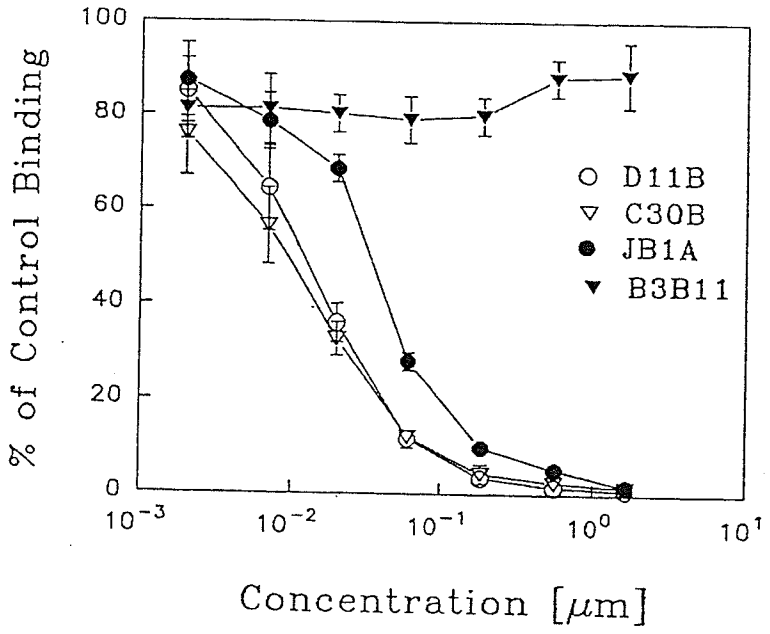


Fig. 3.22b The effects of β1 peptide 82-87 on antibody binding to purified β1 integrin. Antibodies were mixed with the indicated concentrations of peptide, incubated and assesed for their residual binding to immobilized purified β1 integrin. The antibodies JB1A, C30B, and D11B were inhibited from binding to the integrin, in contrast B3B11 binding was not influenced.

Figure 3.22c

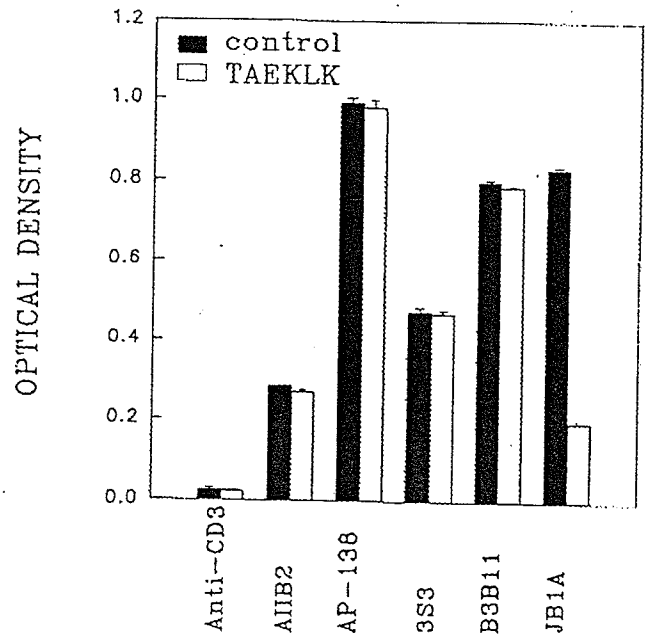


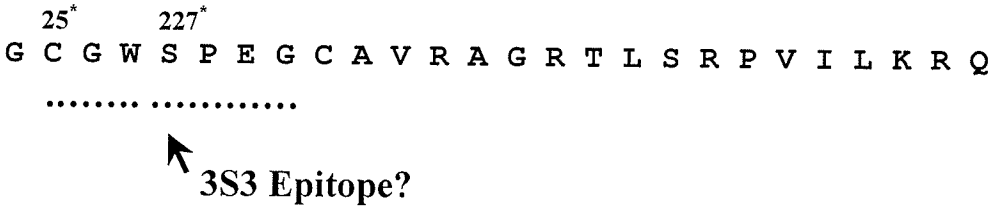
Fig. 3.22c A comparison of the effects of β_1 peptide 82-87 on the binding of other antibodies to purified β_1. Antibodies to β_1 were incubated with the peptide TAEKLK and assessed for binding to purified β_1. The anti-CD3 monoclonal was included as a specificity control for antibody binding to integrin.

3.23. Localization of 3S3 epitope with a 26-mer phage peptide display library:

3S3 is one of the strongest inhibitory antibodies. It recognizes a discontinuous epitope on β_1 integrin (Wilkins JA et al, data not shown). Both the NovaTope library (Li A, data not shown) and 15mer-phage peptide display library (did by myself) failed to provide information of its epitope. However, screening of a 26mer-phage peptide display library with biotinylated 3S3 resulted in the isolation of several clones. Sequencing of 21 clones revealed that 18 clones were identical. These 18 clones have slightly higher O.D. values than other clones and wild type M13 in a phage ELISA although all O.D. values less than 0.06 (O.D. value > 0.1 was usually considered as a positive signal in our phage ELISA) (data not shown). Figure 3.23a shows the insert amino acid sequence of the dominant isolates (18 out of 21 isolates).

Figure 3.23a

Amino Acid Sequence Deduced from Insert of 3S3 Phage



* *indicated the residue 25 and 227 of the β_1 chain*

The sequence of SPEG is present in the second conserved region of integrin β_1 chain that may be homologous to one of suggested ligand binding sites in β_3 chain (53).

Furthermore, the SPEG homologous region in $ß_3$ chain immediately follows a region that is involved in an intra chain interaction (130). In order to address a possible role of this conserved region in adhesion, it was examined for its capacity to block Mn^{2+} activated IM9 cell adhesion to fibronectin. My preliminary data showed that both the first (D^{120}YPIDLYYLMDLSYSMKDDL^{139}) and the second (I^{221}SGNLDSPEGGFDAIMQVAVC^{241}) conserved regions were able to block cell adhesion (***Figure 3.23b***). Therefore, the reasons for 3S3 blocking cell adhesion may be its occupancy of the ligand-binding site and/or obstructing the $ß_1$ integrin from forming the ligand-binding pocket.

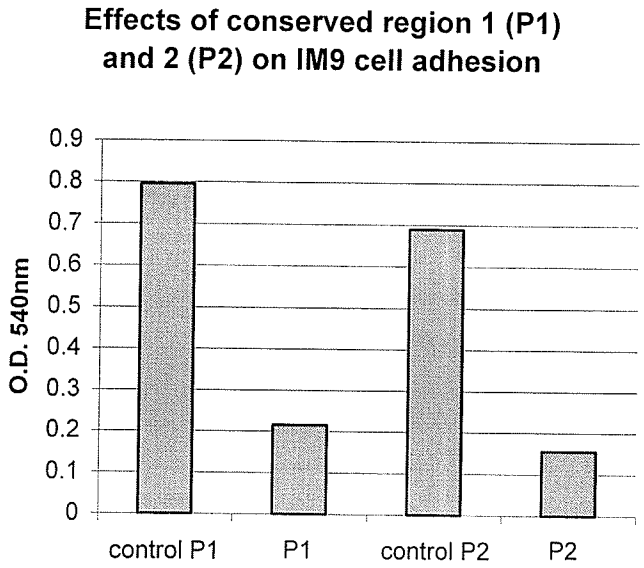


Figure 3.23b. The effects of two-conserved region peptides on IM9 cell adhesion. IM9 cells were pretreated with 2mM Mn^{2+} and 50μM peptide from conserved region 1(P1) or 2 (P2) at room temperature for 30 minutes. Cells were then added to fibronectin coated plate at 37°C for 60 minutes. Adherent cells were stained and quantitated as described in materials and methods. Control P1 and control P2 represent cells treated with same amount of H_2O or DMSO of experimental groups (P1 was dissolved in H_2O and P2 was dissolved in DMSO). Means of six wells/each group were presented.

3.24. Detailed localization of epitopes of B3B11 group antibodies

The B3B11 group of antibodies (B3B11, JB1B and 21C8) are stimulatory. Because they are capable of inhibiting each other in cell binding assays, their epitopes may be close to each other. B3B11 and JB1B epitopes are not lost upon reduction suggesting they be linear epitopes (Wilkins JA et al, data not shown). These two epitopes have been localized to the membrane proximal region of the $\beta_1$chain (636-705) with the NovaTope library (ref. 122,Caixia Shen and Anli Li data not shown, see the appendix of published paper I). The B3B11 epitope was further localized to residues 657-670 with peptide ELISA and peptide competitive ELISA (*Figure 3.24a &b*).

Figure 3.24a & b

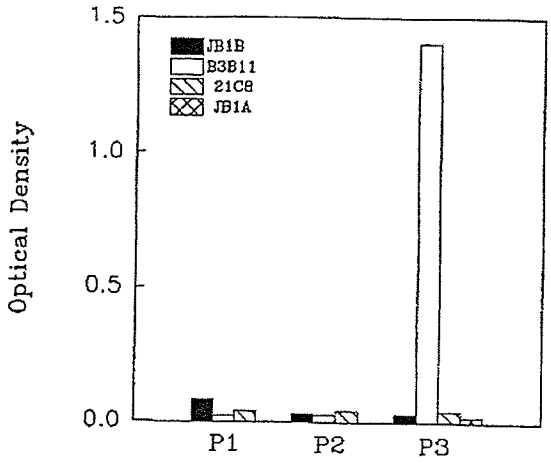


Fig.3.24a **The reaction of anti β_1 antibodies with synthetic peptides corresponding to the B3B11 epitope of β_1.** Three overlapping peptides corresponding to residues 636–649 (P1), 646–659 (P2), and 657–670 (P3) were examined for their reactivities with JB1B, B3B11, and 21C8. These fragments spanned the entire sequence of the B3 clone isolated from the epitope fusion library *(ref. 122)*.

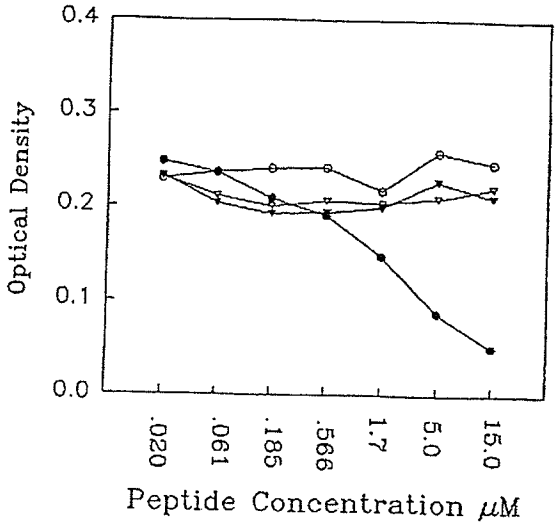


Fig.3.24b. **Competitive inhibition of B3B11 binding to purified β_1 by P3.** B3B11 was preincubated with peptides 1 (▼), 2 (▽), or 3 (●) and assayed for residual binding to immobilized purified β_1 integrin. The effects of P3 on JB1A (○) were also determined.

Further localization of B3B11 epitope with a 15mer phage peptide display library: To identify B3B11 epitope, a random peptide phage display library was screened with biotinylated B3B11. After a three rounds of selection, individual isolates were amplified and 10 out of 19 selected isolates showed significant reaction with B3B11 in phage ELISA (***Figure 3.24c)***. DNA sequencing indicated all these positive phages have the same insert peptide (***Figure 3.24d***). Comparison of the phage insert and the ß1 amino acid sequences indicated the pentameric peptide PQPVQ of the phage insert exists in the $ß_1$chain (residue 660-664), NovaTope library positive clones and the positive peptide. The synthetic peptide KLPQPVQPD (residue 658-666) is able to block B3B11/native $ß_1$chain interaction (***Figure 3.24e***). Thus, residue 660-664 is the core of the B3B11 epitope.

Figure 3.24c

Figure 3.24c. Comparison of the binding of B3B11 and C30B to B3B11 immunoselected phage. 3x 10^{10} individual cloned phage from three rounds of immunoselection were coated onto microtiter wells. The indicated antibodies (5μg/ml) were added and the binding was quantitated using a peroxidase-rabbit anti mouse IgG conjugate. 10 out of 20 clones specifically recognized by B3B11. The O.D. values of clone 8 and 12 were less than other positive clones might result from the different amount of phage were coated.

Figure 3.24d

Clone No.	Sequence
1,3,7,8,9,10,12,13,14,16	T I H H P Q P V Q G L S M P N
4,5,15	I F N V K K L L F A I P L V V
18,19	L N R T W S W L S P F G R N V
2	S N P S M P W N T R F P I W S
6	P E F L H K G L F L N Y R S S
11	A N S Y F L T L P T L S M K R
17	N S A T F I K N L L P H T S L
20	T R C T T S T P R S T P T Q T
Beta-1 Seq.	R D K L P Q P V Q P D P S H C

Figure 3.24d. Comparison of insert amino acid sequences of B3B11 immunoselected phages and human β_1 integrin chain.

Figure 3.24e

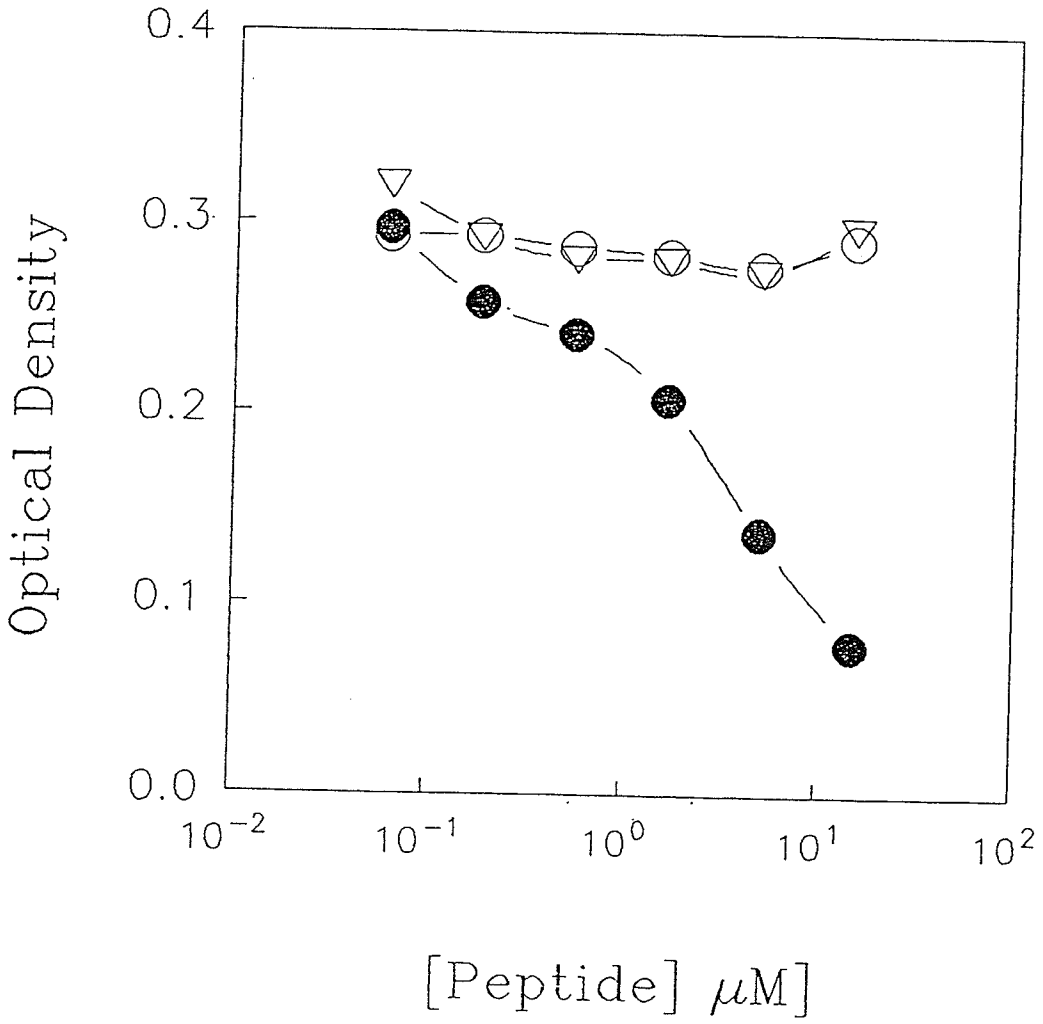


Figure 3.24e. Synthetic peptide (KLPQPVQPD) derived from β_1 integrin specifically inhibited B3B11/β_1 integrin interaction. Control peptide SGSGTAEKLK (triangle) had no effect on B3B11/β_1 interaction. KLPQPVQPD had no effect on control antibody JB1A/β_1 interaction (empty circle).

3.25. Localization of the B44 epitope with chimeric $ß_1$ integrins:

B44 is one of the most potent stimulatory antibodies of Jurkat cell adherence to fibronectin (see *Figure 3.11*). Previous studies in this laboratory have found: 1) the B44 epitope can be detected by B44 antibody under reducing conditions suggesting that B44 recognizes a linear epitope; 2) B44 binding to cells can be blocked by another stimulatory antibody 13B9 (see table 2.1), suggesting their epitopes may be close to each other; 3) Trifluoromethanesulfonic acid (TFMS) but not N-glycosidase can abolish B44 recognition, suggesting this epitope may be an O-linked carbohydrate dependent epitope (Anli Li and Wilkins data not shown).

As described above, we have previously localized several regulatory epitopes of $ß_1$ integrin with NovaTope libraries and phage peptide display libraries. However, these techniques failed to provide any information about the B44 epitope although it has been confirmed to be a linear epitope.

Chimeric $ß_1$ integrin CHO (chinese hamster ovary) cell transfectants have provided some information for several other regulatory epitopes (129, 131, 132). This approach was therefore undertaken for the localization of the B44 epitope. The chimeric $ß_1$ integrin CHO cell transfectants used in this study were provided by Dr. Takada (111) and shown in Table 3.2. Transfectant cells (10^7) expressing chimeric $ß_1$ chains were lysed in 1 ml of 20mM Tris, 150mM NaCl, 1% Triton X-100, 0.05% Tween 20, PH 7.4.

Table 3.2 The chimeric ß$_1$ integrin CHO cell transfectants used to localize B44 epitope

Chimeric ß$_1$ integrin CHO cell transfectants	Description
h189/c	N-terminal 189aa of human ß$_1$ chain / C-terminal chicken integrin ß$_1$ chain
h304/c	N-terminal 304aa of human ß$_1$ chain / C-terminal chicken integrin ß$_1$ chain
h354/m	N-terminal 354aa of human ß$_1$ chain / C-terminal mouse integrin ß$_1$ chain
h425/m	N-terminal 425aa of human ß$_1$ chain / C-terminal mouse integrin ß$_1$ chain

Thus, by examining transfectants for the presence of B44 reactivity, we can localize the B44 epitope. Since B44 recognized CHO cell lysates, in our experiments, all cell lysates from different chimeric ß$_1$ integrin transfectants were immunoprecipitated with JB1A before western blot analysis. Biotinylated B44 did not recognize chimeric ß$_1$ integrin until residue 355-425 was involved (***Figure 3.25a***). This result was confirmed by western blot with B44 and JB1A in which both h354/m and h425/m could recognize JB1A, but only h425/m contained the B44 recognition site (***Figure 3.25b***). B44 did not recognize chicken and mouse ß$_1$ integrins but did strongly recognize CHO cell lysate (***Figure3.25a, 3.25c***) suggesting that this epitope also existed on hamster ß$_1$ integrin. The origin of the common band seen in the lanes containing CHO lysates (Fig. 3.25a) is unknown. However, the bands recognized by B44 were specific . In Fig. 3.25c the CHO cells had also been co-transfected with α5 to increase the levels of ß$_1$ chain expression.

Figure 3.25a,b,c

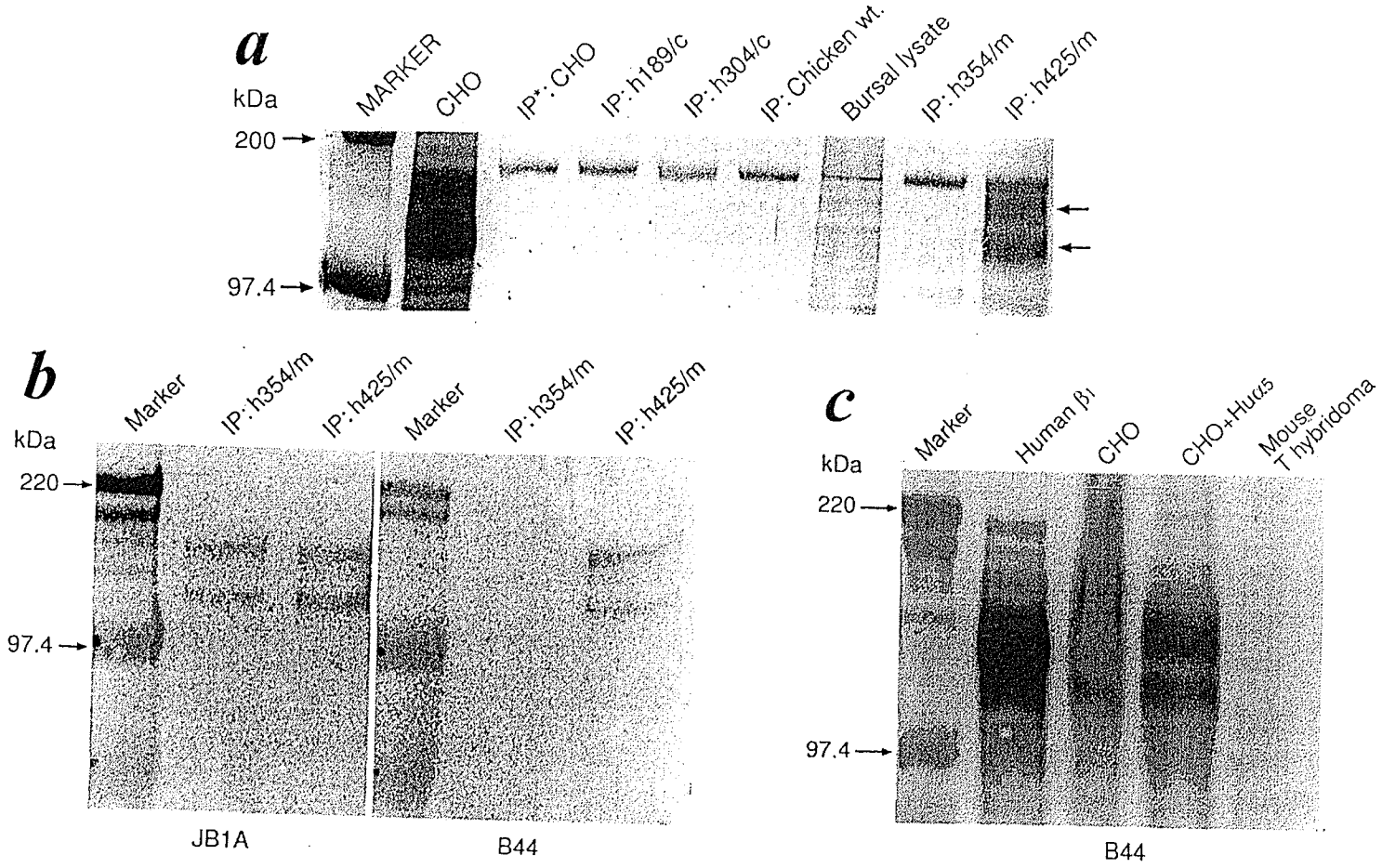


Fig. 3.25. (a) Localization of the B44 epitope to the 354 to 425 region of the β1 chain. *Cell lysates of wild type (CHO) or transfected CHO cells expressing the indicated interspecies chimeras were immunopreciptated with the anti-β1, JB1A, separated by SDS-PAGE under reducing conditions and examined by western blot for reactivity with B44. The lanes marked IP were treated in this fashion. The lanes marked CHO and Bursal lysates were direct blots of whole cell lysates of wild type CHO cells and chicken bursa of fabrica lymphocytes. Note that B44 reacted with the wild type CHO integrin but not the chicken integrin. The arrows indicate the positions of mature and pre β1 integrins. (b) Comparison of integrin levels of JB1A precipitable integrins in CHO cells transfected with h354/m or h425/m chimeric integrins. Lysates of the indicated cells were immunoprecipitated with JB1A and seperated on SDS-PAGE and transferred to nitrocellulose. The blots were examined for reactivity with JB1A or B44. Note that although there were comparable levels of both h354/m and h425/m, as revealed by reaction with JB1A, only the h425/m construct react with B44. (c)B44 doesnot react with cell lysates of a clone of mouse T cell hybridoma.*

The epitope recognized by stimulatory antibody HUTS-21 has also been localized to the same region, it was therefore questioned whether this antibody (HUTS-21 recognizes a discontinuous epitope) was able to competitively inhibit B44 binding $ß_1$ integrin. ***Fig.3.25d*** shows that both HUTS-21 and 13B9 partially inhibit biotinylated B44 binding to purified $ß_1$ integrin. This result suggests their epitopes may be in spatial proximity. Thus, residues 355-425 define a cluster of stimulatory antibody binding sites.

Figure 3.25d

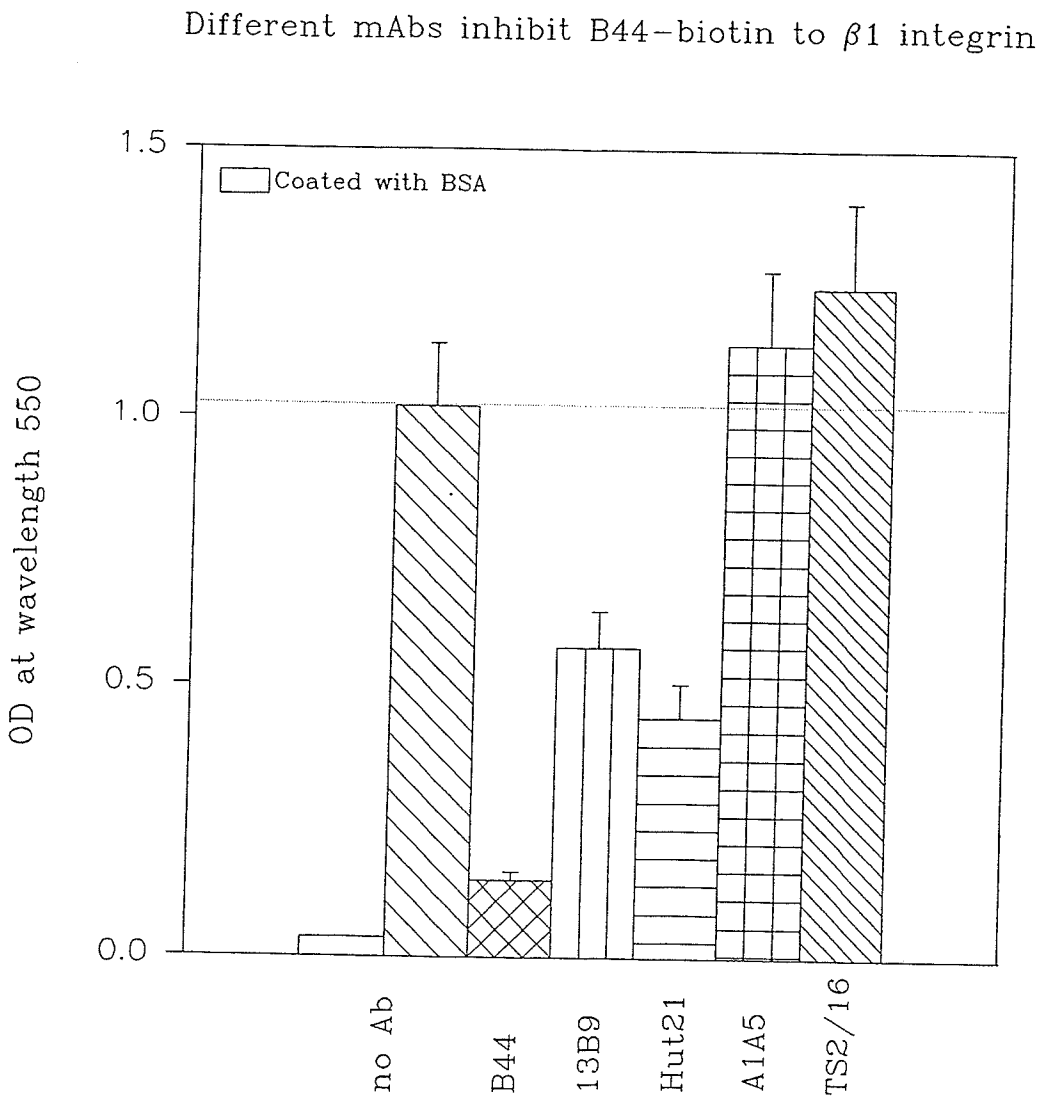


Fig. 3.25d. The effects of monoclonal antibodies to β1 CLIBS on B44 binding. *Microtiter wells were precoated with purified human β1 integrin and incubated with biotinylated B44 in the presence of a 10-fold excess of non labeled competitor antibody. The levels of residual B44 binding were assessed following the addition of avidin-alkaline phosphatase conjugate and substrate.*

B44 epitope may be an O-linked carbohydrate dependent epitope

A homology comparison of amino acid sequences among human, mouse and chicken suggested that two regions could be B44 epitope candidate sites for this linear epitope. Three biotinylated peptides (dotted underline) were synthesized by Research Genetics corresponding to these two regions (*Figure 3.25e*) and peptide ELISA was performed to further localize the B44 epitope. None of the peptides was able to bind B44 or to inhibit B44 binding to native β_1 integrin (data not shown) suggesting that other factors such as postranslational modification might contribute to the formation of this epitope.

Figure 3.25e

Homology Comparison of aa Sequence of ß1 Subunits from Different Species

C = chicken M = mouse H = human

As discussed above, deglycosylation of native β_1 integrin with N-glycosidase F that removed N-linked carbohydrates can not prevent B44 recognition. However, TFMS that non-selectively removed both N- and O-linked carbohydrates did eliminate B44 reactivity. These data suggested that the B44 epitope might be an O-linked carbohydrate

or O-linked carbohydrate dependent epitope (Anli Li and Wilkins JA, data not shown). My results further demonstrated this epitope was also sensitive to O-sialoglycoprotein endopeptidase that digested O-linked carbohydrate proteins (***Figure 3.25f***). Collectively, it seems that this epitope was an O-linked carbohydrate dependent epitope. However, since there are no known consensus sequences for identifying O-glycosylation sites it is difficult to predict where the B44 site may be.

Figure 3.25f

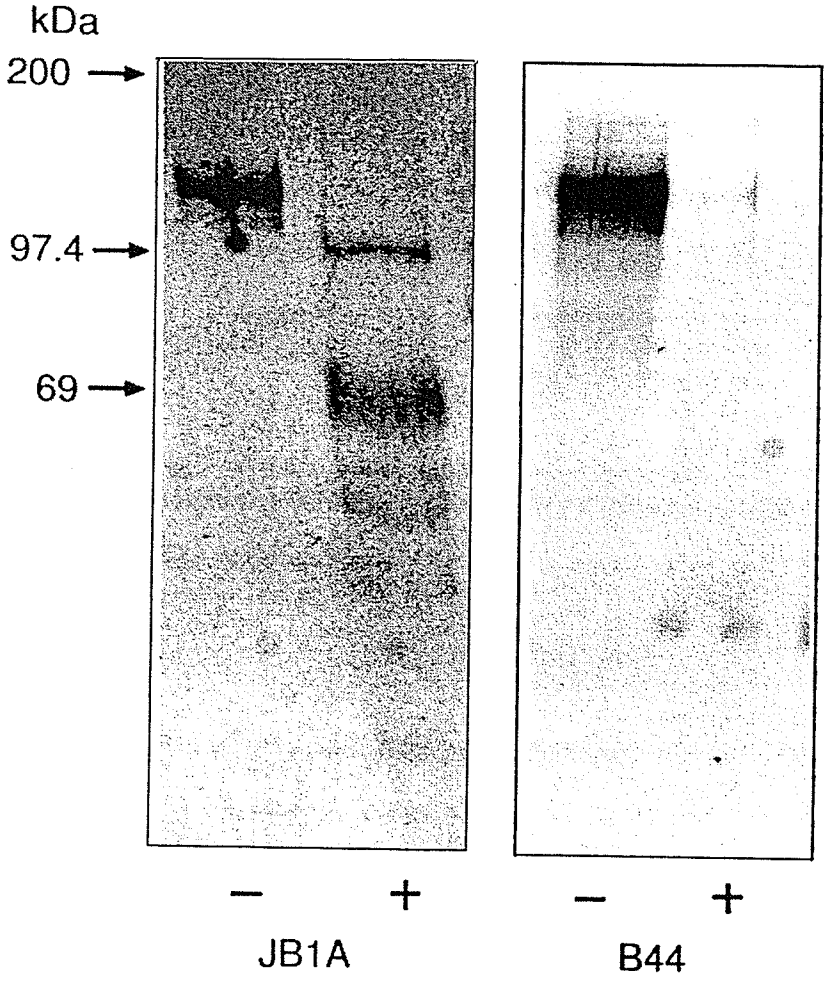


Fig. 3.25f. Digestion of human β1 integrin with O-sialoglycoprotein endopeptidase. *Human purified β1 integrin (about 20μg) were boiled 5 minutes and then digested with 20μl (2.4mg/ml) O-sialoglycoprotein endopeptidase (Cedarlane Laboratories, Inc) 37°C overnight (+). Protein fragments were separated by 10% SDS-PAGE under reducing condition and transferred to nitrocellulose membranes. The blots were examined for reactivity with JB1A and B44. Integrin treated with PBS (-) as a control.*

3.3 CHARACTERIZATION OF REGULATORY EPITOPES

3.31. Characterization of the N29 epitope

The N29 epitope has been localized to the NH2- terminal cysteine rich domain (residue 15-55) as described above. To understand the regulatory role of this domain, the N29 epitope was characterized by correlation of its expression and integrin functional states in several different systems.

3.31a. N29 epitope expression correlated with integrin function in different cell lines. K562 (erythroleukemia) and Jurkat (T leukemia) are two spontaneously adherent cells lines; IM9 (B leukemia) is a cell non-adherent line, to fibronectin (data not shown). The N29 epitope was expressed on all $ß_1$ integrins of K562 cells (see *Figure 3.31c*) and significantly expressed (about 80%) on $ß_1$ integrins of Jurkat cells but it was only expressed on a few (less than 10%) $ß_1$ integrins of IM9 cells. ***Figure 3.31a*** shows the different expressions levels of the N29 epitope on Jurkat and IM9 cells. Thus, N29 epitope expression appeared to correlate with the spontaneous adherence status of the expressing cell type.

Figure 3.31a

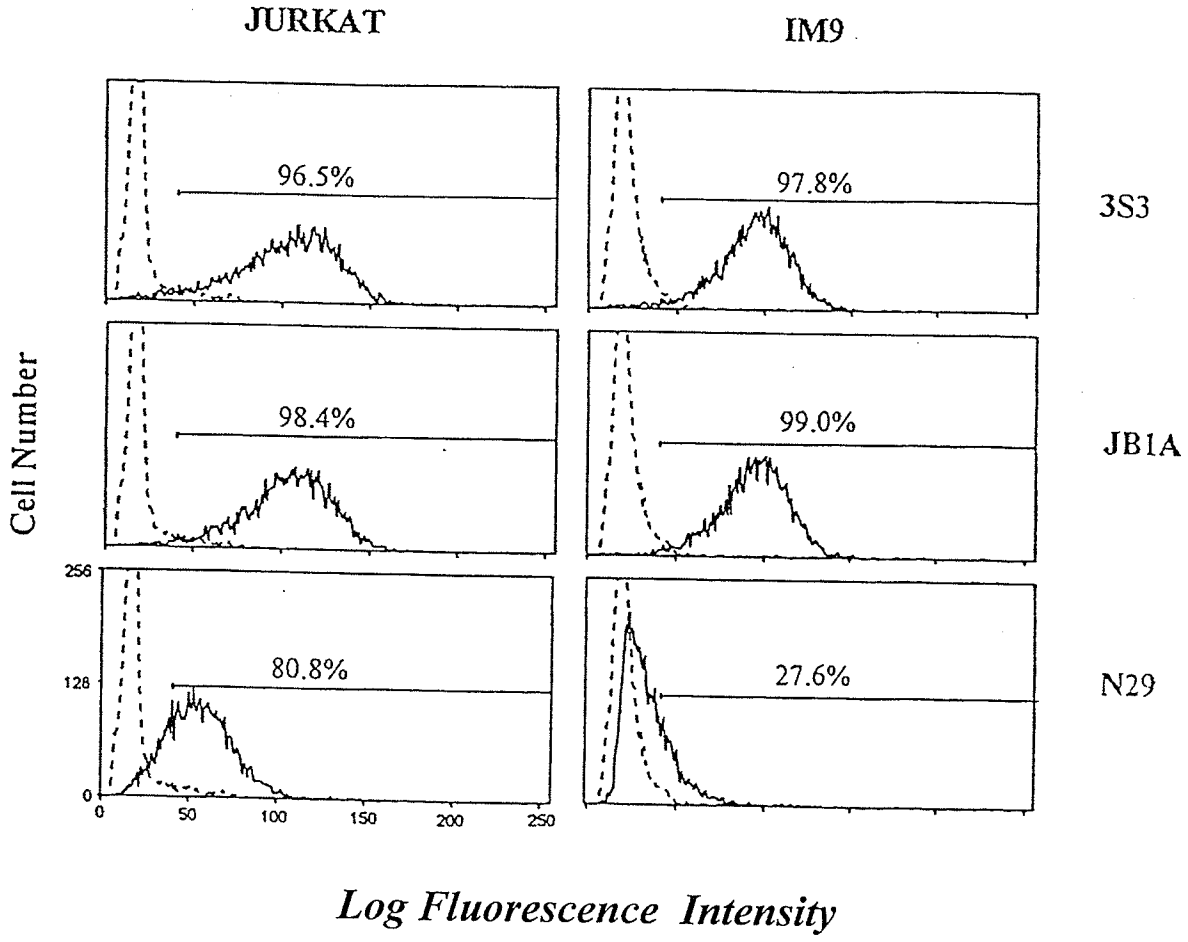


Figure 3.31a. Comparison of N29 epitope expression between Jurkat and IM9 cells with flow cytometry. Cells were first incubated with indicated antibodies (5μg/ml) 4°C for 60 minutes. The cells were washed twice with phosphate-buffered saline and incubated in 4°C for 60 minutes with FITC labeled goat anti-mouse IgG. All assays included cells incubated with the second antibody alone (dashed line) as a control for non-specific binding.

3.31b. N29 epitope expression correlated with integrin function in stimuli activated cells.

As discussed, divalent cations, reducing agent dithiothreitol (DTT) and inside-out signaling are capable of activating integrins. Since the N29 epitope is most highly expressed on spontaneously adherent cells, it is suggested that the epitope might be activation related, i.e. the N29 epitope may be expressed exclusively on activated β_1 integrins. To test this hypothesis, non-adherent cells IM9 (B cell line) were treated with divalent cations or DTT, and non adherent IL-2 dependent peripheral blood lymphocytes from health donors were treated with cross-linking CD3 (i.e. triggering T cell receptor), and examined for changes in cell adhesion and epitope expression.

N29 epitope expression correlated with integrin function induced by Mn^{2+}. IM9 cells (non-adherent B cell in RPMI-1640 cell medium) were treated with different concentration of Mn^{2+}, Mg^{2+} , and Ca^{2+} in Puck's saline buffer. However, since the concentrations of Mg^{2+}, and Ca^{2+} required to induce IM9 cell adhesion were very high, t $Mn^{2+}$was chosen for next experiments. ***Figure 3.31bI*** shows that Mn^{2+} induces IM9 cell adhesion. Its half-maximal stimulatory concentration is around 70 μM. The adhesion induced by divalent cations can be inhibited by more than 60% by combined anti-α_4 and anti-$\beta_1$antibodies, suggesting that $\alpha_4\beta_1$ is mediating a significant proportion of the induced adhesion on IM9 cells (data not shown). It was observed that Mn^{2+} induced cell adhesion could only be effectively inhibited with a combination of anti-α_4 and anti-$\beta_1$antibodies. Thus, Mn^{2+} activated $\alpha_4\beta_1$ integrin, which led to cell adhesion. ***Figure 3.31bII*** shows that Mn^{2+} also induces N29 epitope expression.

Figure 3.31bI

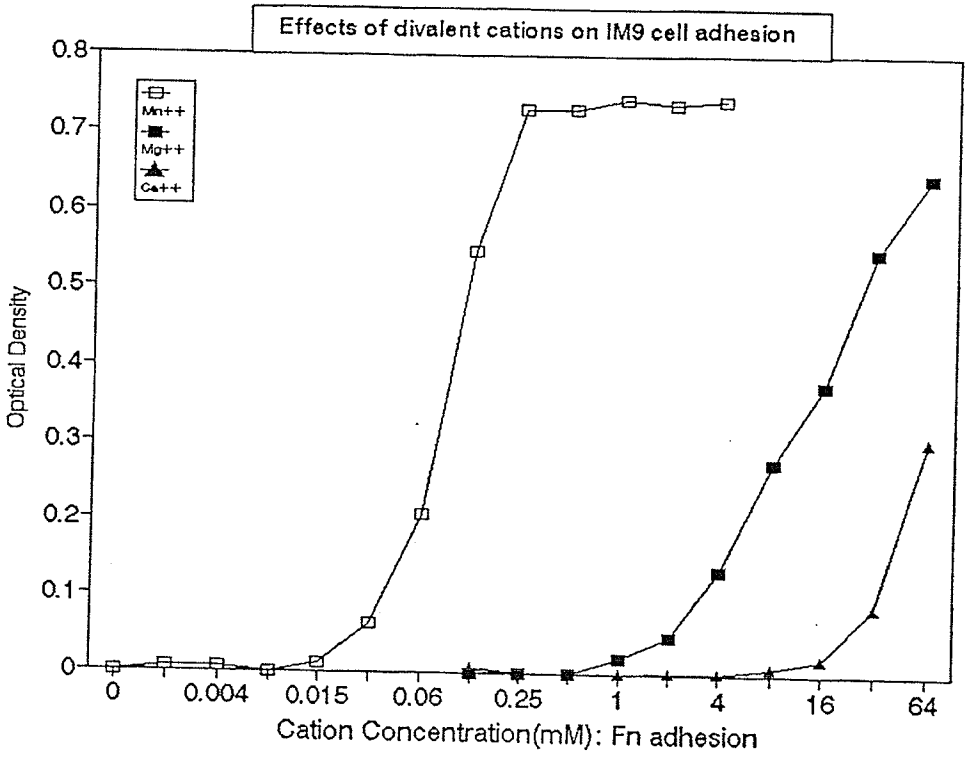


Figure 3.31bI. The induction of IM9 adherence to fibronectin by divalent cations. IM9 cells were incubated with the indicated concentration of divalent cations in Puck's saline and tested for adherence to immobilized fibronectin in microtiter wells. The results are the means of absorbance *in triplicate wells of three experiments.*

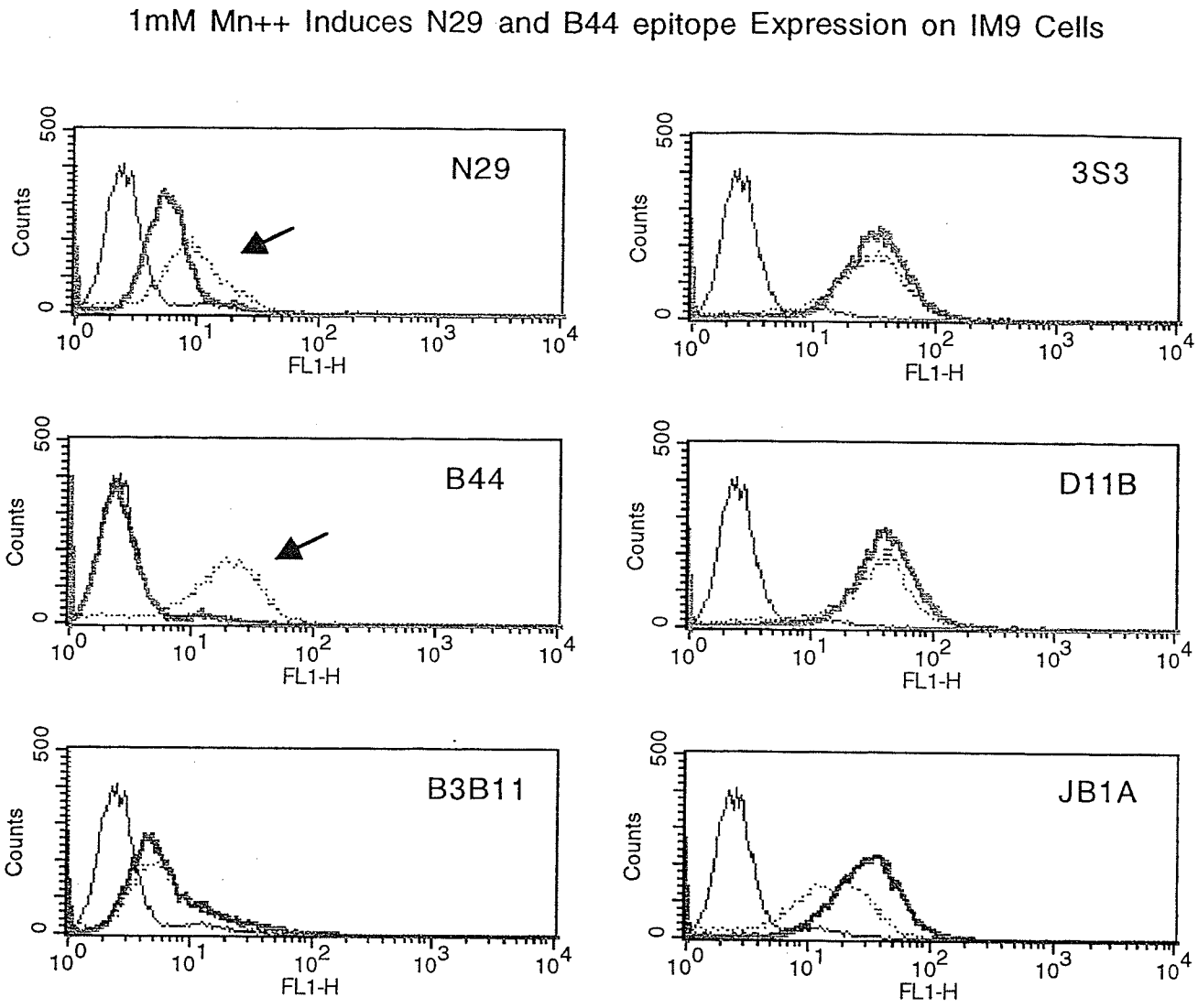


Figure 3.31bII. The effects of Mn^{2+} treatment of IM9 cells on β_1 epitope expression. Cells were treated with Mn^{2+} (1mM), stained with the indicated antibodies and analyzed by flow cytometry. The control, untreated, and Mn^{2-} –treated cell profiles are represented by the dashed, solid, and dotted lines, respectively. The arrows indicated the profiles of the Mn^{2-} –treated cells. D11B and 3S3 used here as internal negative controls. JB1A itself is sensitive to Mn^{2+} will be discussed in 3.32. Mn^{2+} does not induce B3B11 epitope expression.

N29 epitope expression correlated with integrin function induced by DTT.

Similar to Mn^{2+}, DTT can induce $\alpha_4\beta_1$ integrin mediated IM9 cell adhesion (see section 3.4). This reagent strongly induced N29 epitope expression, as shown in ***Figure 3.31bIII***, 10mM DTT induced this epitope expression on 100% β_1 integrin on IM9 surface.

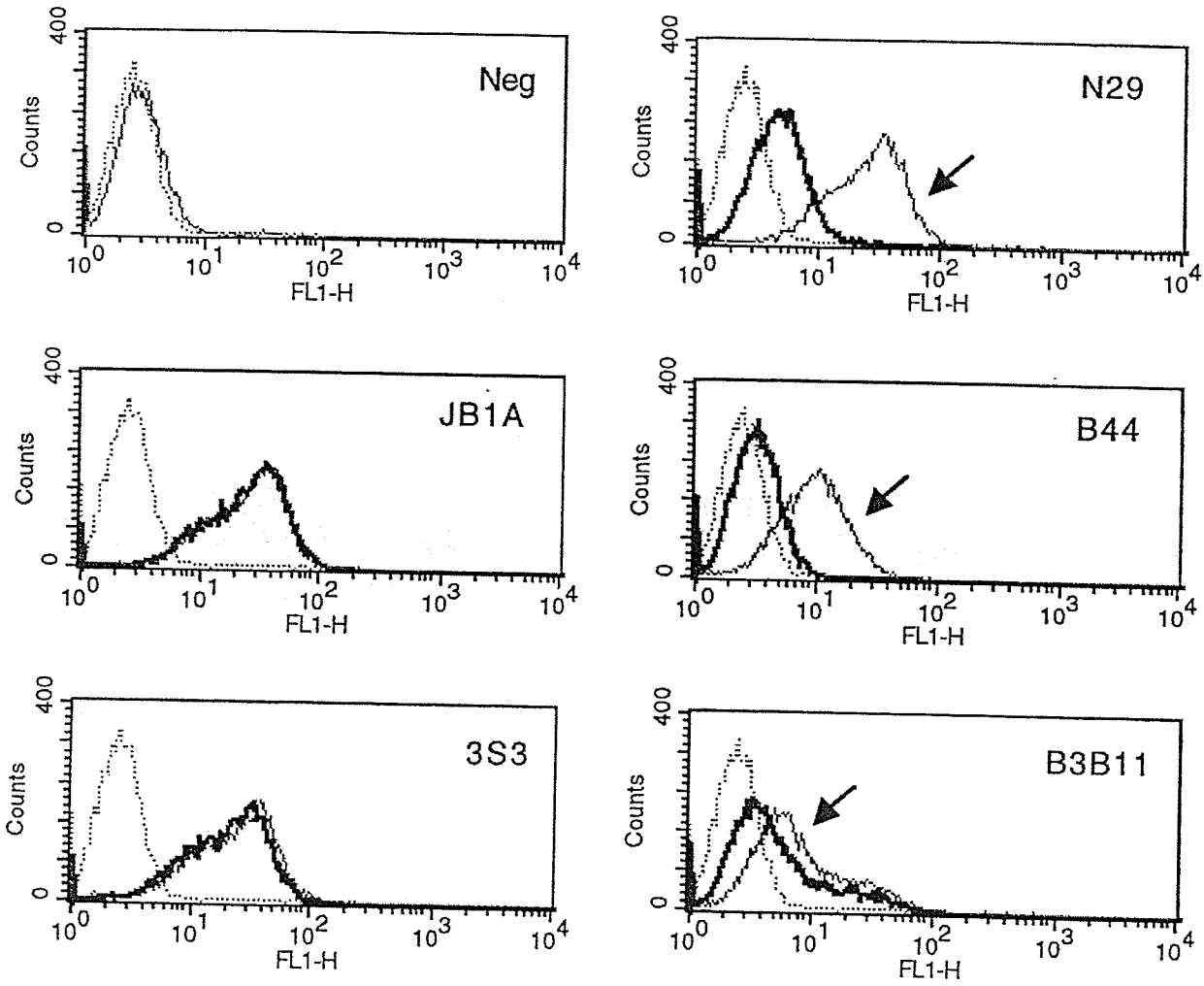


Figure 3.31bIII. The effects of DTT treatment of IM9 cells on β_1 epitope expression. Cells were treated with DTT (10mM), stained with the indicated antibodies and analyzed by flow cytometry. The control, untreated, and DTT–treated cell profiles are represented by the dashed, solid, and dotted lines, respectively. The arrows indicated the profiles of the DTT–treated cells. JB1A and 3S3 epitopes have no changes (negative control) whereas expression of the B3B11 epitope has a little increase.

N29 epitope expression correlated with integrin function induced by inside-out signaling. Because cross-linking CD3 (triggering T cell receptor) can induce T cell adhesion (102, and our unpublished data), I examined the effect of anti-CD3 on N29 epitope expression on IL-2 dependent T cells expanded from peripheral blood lymphocytes of health donors. As shown in ***Figure 3.31bIV***, triggering T cell receptor can induce both N29 and B44 epitope expression. Although the variation of N29 epitope expression levels are higher than those of B44 among different donors and different states of cell cycle, the cross-linking of CD3 induces an increase in N29 epitope expression appears in most of our FACS data. These preliminary results suggested that human $ß_1$ integrin conformational changes could be induced by physiological stimuli. To further demonstrate this "inside-out" signaling would require the use inhibitors of intracellular signaling pathways.

Figure 3.31bIV

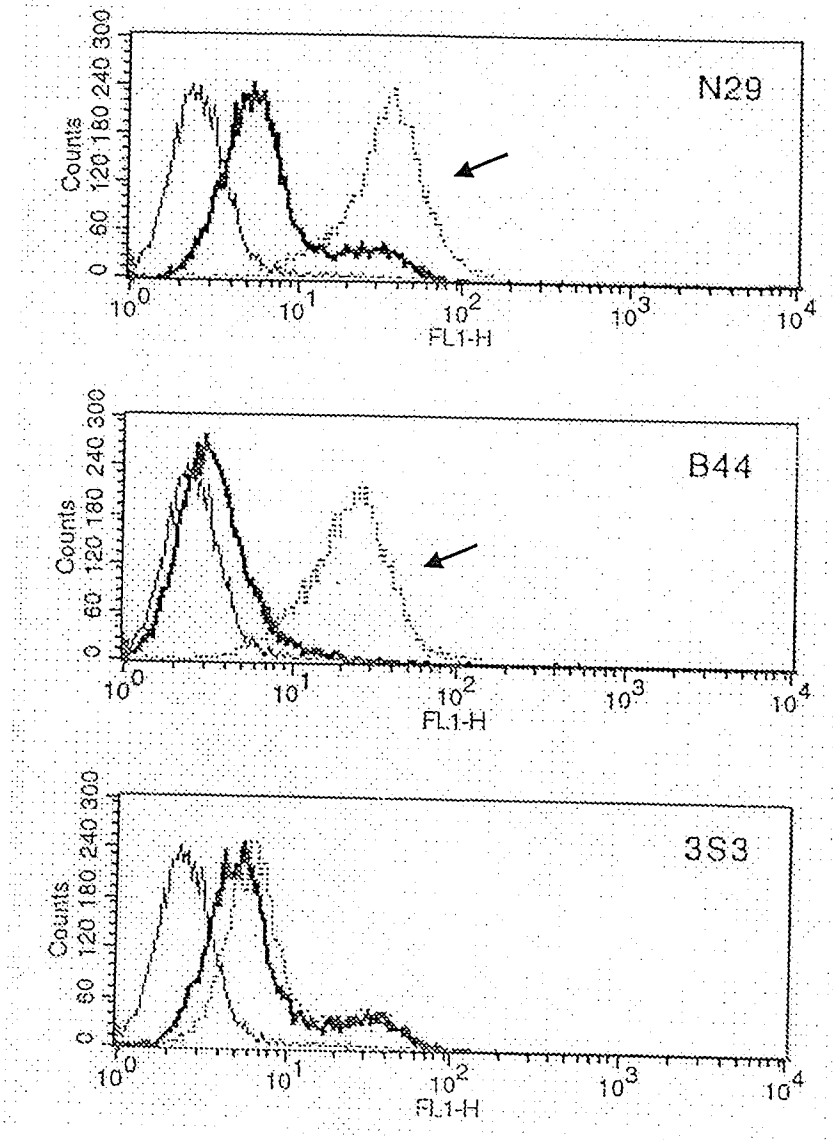


Figure 3.31bIV. Cross-linking CD3 induced β_1 integrin conformational changes on T lymphocytes. IL-2 – dependent health donors T cells (see materials and methods in appendix paper IV, ref 134) were first incubated with mouse antihuman CD3 (10μg/ml) at 4°C for 30 minutes, then incubated with rabbit antimouse IgG (1μg/ml) to cross-link CD3 at 4°C for 60 minutes. Regulatory epitopes of β_1 integrin were examined with biotinylated antibodies (37°C 30 min) by flow cytometry analysis (FACS). The arrows indicated the profiles of the cross-linking CD3 treated cells.

3.31c. N29 epitope expression on K562 was not influenced by ligand binding

Because N29 is a stimulatory antibody and its epitope expression correlated with integrin functional states, I therefore examined whether the N29 epitope is a cation ligand induced binding site (CLIBS).

K562 cells, which spontaneously bind to fibronectin, were preincubated with RGD or RGE (control) peptides (1mM) or fibronectin (100μg/ml) for 1 hour at room temperature. N29 and other antibodies were then added to this mixture for 30 min at 37°C, and the cells were processed for flow cytometry analysis as described above. Figure 3.31c shows that stimulatory antibody B44 recognizes a CLIBS, but the expression levels of other epitopes including N29 were not significantly changed by this treatment. It seems that N29 epitope is not a CLIBS because this epitope is fully expressed on K562 cell without ligand occupancy and ligand occupancy has no effect on its expression. In order to examine whether N29 epitope is a CLIBS on other cells, further studies with different ligands on Jurkat cells or partially activated IM9 cells, on which N29 epitope is only partially expressed, may be required.

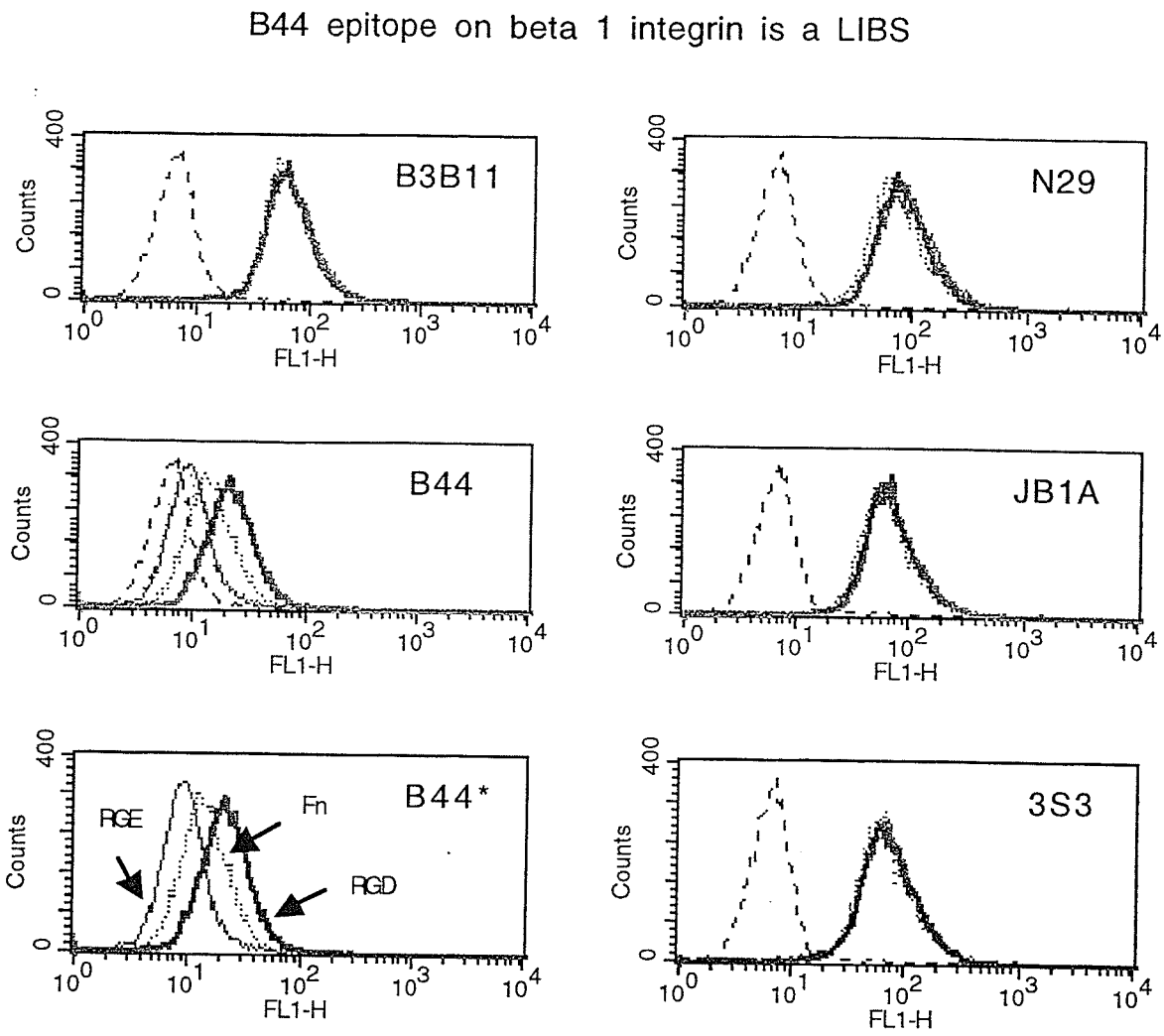


Figure 3.31c. The effects of ligand binding on the β_1 epitope expression of K562 cells. Cells were treated with fibronectin (100μg/ml) or RGDS / RGES peptides (1mM), and stained with the indicated antibodies and analyzed by flow cytometry. The panel labeled B44 indicates the line patterns used for each treatment group. Note that the negative control cells are omitted from this panel for clarity. Control binding is indicated in all other panels by the line with large dashes.*

3.32. Characterization of JB1A group antibodies

3.32a. JB1A, C30B and D11B have different affinities with phage isolates

The fact that JB1A, C30B and D11B recognize the same region (residue 82-87) of the $ß_1$ integrin chain raises the question that these antibodies may be identical although they are from different hybridoma preparations. To target this problem, individual JB1A phage isolates were coated in ELISA plate and reacted with the individual antibodies. As shown in ***Figure 3.32a***, although in many cases all three antibodies recognized these isolates, the patterns of antibody reactivity to each phage were quite distinctive. The apparent differences in binding patterns to individual phage may result from the contributions of the different flanking amino acids around the major motif. That these are indeed different antibodies is further supported by several observations. JB1A is sensitive to Mn^{2+} inhibition while neither C30B nor D11B were influenced by this cation, (see following figures).

Figure 3.32a

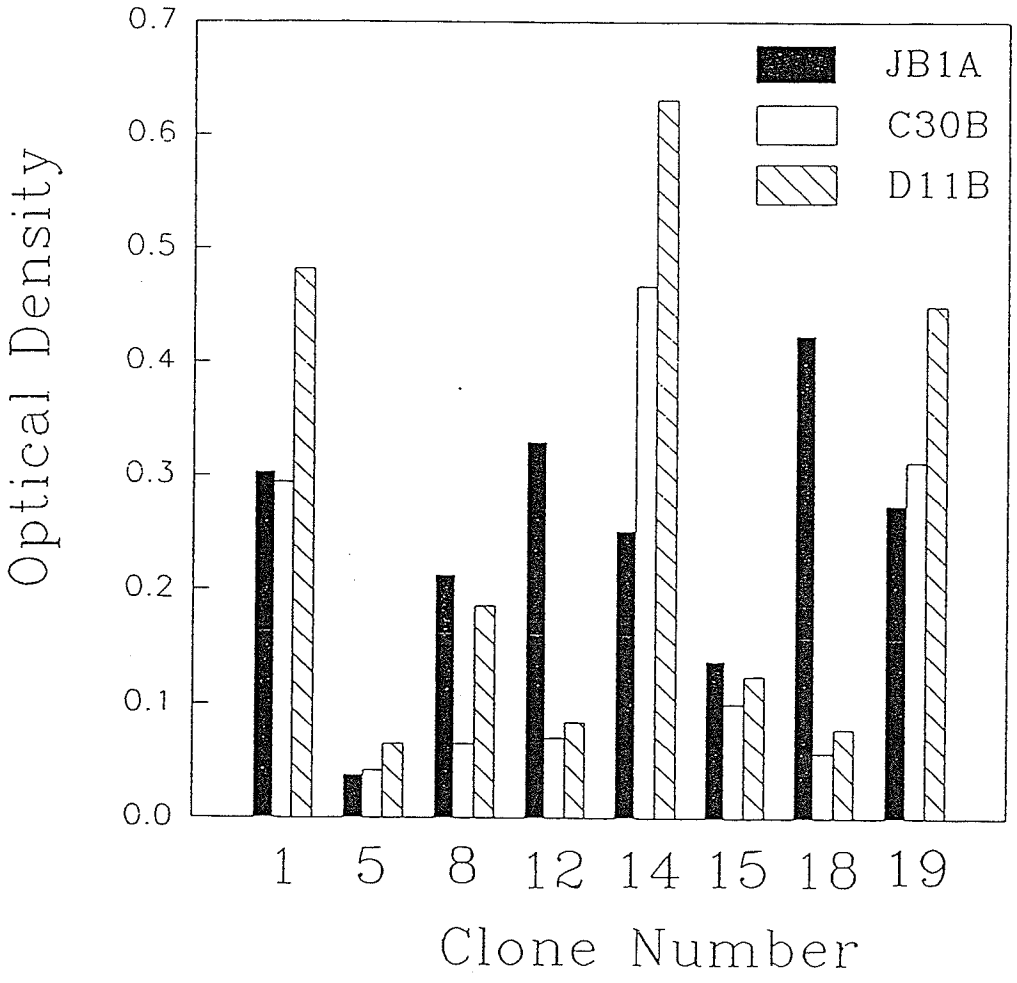


Figure 3.32a. Comparison of the binding of C30B, JB1A and D11B to JB1A immunoselected phage. Microtitre wells were coated with phage isolated from the third round of selection of a random peptide display library and tested for reactivity with JB1A, C30B or D11B in an ELISA. The reactivity of these isolates with AIIB2, 3S3 and B3B11 was less than 0.03.

3.32b. Mn^{2+} decreased JB1A binding to IM9 cells results from decreasing JB1A affinity rather than decreasing JB1A epitope expression.

Mn^{2+} is able to induce $ß_1$ integrin conformational changes and cell adhesion (see above), It was therefore questioned whether this stimulator could influence JB1A epitope expression. Flow cytometric analysis, demonstrated that JB1A binding to IM9 cells dramatically decreased following exposure of these cells to increasing Mn^{2+} concentration (***Figure 3.32bI***). In contrast, C30B or D11B (recognize same epitope) binding was not influenced by this cation (***Figure 3.32bII***). Thus, JB1A itself may be sensitive to Mn^{2+}.

Figure 3.32bI

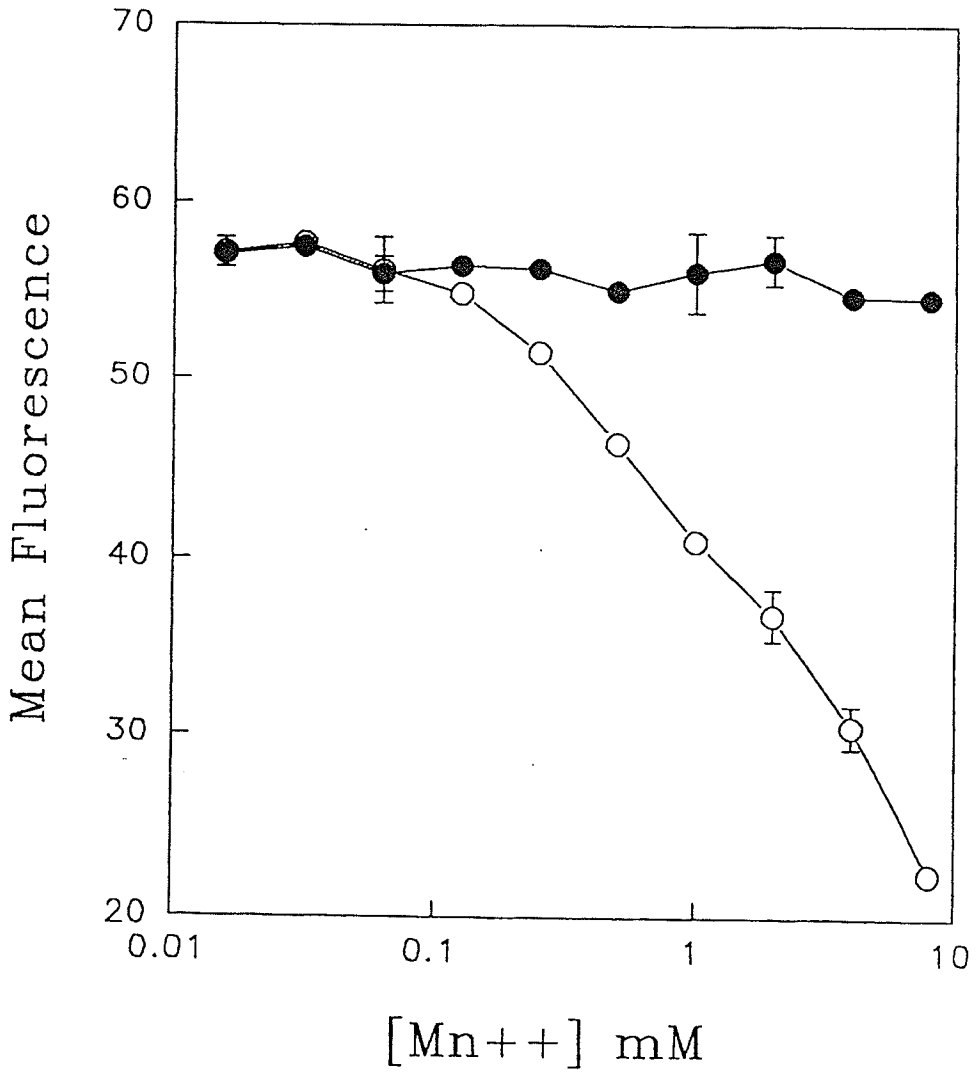


Fig. 3.32bI The concentration dependence of Mn^{++} mediated inhibition of JB1A binding. IM9 cells were pretreated with the indicated concentration of Mn^{++} and reacted with either JB1A (open circles) or 3S3 (solid circles) and the mean fluorescence intensity of each sample was determined by flow cytometry.

Figure 3.32bII

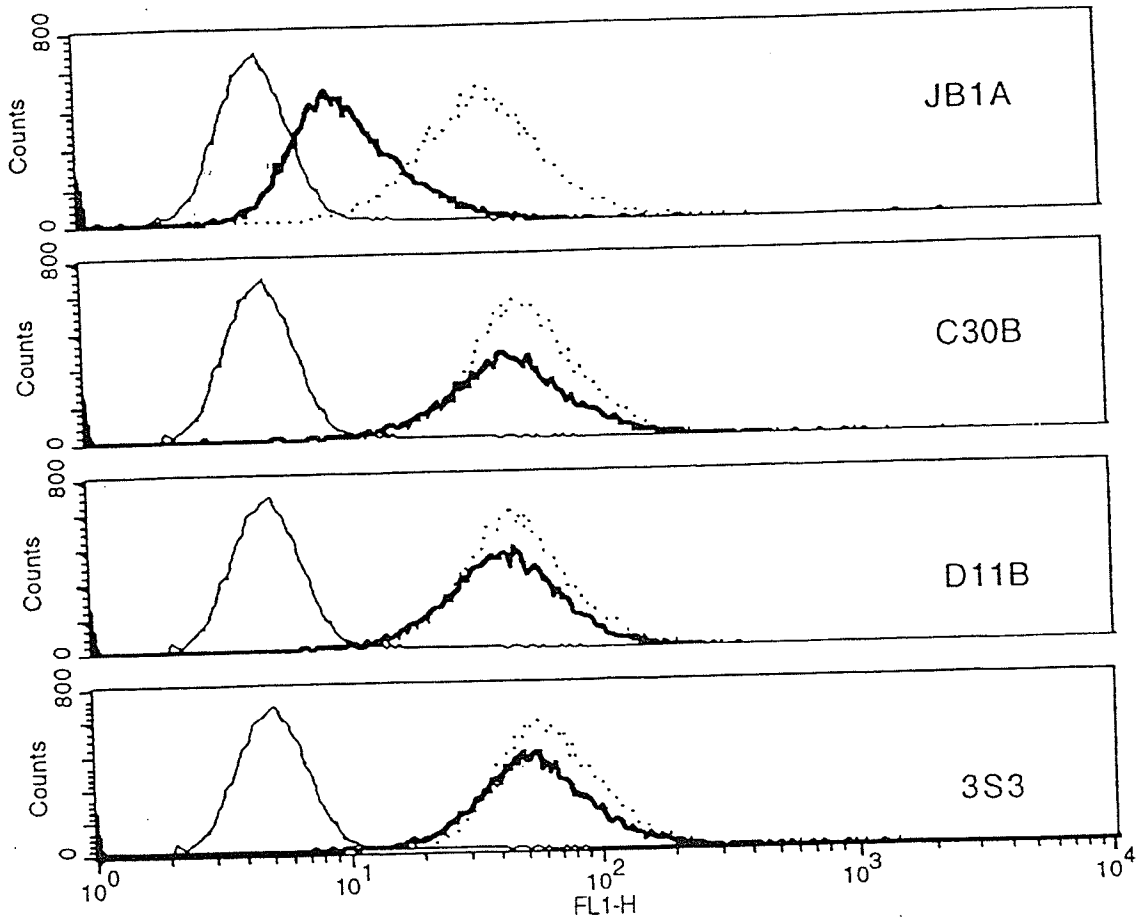


Fig. 3.32bII The effects of Mn^{++} on JB1A binding to IM9 cells. IM9 cells were washed in Puck's saline and resuspended in the same in absence (dotted line) or presence (solid line) of 8mM Mn^{++}. The cells were stained with the indicated antibodies and analyzed by flow cytometry. The negative control (light solid line) is included with each histogram.

To further characterize this phenomenon, JB1A or B3B11 (control antibody which recognizes residue 658-666 of the $ß_1$ chain) were preincubated with different concentrations of Mn^{2+}, then added to purified $ß_1$ integrin or peptide (residue 80-90, i.e. KGTAEKLKPED or 658-666, i.e. KLPQPVQPD) coated plates. Only JB1A was sensitive to Mn^{2+}(***Figure 3.32bIII***). Similar studies revealed that C30B and D11B binding to purified integrin and peptide were not influenced by Mn^{2+}(***Figure 3.32bIV***). Thus, Mn^{2+}does not decrease JB1A epitope expression on IM9 cell surface but it acts directly on JB1A and decrease antibody affinity.

Figure 3.32bIII

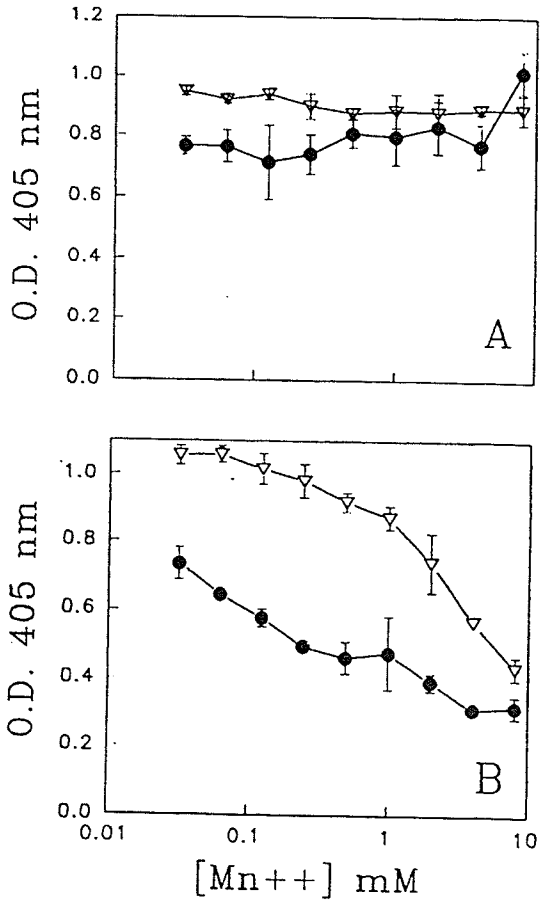


Fig.3.32bIII The effects of Mn^{++} on antibody binding to purified β_1 and peptides. (A) B3B11 or (B) JB1A were mixed with the indicated concentrations of Mn^{++} and assessed for their binding to β_1 (open triangles) or β_1 peptides (solid circles) 658-666 for B3B11 (A) or 80-90 for JB1A (B).

Figure 3.32bIV

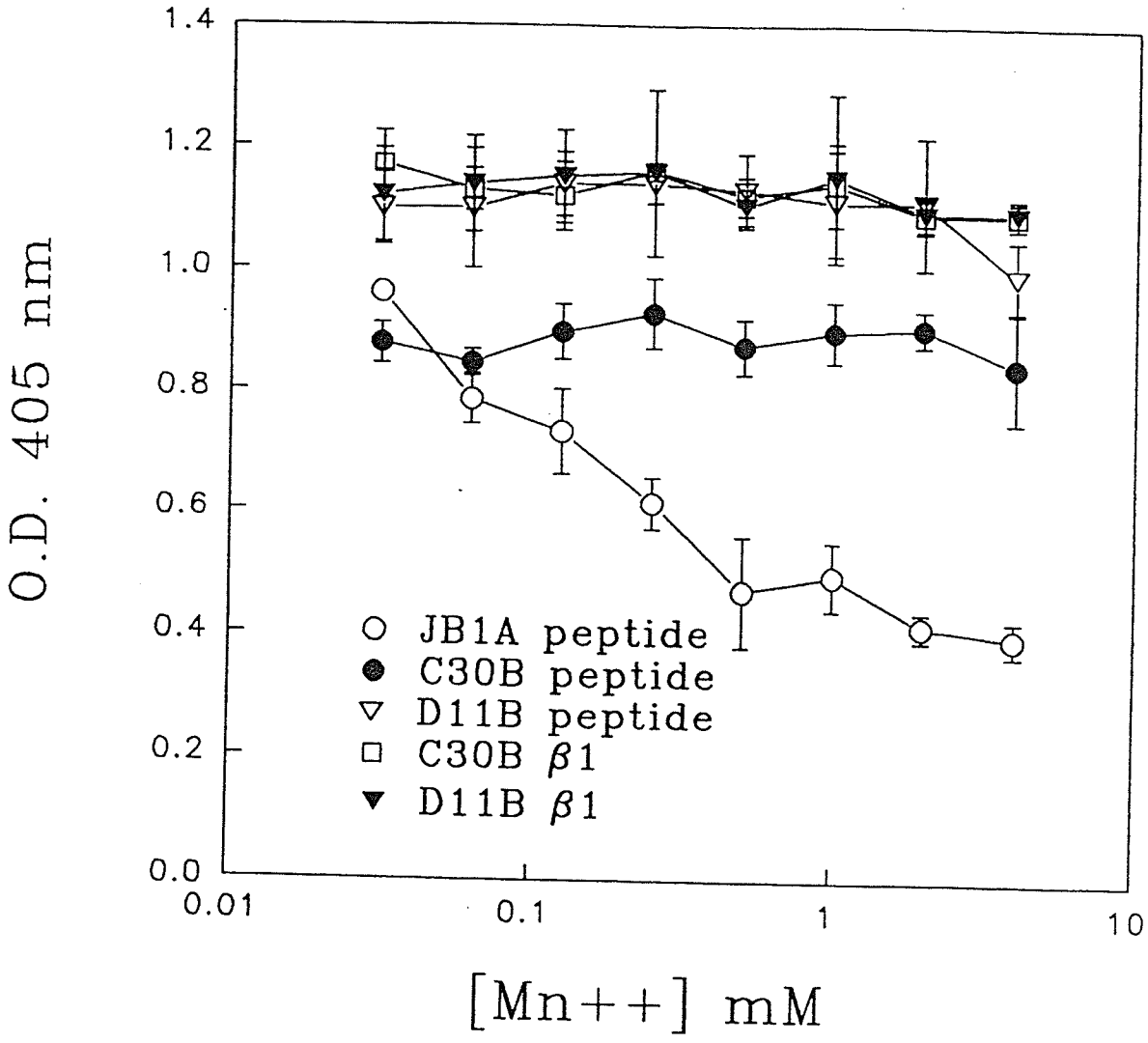


Fig.3.32bIV. A comparison of the effects of Mn^{++} on the binding of JB1A, D11B and C30B to purified β_1 and to β_1 peptide 82-90. Antibodies were mixed with the indicated concentrations of Mn^{++} and assessed for binding to peptide or to β_1.

3.32c. JB1A inhibits stimulatory epitope expression on cell surface

JB1A group antibodies are capable of blocking Jurkat cell adhesion to fibronectin. The simplest explanation for this phenomenon is that this epitope (residues 82-87) is at or close to the ligand-binding site. However, my preliminary data failed to demonstrate either the direct binding of the JB1A peptide to fibronectin or an effect of this peptide on Jurkat/fibronectin adhesion (data not shown). The alternative explanation is that JB1A induces allosteric changes of $ß_1$ integrin, which switches integrin conformation to an inactive form. ***Figure 3.32c*** demonstrates that JB1A as well as the inhibitory antibody 3S3 inhibits the expression of the stimulatory epitope N29. JB1A and 3S3 also inhibit stimulatory epitope B44 expression (see section 3.33). Thus, blocking cell adhesion by JB1A group antibodies may result from allosteric changes of integrin conformation.

Figure 3.32c

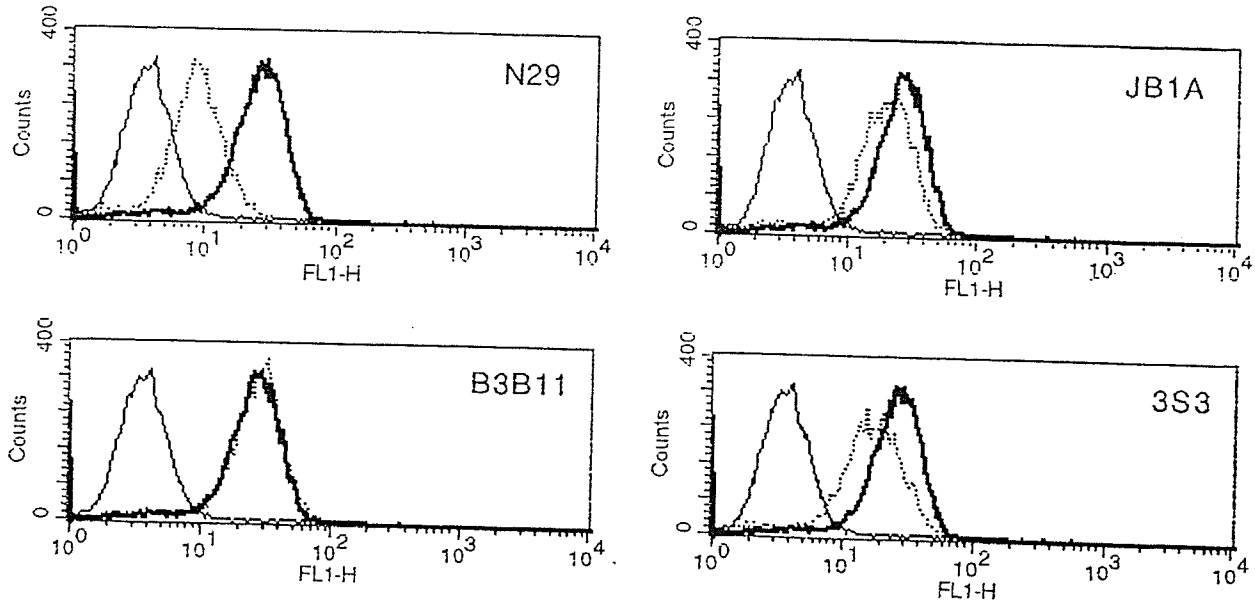


Figure 3.32c. The effects of binding of antibodies on the expression of the N29 epitope. Jurkat cells were pre-incubated with buffer (thick solid line) or the indicated antibodies (dotted line) at 15μg/ml and subsequently assessed for binding of biotinylated N29 by flow cytometry. Cells without incubating with any biotinylated antibody were used as negative control (light solid line). N29 inhibited biotinylated N29 cell binding was used as positive control. That B3B11 did not influence biotinylated N29 cell binding was a negative control. Both JB1A and 3S3 inhibited biotinylated N29 cell binding.

3.33. Characterization of B44 and its epitope:

My previous data have demonstrated that B44 epitope expression could be induced by Mn^{2+} or DTT on IM9 cells. That this epitope was a cation ligand induced binding site (CLIBS) was also demonstrated with K562 cells (*Figure 3.31c*). These results were further confirmed on Jurkat cells. ***Figure 3.33a*** indicated Mn^{2+} was more efficient than Mg^{2+} at inducing B44 epitope expression. Interestingly, although all three divalent cations supported Jurkat cell/ Fibronectin adhesion in Puck's saline A buffer (***Figure 3.33b***), Ca^{2+} markedly decreased the expression of this epitope. This may suggest that Ca^{2+} plays a bifunctional role in β_1 integrin function, i.e. maintenance of basal level cell adhesion but inhibition of maximal cell adhesion mediated by Mn^{2+}. Both B44 and N29 epitopes were sensitive to DTT in Jurkat cells (data not shown) and the B44 epitope can be remarkably induced by RGD peptide in a divalent cation dependent fashion (***Figure 3.33c***).

Figure 3.33aa

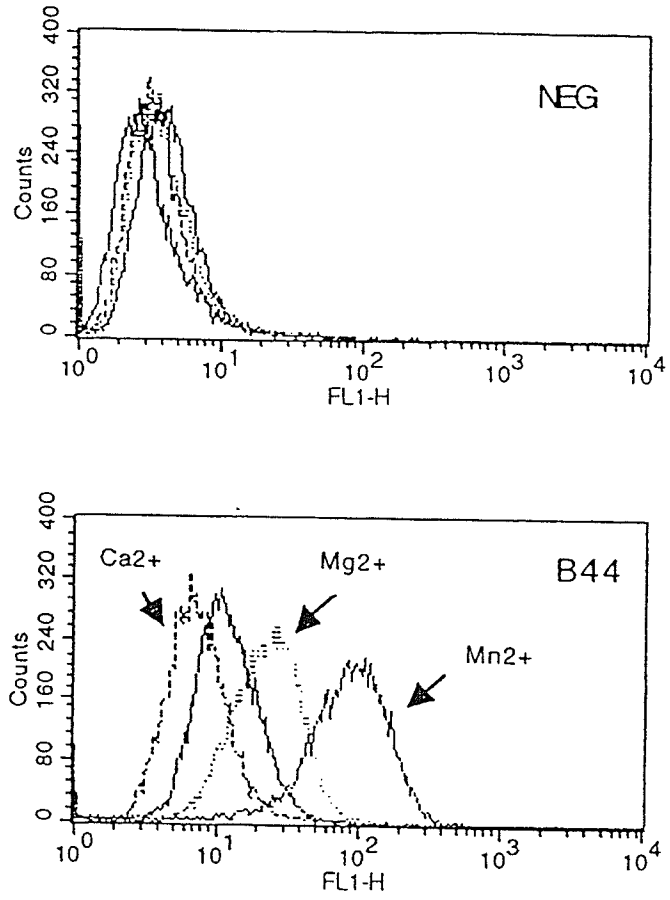


Figure 3.33a. *The effects of divalent cations on B44 epitope expression. Cells were incubated in Puck's saline with either 2mM Mn^{2+}, Mg^{2+}, Ca^{2+}, and examined by FACS for B44 expression levels. The upper panel was negative control in which cells were treated with different divalent. cations but no first antibody was added. Note exposure to Ca^{2+} causes a reduction in the level of. B44 expression (as compare to no cation) to near background (as compare to upper panel figure).*

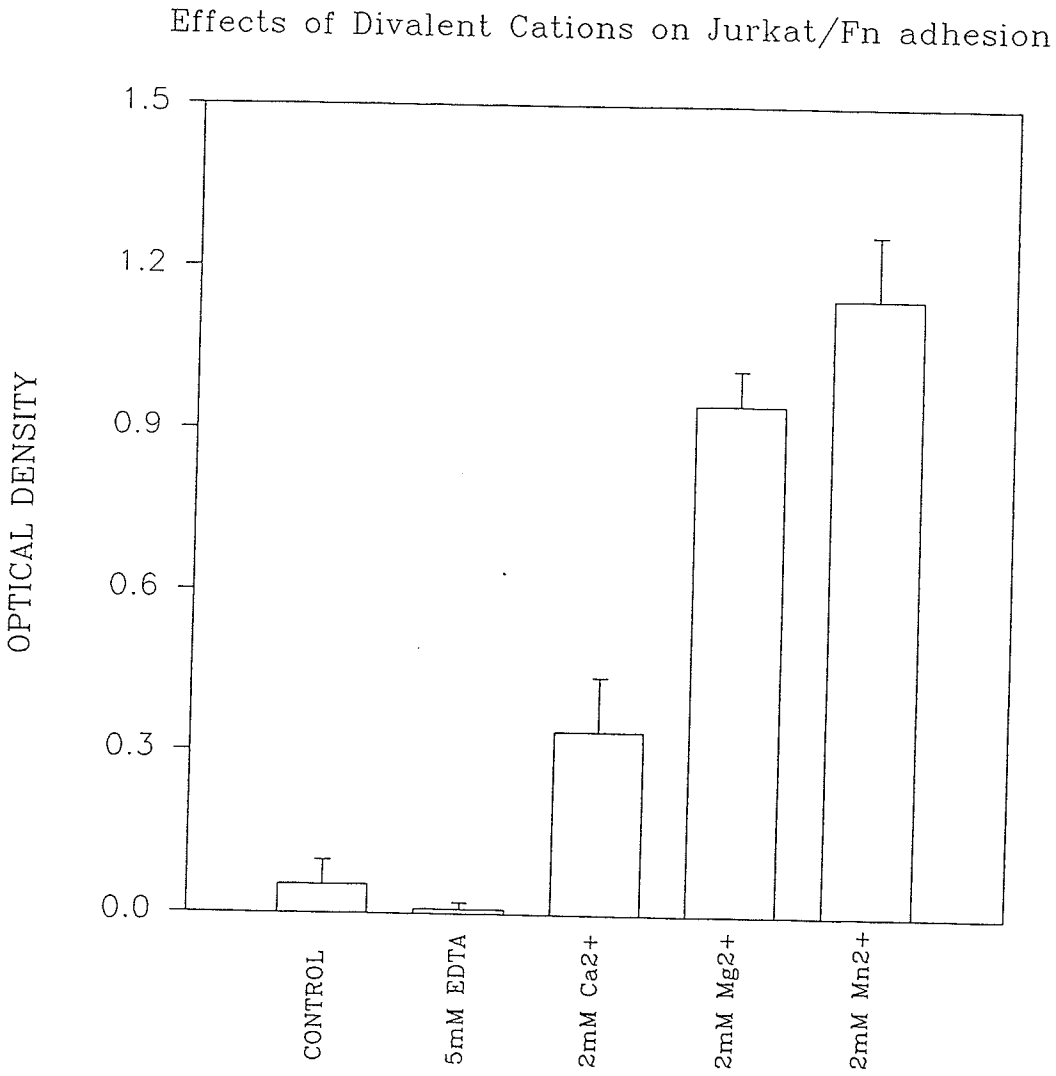


Figure 3.33b. Divalent cation requirements for Jurkat cell adhesion. Jurkat cells and microtiter wells were washed twice with Puck's saline (no divalent cations), cells then incubated in Puck's saline with either 2 mM Ca^{2+}, Mg^{2+}, or Mn^{2+} or 5mM EDTA and examined for their capacity of adhesion to fibronectin. The results are the means of sextuplicate assays and the experiments were carried out at least five times.

Figure 3.33c

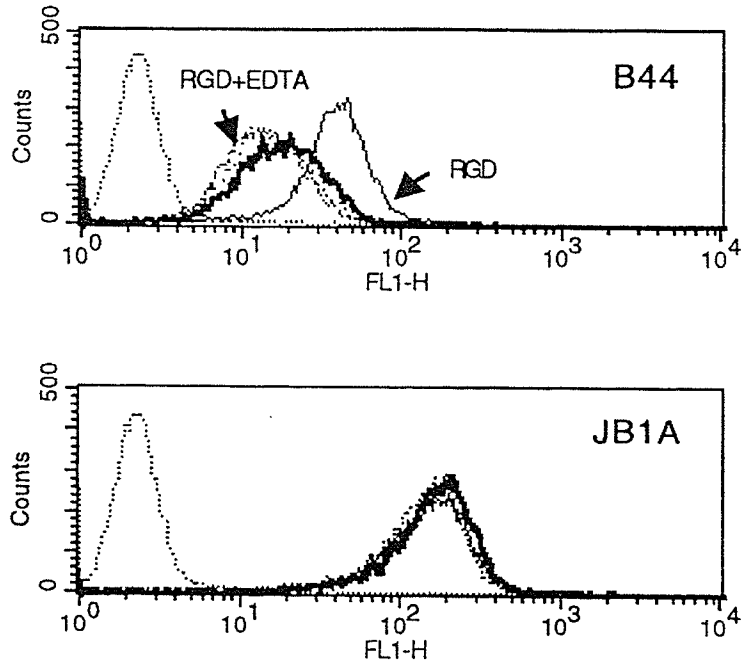


Figure 3.33c. The B44 epitope is induced on Jurkat cells by ligand. Cells were washed and suspended in cell medium (RPMI-1640), then treated with RGES, or RGDS in the presence or absence of 1mM EDTA and stained with B44 or JB1A. RGDS alone caused an increase in B44 levels which was inhibited by the addition of EDTA. Note these treatments did not affect JB1A levels. The JB1A and B44 FACS profiles for cells treated with the control peptide RGES were almost identical to those for untreated cells and they are overlapped by the control profiles.

In order to examine the response of this epitope to intracellular signals, stimulating with phorbol 12-myristate 13-acetate (PMA) (data not shown) and cross-linking CD3 (see *Figure 3.31bIV*) were performed on human peripheral blood lymphocytes with flow cytometry analysis. B44 epitopes can be dramatically induced by these signals. Since cells were washed twice with RPMI-1640 (serum free) before exposure to antibody and reaction with antibody only lasted 30 minutes, it seems unlikely this epitope expression results from ligand occupancy because preexisting ligand(s) or newly secreted ligand(s) contributed to the exposure of this epitope. In other words, a CLIBS like conformation can be expressed in apparently ligand independent fashion (i.e. before ligand occupancy).

Because the B44 epitope was one of the best reporters of integrin functional states in our laboratory, we then monitored by FACS, β_1 integrin conformational changes after different regulatory mAb treatments by with biotinylated B44. Surprisingly, all stimulatory mAbs (such as N29, A1A5, TS2/16 and B3B11) increased the epitope expression except 13B9 and HUTS-21 that bind to the same region as B44 binding site (see section 3.25), whereas all inhibitory mAbs decreased its expression although the changes were not dramatically (***Figure 3.33d***). N29 epitope (residue 15-55), A1A5, TS2/16 epitope (207-218) and B3B11 epitope (residue 660-668) are far from each other in the primary aa sequence of β_1 integrin, so are JB1A epitope (residue 82-87) and 3S3 epitope (discontinue epitope), but their effects on β_1 integrin conformation (B44 epitope expression) are highly harmonious with their behaviors on integrin function. These data not only provide a possible explanation for the role of inhibitory mAb JB1A in β_1 integrin but also provide a simplified outline for integrin structure on cell surface. It will be interesting to determine if

other regulatory antibodies provide a consistent correlation between functional activity and effects on epitope expression patterns.

Figure 3.33d

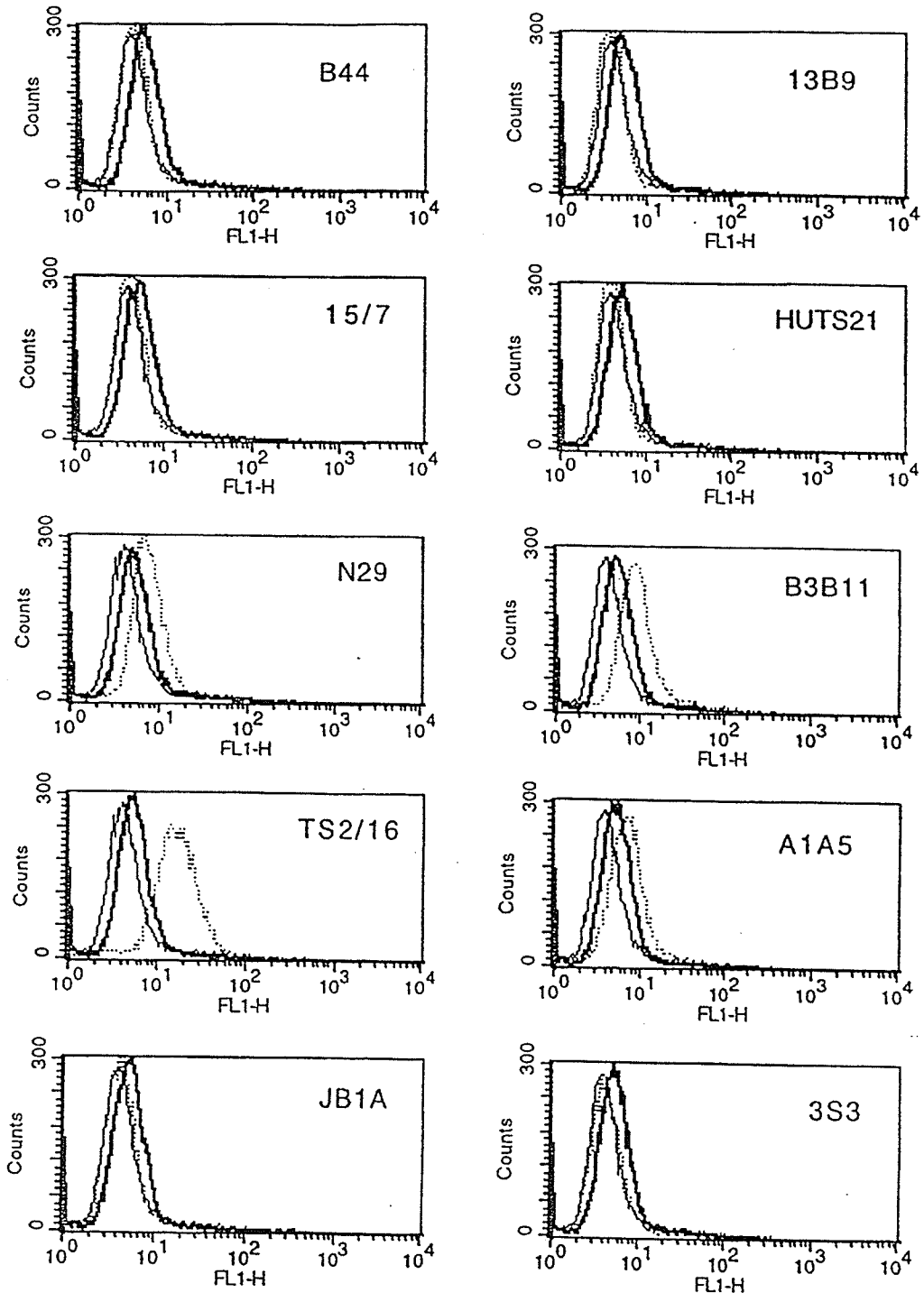


Figure 3.33d. The effects of binding of antibodies to β1 on the expression of the B44 epitope. Jurkat cells were pre-incubated with buffer (thick solid line) or the indicated antibodies (dotted line) at 15μg/ml and subsequently assessed for binding of biotinylated B44. The antibodies HUTS-21, 15/7 and 13B9 block B44 binding.

3.4. REGULATING INTEGRIN FUNCTION BY DITHIOTHREITOL (DTT) AND PROTEIN DISULFIDE ISOMERASE (PDI) INHIBITOR BACITRACIN.

Disulfide bonds appear to play an important role in integrin function. That DTT induced $ß_3$ integrin mediated platelet aggregation has been reported (see Chapter 1) and many stimulatory epitopes including our N29, B3B11, and B44 epitopes have been mapped adjacent to long range disulphide bonds (122, 114). In this study, I used DTT as a probe to examine the role of disulphide bonding in human $ß_1$ integrin function.

As previously discussed DTT induced changes in the regulatory epitope expression patterns on the cell surface. The relationship between DTT induced cell adhesion and the induction of free sulfhydryls on ß1 integrins were examined. In a related series of experiments the potential mechanism of disulfide bond exchange *in vivo* was examined using the PDI inhibitor, bacitracin.

Cell adhesion was induced by DTT and measured as outlined in Section 3.1. Cells were preincubated with DTT in RPMI-1640 for 30 minutes at room temperature and then washed twice with RPMI-1640 before addition to fibronectin coated wells. Both Jurkat (data not shown) and IM9 cells adhesion were enhanced by DTT. ***Figure 3.4a*** shows that the concentration of DTT for half-maximal IM9 adhesion is around 2-5 mM, for maximal IM9 adhesion the concentration is around 25 mM. The adhesion activity began to decrease at concentrations in excess of 50 mM. This adhesion appears to be mainly mediated by $\alpha_4ß_1$ integrin because it can be inhibited by combined anti-α_4 and anti-$ß_1$antibodies although neither antibody one its own can inhibit this type of cell adhesion (data not shown).

Figure 3.4a

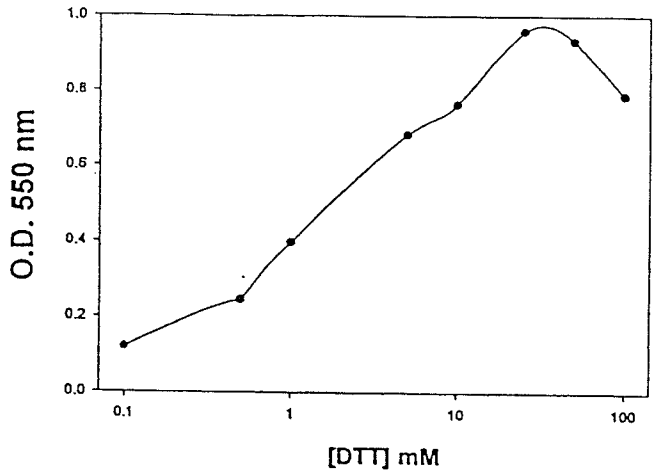


Figure 3.4a. The induction of IM9 adherence to fibronectin following treatment with DTT. IM9 cells were incubated with indicated concentration of DTT and tested for adherence to immobilized fibronectin. Representative results of one of at least two independent experiments are shown. The O.D. values are the means of absorbance *in sextuplicate wells.*

DTT induced exposure of free sulfhydryls in $ß_1$ integrin. This experiment was designed to determine whether there was a correlation between the induction of adhesion and the exposure of free –SH groups of $ß_1$ integrin. Alternatively DTT might indirectly activate integrins by other mechanism. DTT, EDTA and Mn^{2+} treated Jurkat and IM9 cells (as shown in ***Figure 3.4b***) were labeled with biotinylated BMCC, which specifically reacts with free –SH groups. Cell lysates were then immunoprecipitated with JB1A and analyzed by western blot with streptavidin-AP. As indicated in ***Figure 3.4b***, 10mM DTT significantly increased the number of free –SH groups on integrin $ß_1$chain. However, it was also apparent that both adhesive cells (Jurkat) and nonadhesive cell (IM9) contained free –SH groups on integrin $ß_1$chains and the presence of these –SH groups was independent of the presence of divalent cations. However, these data do not prove that DTT effects on cell adhesion result from the SH changes on integrins. Mapping and point mutation of cysteine residues that can be labeled by BMCC may be able to provide some direct evidence to address this question. The present data also suggests that the predicted pairing patterns for ß1 may not be consistent with the model proposed for ß3 by Dr. Calvete (see chapter 1) because free –SH groups exist in both inactive and active form of $ß_1$chain.

It will be of interest to determine if regulatory antibodies affect biotin-BMCC labeling of $ß_1$chain. I hypothesise that $ß_1$ integrins treated with stimulatory antibodies N29, B3B11, JB1B, and B44 will generate more free –SH groups because these antibodies may block the reforming of long range disulfide bonds in $ß_1$ integrins. It also should be addressed why DTT treated cells have some higher molecular weight proteins which can be purified by JB1A. Whether these molecules are different level biotin-

BMCC (Mw about 0.533 kD) labeled $\beta_1$chain or integrin associated proteins which contain reduceable disulfide bonds remains to be determined.

Figure 3.4b

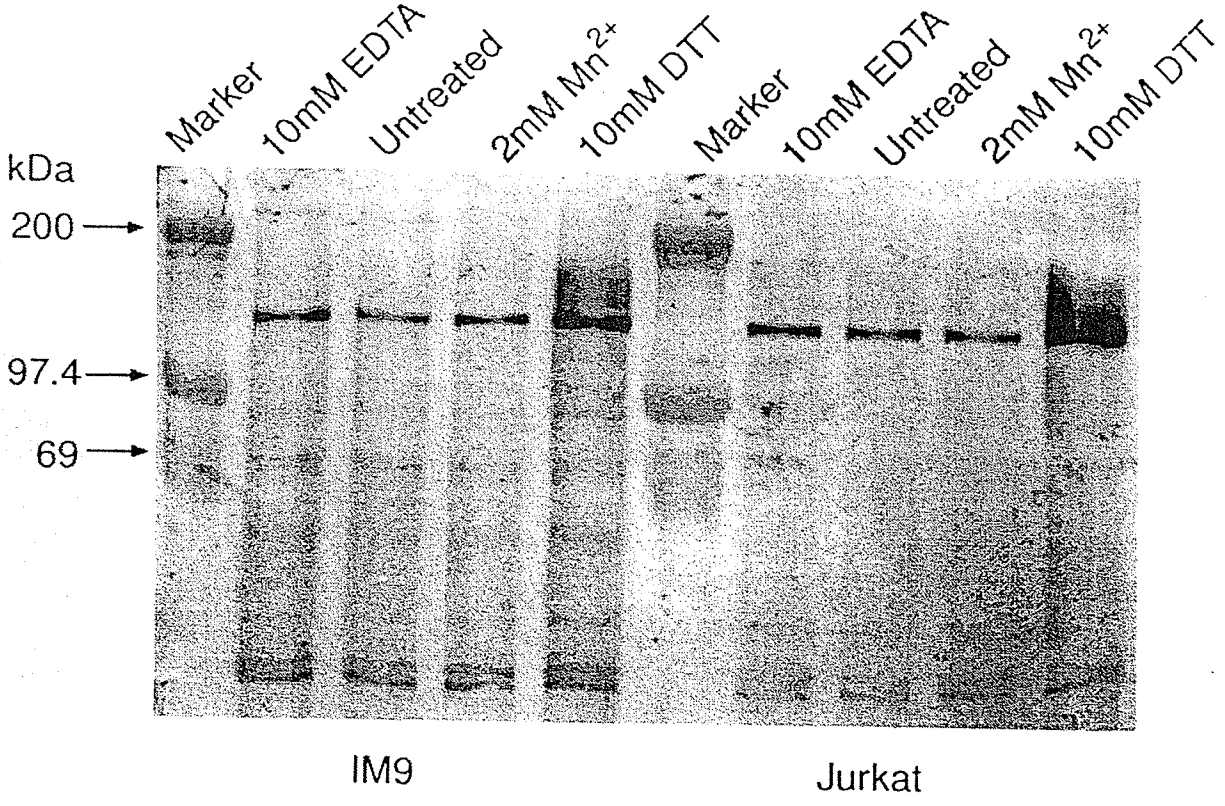


Figure 3.4b. Labeling free –SH groups of integrin β_1 chain with biotin-BMCC on IM9 cells and Jurkat cells. As described in materials and methods, cells were first treated with 10mM EDTA or 2mM Mn^{2+} or 10mM DTT at room temperature for 30 minutes, then biotin-BMCC dissolved in DMSO was added to each tube at a concentration 1 [μM]. After labeling, cells were lysed and β_1 integrins were immunoprecipitated with JB1A coupled Sepharose 4B beads and analyzed with western blot. The biotinylated free –SH groups were detected with streptavidin alkaline phosphatase and color was developed with NBT and BCIP.

Role of bacitracin, the protein disulfide isomerase (PDI) inhibitor, in integrin mediated cell adhesion. There are several pieces of data which suggest that de-association of long-range disulfide bonds may be a mechanism for keeping $ß_1$integrin in an active conformation. These include 1) DTT induces $ß_1$ integrin mediated cell adhesion; 2) DTT induces exposure of free sulfhydryls in $ß_1$ integrin; 3) DTT induces the expression of several stimulatory epitopes (N29, B44 and B3B11) and, these epitopes are localized in close proximity to predicted long-range disulfide bonds (Cys^{7}-Cys^{444}, Cys^{415} –Cys^{671}) (see section 3.2 & 3.31); 4). A most reasonable explanation of stimulatory roles of N29, B44, and B3B11 is their possible roles in blocking the reforming of these two long-range disulfide bonds. However, it is not clear whether similar mechanisms that control integrin function by dissociation of disulfide bond(s) exist under physiological conditions *in vivo* and if so, which molecule(s) mediate integrin disulfide bond exchange.

Protein disulfide isomerase (PDI), which catalyzes protein disulfide bond exchange, has been found on lymphocyte and platelet surfaces (82, 133). To determine whether this enzyme is the functional surrogate of DTT, Bacitracin, a PDI inhibitor, was examined for effects on cell adhesion. Strangely, Bacitracin strongly inhibited $ß_1$ integrin mediated cell adhesion (Mou's data, ref.134) without decreasing N29 and B44 epitope expression (my FACS data), which contradicts my previous prediction because the expression of these two epitopes was highly correlated with dissociation of disulfide bonds and cell adhesion status. If bacitracin inhibited cell adhesion results from inhibiting PDI function, i.e. inhibiting disulfide bonds exchanges, N29 and B44 epitope should be eliminated. In contrast, after treatment with 3.5mM Bacitracin, B44 epitope (CLIBS) expression was dramatically increased on different cells such as Jurkat, K562, IM9, and

peripheral blood lymphocytes, whereas N29 epitope, one of the most sensitive indicators to DTT activation, did not change (data not shown except Jurkat cell in ***Figure 3.4c***). My experiments further demonstrated that Bacitracin is able to block Mn^{2+} induced IM9 cell adhesion (data not shown) and block soluble fibronectin binding to K562 cells which express fibronectin receptor $\alpha_5\beta_1$ integrin on the cell surface (***Figure 3.4d***). Thus, Bacitracin may be the antagonist of β_1 integrin that directly interacts with integrin ligand binding pocket.

Although Bacitracin slightly inhibited PDI expression (data not shown), the role of PDI in integrin activation may be minor because Yanglong Mou demonstrated that anti-PDI antibody and other PDI inhibitors failed to show the inhibition of cell adhesion (134).

Figure 3.4c

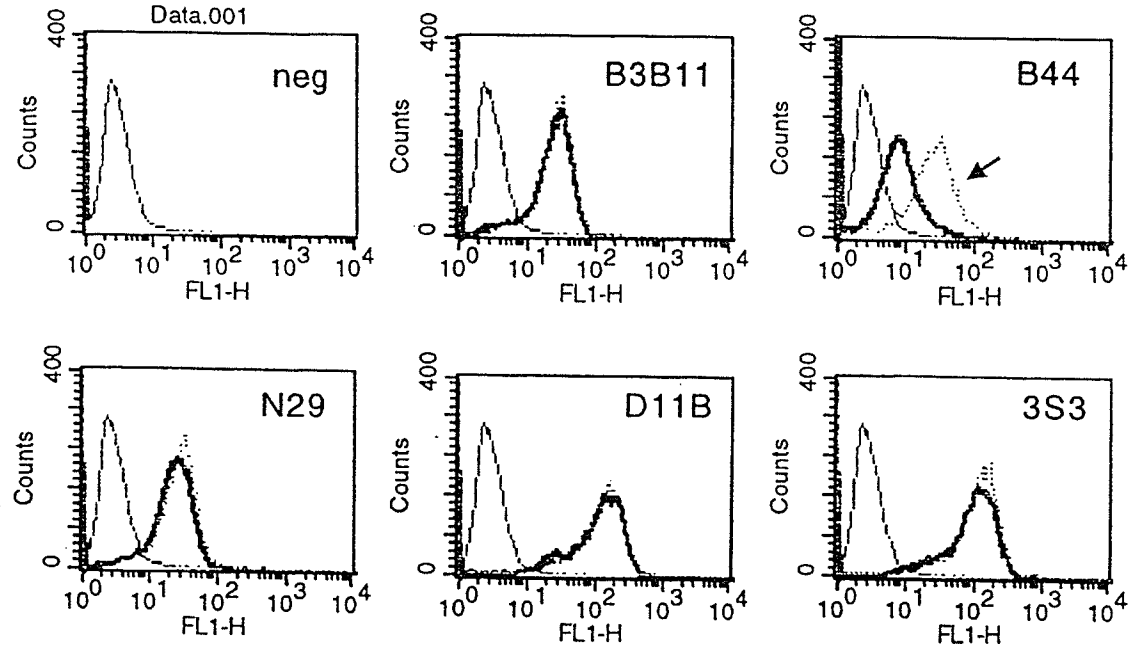


Figure 3.4c. *Effects of bacitracin on different β_1 epitope expressions on Jurkat cell. Cells were pretreated with (dotted line) or without (thick solid line) 5mg/ml bacitracin at 37°C for 30 minutes, then incubated with indicated monoclonal antibodies (5μg/ml) at 37°C for 30 minutes. Antibody binding was detected with FITC labeled goat antimouse IgG by flow cytometry analysis. The arrow shows the expression level of B44 epitope on Jurkat cells after treated with bacitracin. Cells incubated with second FITC labeled antibody alone was used as negative control.*

Figure 3.4d

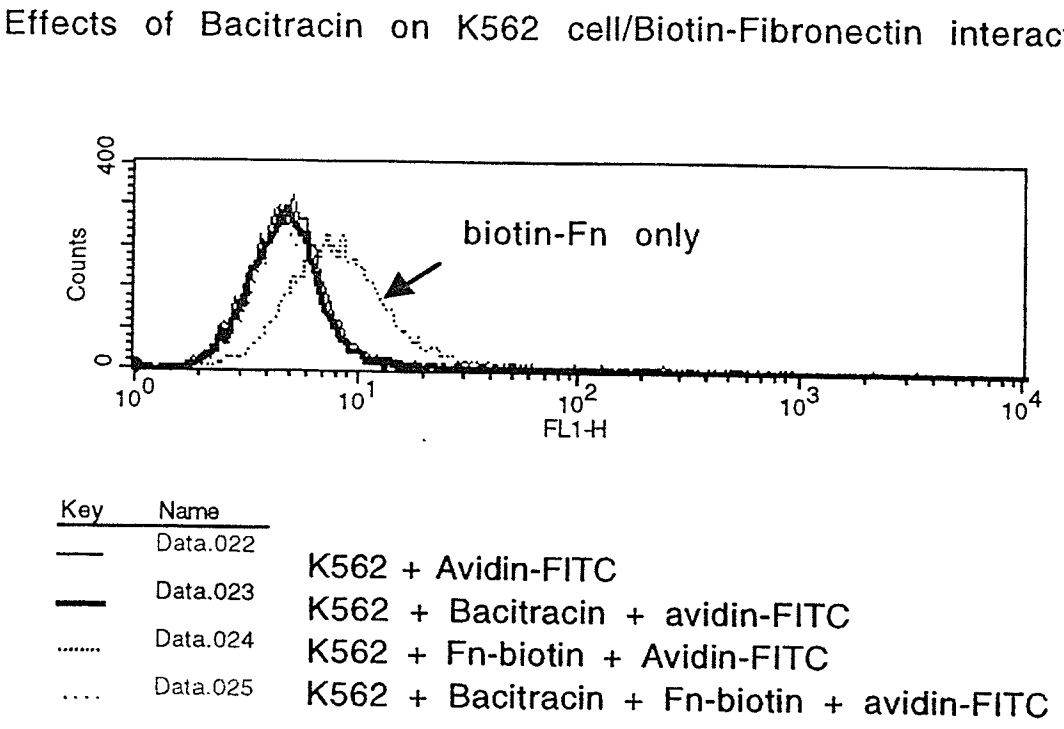


Figure 3.4d. *The effect of bacitracin on the binding of soluble fibronectin to cell surface $\alpha_5\beta_1$. K562 cells were incubated with biotinylated fibronectin in the presence or absence of 3.5 mM bacitracin for 30 minutes. The cells were then reacted with FITC-labeled avidin and examined for staining by FACS. The control binding of fibronectin is marked with the dotted line(the original color is blue); the binding in the presence of bacitracin is indicated by the line of dots with more space in between (the original color is brown); and the negative control binding is indicated by the solid lines. Note that except for control binding of fibronectin (arrow), all other profiles were identical, and bacitracin completely blocked fibronectin binding to K562 cells.*

CHAPTER 4: DISCUSSION

In this study, I have localized several regulatory epitopes on the human $ß_1$ chain and monitored their expression under different states of integrin functionality. Collectively these results indicate that there are multiple conformations of integrins, which correlate with the functional status of the molecule. Furthermore, it appears that it is possible to generate functional integrins with conformations that do not necessarily represent physiologically relevant structures. However, such information can provide insight into the structural changes that are required for the acquisition of ligand receptivity. These results have allowed me to develop a structural model of integrin regulation (*Fig.4.1).*

Figure 4.1

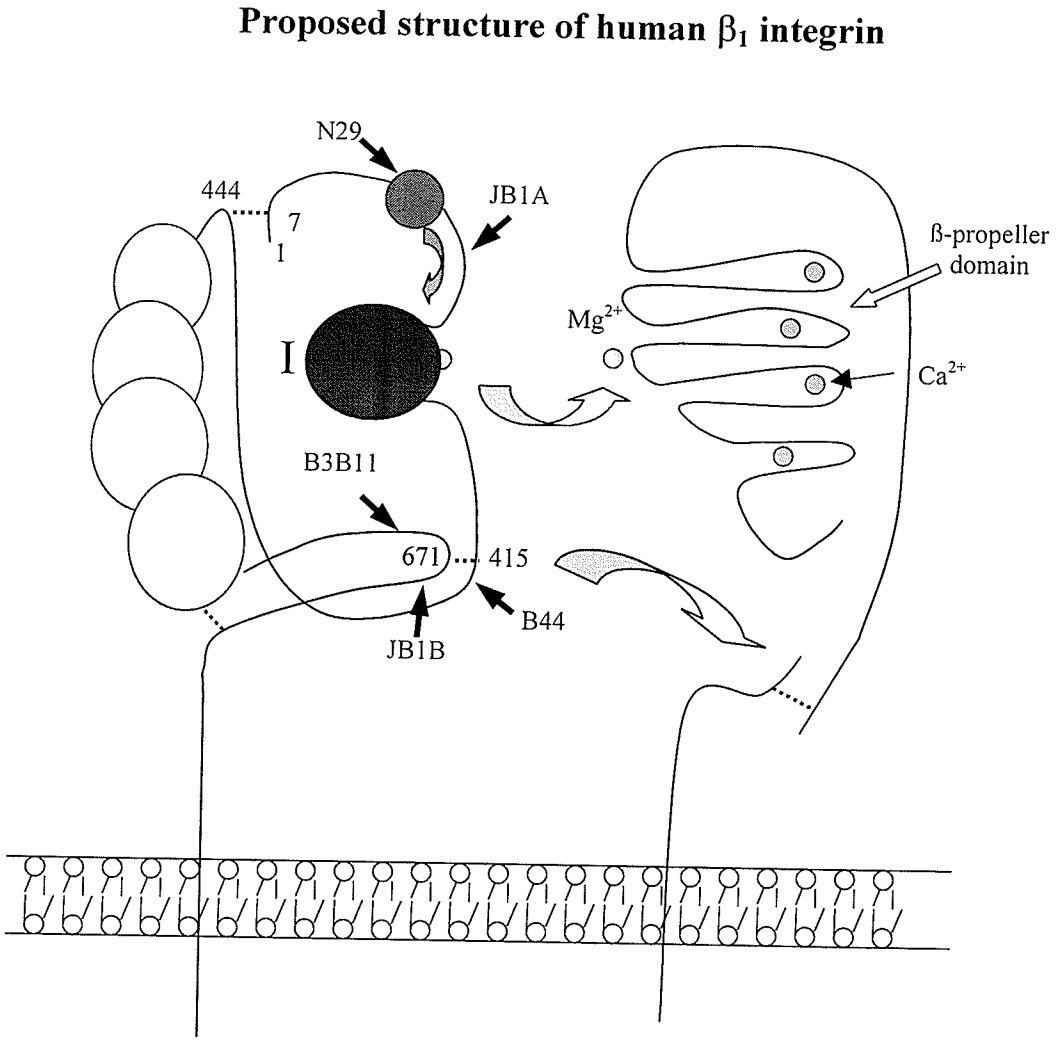


Figure 4.1. *Proposed location of the human β_1 epitopes studied in this investigation. B3B11 group (B3B11 and JB1B) and B44 group (B44 and 13B9) antibodies bind to the region closing to the long-range disulfide bond (671-415). N29 binds to the region closing to another long-range disulfide. bond (7-444). These suggest that some stimulatory antibodies control cell adhesion may through blocking reforming long- range disulfide bonds. N domain may be a "door" of integrin family which control integrin ligand entry by its interaction with I-like domain. JB1A inhibits cell adhesion may through its effect on regulating of N domain/I-like domain interaction. Whereas 3S3 blocking cell adhesion not only through blocking ligand binding site but also through fixing the N domain to I-like domain, i.e. locking the" door". After the "door" opening, and ligand binding, B44 epitope (CLIBS) then exposes to outside*

4.1 Characterization of JB1A Group Epitopes:

Three monoclonal antibodies, JB1A, C30B and D11B recognize the same epitope sequence, TAEKLK (residues 82-87), on the human $ß_1$ chain. These antibodies also recognize TPAKLR (residue 179-184) with a lower affinity. However, other properties of these antibodies such as binding patterns to different phage inserts and their capacities to inhibit cell adhesion are different. Only JB1A is sensitive to Mn^{2+} during antibody/epitope interaction. Thus, they are different antibodies. That three different antibodies recognizing the same region were cloned in our laboratory may be a reflection of the high levels of sequence homology between human and murine $ß_1$ chain. Thus, only limited regions have immunogenicity, which can be recognized by B cell clones.

There are several explanations for their inhibition of cell adhesion: **1)** TAEKLK is one of the ligand binding sites on human $ß_1$ chain i.e., Antibody binding to this site blocks ligand interaction. However, my preliminary studies have failed to demonstrate either direct interaction between this peptide and fibronectin or this peptide blocking cells adhesion (data not shown). This might be the case, because the JB1A epitope does not correspond to the homologous ligand contact sites proposed for the $ß_3$ chain (108, 109, 113). **2)** The blocking of cell adhesion is a result of antibody binding TPAKLR (residue 179-184 of human $ß_1$ chain). Although these antibodies bind TPAKLR with relative low affinity (data from phage display library and peptide ELISA of ***Fig.3.22a*** and ref. 115), the possibility that the antibody binds with one Fab binding this site and with the other one to TAEKLK cannot be excluded. TPAKLR is located between two conserved regions (residue 120-139 and 221-241) that have been suggested to be ligand-binding sites (53,

54). Recently, residues 164-202 of the $ß_3$ chain that correspond to the ß1 region which contains TPAKLR have been shown to be involved in the control of ligand binding specificity (135). However, similar to TAEKLK, I failed to demonstrate that TPAKLR bound to fibronectin or blocked cell adhesion. Furthermore, JB1A is not able to inhibit the adhesion of a mouse T hybridoma to fibronectin or to bind to these cells, despite the fact that the TPAKLR sequence is also present on murine $ß_1$ integrin (data not show). **3)** JB1A binding alters integrin conformation to an inactive form. TAEKLK (residues 82-87) is located between the N29 epitope (NH_2 terminal cysteine rich domain, residues 15-55) and the proposed I domain structure (around residues 110-330). The NH_2 terminal cysteine rich domain (designated as N domain) has been hypothesized in this study as a "door" on the integrin which may control the entrance of ligand to the ligand binding groove (see discussion below). The I domain structure contains divalent cation and ligand binding sites and the sites required for α/ß subunit interaction (50, 52, 56). Thus, the JB1A group of antibodies blocking of cell adhesion may be the result of effects on this domain. This hypothesis has been partially supported by the preliminary data which indicate that JB1A inhibits stimulatory N29 and B44 epitope expression. (***Figure 3.32c***).

4.2 Roles of NH2 terminal cysteine rich domain: fact or fiction?

In this study, I localized the N29 epitope to the residues 15-55, the NH_2 terminal cysteine rich domain (N domain). Although this epitope can be detected by western blot under reducing condition in both $ß_1$ integrins and two fusion proteins (residues 1-55 and residues 1-105) expressed from distinct vectors in *E.coli*, the further identification of its epitope with synthetic peptides or phage peptide display libraries has failed. These results

may suggest that the N29 epitope is dependent on local conformation, i.e. partial oxidation of –SH groups within this cysteine rich domain may be required for this epitope formation. In this case, reducing agents in the loading buffer may not be sufficient to prevent protein partially renaturation during protein transfer and other steps in the western blot procedures.

The regulatory role of N domain in integrin family has not been reported before although the regulatory epitope of AP5 in the ß3 chain (residue 1-6) is close to this domain (129). N29 binding to this domain strongly enhances cell adhesion indicating that it is important for integrin function. The N29 epitope is expressed on both spontaneously adhesive cells such as K562 and Jurkat and on stimulus activated non-adhesive cells such as IM9 and peripheral blood lymphocytes but it is not significantly expressed on non-adhesive cells. These observations suggest that the expression of N29 epitope may be a prerequisite for normal cell adhesion, or maintenance of integrin active states.

Possibly the most interesting data for understanding N domain function is from mapping the discontinuous 3S3 epitope with 26mer-phage peptide display library. 3S3 is the strongest inhibitory antibody generated in this laboratory. It does not recognize the $ß_1$ chain under reducing conditions. After three rounds of panning with biotinylated 3S3, 18 out of 21 phage isolates had the identical insert peptide that contains CGWSPEG residues. Comparison with amino acid sequence of $ß_1$ chain revealed that the CGW (residues 25-27 of $ß_1$ chain) and SPEG (residues 227-230 of $ß_1$ chain) respectively exist in the N domain and in the second conserved region.

Several pieces of information suggest that the SPEG region might be a ligand-binding site. These include:1) the corresponding region in the $ß_3$ chain is a fibrinogen-binding site (53). 2) A point mutation at E^{229} (112) in the $ß_1$ chain results in a complete loss of ligand binding capacity. 3) My preliminary studies demonstrated that the peptide derived from this $ß_1$ conserved region can block Mn^{2+} activated IM9 cell adhesion (***Figure 3.23b***). Thus, binding to this region and retention of the N29 domain in this area may be the basis of the 3S3 inhibitory function. These results suggest the spatial close proximity between the ligand bind site and the NH_2 terminal cysteine rich domain.

I hypothesize that the NH_2 terminal cysteine rich domain is the door of integrin family because the cysteine residues in this domain are conserved in all ß integrin chains in different integrin and animal species. This hypothesis suggests that the NH_2 terminal cysteine rich domain "tightly blocks" the ligand binding sites when integrin is in its inactive form i.e., the "door" for ligand entrance is closed. In this conformation, the N29 epitope is a cryptic epitope that hides among other parts of α/ß heterodimer. 3S3 binding inhibits N29 epitope expression suggesting this antibody not only occupies the ligand binding site but also "locks" the "door." It is worth mentioning that this domain may not merely block the SPEG region. By means of its three-dimensional structure, it is possible to block other ligand binding sites because the SPEG region and other ligand and cation binding sites are in spatial close proximity in the I domain structure of $ß_1$ chain (***Figure 1.33a***). This "door" can be opened when integrin is activated by divalent cations, reducing agents such as DTT, or intracellular signals such as cross-linking CD3 on T lymphocytes.

During these processes, the N terminal domain somehow dissociates from ligand binding sites so that ligand can enter into its binding groove and the N29 epitope is expressed on the cell surface. This model can also explain why the N29 epitope is always expressed on the spontaneous adhesion cell surfaces such as K562 and Jurkat cell surface because these cells can not bind integrin ligands without N29 epitope expression.

The spatial close proximity between the N terminal and I domain structure (ligand binding sites) provides another possibility that the N domain catalyzes the disulfide bond exchange within the I domain structure. The CGEC (residues 15-18) and CGWC (residues 25-28) in the N domain contain the consensus sequence Cys-Gly-X-Cys which was found at the active site of the Protein-Disulfide Isomerases (PDI), bacterial thioredoxins and other proteins which have similar enzymatic activity (85, 136, 137). Thus, the NH_2 terminal domain may influence ligand binding through exchange with, for example the I domain disulfide bonds.

Although this model can explain many phenomena, it needs to be further verified. Two major questions remain to be addressed: **1)** whether the NH_2 terminal cysteine-rich domain does make contact with the I domain-like structure in the $ß_1$ chain? Although the residues adjacent to corresponding SPEG region in β_3 (residues 214-218) can bind $\alpha_{IIb}\beta_3$ (53), there is no report that these residues bind the N terminal domain. Although, my preliminary data with biotinylated peptides from the second conserved region (residue 221-241) demonstrated that this biotinylated peptide binds the NH_2 terminal 57 amino acid peptide (data not show), this peptide also binds other control proteins such as BSA. Thus this conserved region seems to be a non-specific binding site. To further

demonstrate the relationship between these two functional domains, protein cross-linking experiment may be required. **2)** Whether CGW*SPEG is the 3S3 epitope? Although phage data and 3S3 inhibitory role support this result, the direct evidence that both residues 25-27 and residues 227-230 are modules of 3S3 epitope have not been obtained. An E^{229}mutant from Takada group (112) and direct mutations of residues 25-27 may provide further support for this hypothesis in the future investigation.

4.3 Localization and characterization of B3B11 and B44 group epitopes.

4.3a The role of long-range disulfide bonds in integrin function.

The B3B11 group stimulatory epitopes have been localized to residues 636-705. The B3B11 epitope was further localized to residues 658-666 with the phage peptide display library in this study. Because JB1B failed to recognize the peptide before residue 670 in my peptide ELISA, this epitope may be located in somewhere of residues 671-705. Thus this group of epitopes encompasses a predicted long-range disulfide bond (Cys^{415}-Cys^{671}). It is very interesting that B44, another strong adhesion stimulatory epitope, is located in the opposite side of this disulfide bond i.e., residues 355-425 (***Figure 4.1***). These data suggest the important role of long-range disulfide bonds in integrin function. This interpretation is further supported by the fact that the N29 epitope that is close to another long-range disulfide bond (Cys^{7}-Cys^{444}).

DTT activation of β_3 integrin on platelet has been known for many years (87). In this study, I found DTT is also capable of inducing IM9 cell adhesion to fibronectin and

this adhesion can be inhibited by antibodies against α_4 and β_1. Furthermore, this reducing agent generates increased numbers of free –SH groups on β_1 integrin (Fig. 3.4b) and induces expression of the epitopes recognized by the stimulatory antibodies N29, B44 and B3B11 and found close to the two predicted long-range disulfide bonds. These data strongly suggest that dissociation or association of disulfide bonds, especially the long-range disulfide bonds, may play an important role in integrin conformational transition.

That DTT induces cell adhesion by directly reducing disulfide bonds of integrin has not been reported before. In contrast, DTT-induced activation of LFA-1 ($\alpha_L ß_2$) mediated adhesion of natural killer cells to ICAM-1 expressing target cells was reportedly not associated with integrin reduction (89). In this case, integrin conformational changes could not be found by flow cytometry analysis with two reporter antibodies and DTT did not upregulate –SH groups detected by sulfo-NHS-biotin. These results clearly differ from my results. There are three possible explanations for these apparent differences: 1) the concentration of DTT used in my experiments (10mM) was 20 times higher than that used on NK cells. The 0.5mM DTT may be sufficient for activation of LFA-1 on NK cell by a signal pathway but was not sufficient to reduce LFA-1. But this reason cannot account for my results with biotin BMCC labeled $ß_1$ integrin where free –SH groups were shown to exist in both the active (on Jurkat cells) and inactive forms (on IM9 cells) even before DTT treatment. 2) $ß_2$ integrin differs from $ß_1$ integrins. There are no free SH groups in $\alpha_L ß_2$. 3) The method used 3-(N-maleimido-propionyl) biocytin (MPB; Molecular Probes), a biotin-conjugated maleimide derivative, to detect free –SH groups in the case of LFA-1 in previous publications (89) were not as sensitive as biotin BMCC

system in my experiments, or my results were due to non-specific reactions. The further comparison of these two -SH group labeling systems may be required to clarify this controversy. However, no matter what is the correct explanation, it is possible that DTT can activate integrins through both signal pathway or by directly reducing integrin disulfide bonds.

The fact that both adhesive (Jurkat) and non-adhesive (IM9) cells express free –SH groups on β_1 chains also contradicts a model for disulfide pairing patterns based on β_3 integrins (56), in which all cysteine are predicted to be in paired disulfide bonds. There appears to be some support for my results in a recent publication where it was shown that ligand binding induced the formation of a new disulfide bond in a fraction of $\alpha_2\beta_1$ on platelet surface (138). Thus the exchange of integrin disulfide bonds on cell surfaces may be an important mechanism of control integrin function, at least in several β_1 integrin families. The further localization of these free –SH groups before and after DTT treatment should be of significance in integrin structure studies in the future. Several methods including the point mutations of putative cysteines and proteinase digestion of biotin-BMCC labeled β_1 chain may be useful to address this question.

To identify possible functional reductase systems *in vivo* that control disulfide bond exchange is another important theme. Although we failed to demonstrate the role of cell surface PDI in integrin mediated cell adhesion (134), other molecules such as fibronectin and integrins themselves as suggested above might play similar role of DTT in facilitating disulfide exchange in vivo. This work, by accident, found a potential application of

bacitracin, an antibiotics, in regulating β_1 integrin function. Bacitracin and its derivatives may have some clinical significance in control of inflammation and tumor in future.

4.3b The region around B3B11 and B44 epitope may involve in α/β subunits interaction.

In addition to the possible effects on ß1 intrachain disulfide bonds association, B3B11 and B44 group antibodies may also influence the interaction of α/β subunits. Two α/β chain interacting regions on β_3 chain corresponding to the I domain and the membrane proximal region have been suggested by Calvete (56), The B3B11 and B44 epitopes are located in the latter region. It has been suggested that some LIBS may be present at the α/β interface (100). Protein cross-linking studies may be useful in obtaining information about α/β_1 chain interactions in this region.

4.3c The role of O-linked carbohydrate in β_1 integrin.

Integrins are glycoproteins, which contain 14 and 12 potential N-linked glycosylation sites on α and β subunits, respectively. The roles of N-glycosylation have been reported before (59, 60). Although one O-glycosylation site has been localized to Ser^{847} in α_{IIb} and the effects of O-linked glycosylation on VLA-4 and VLA-5 dependent HL60 cell adhesion has been reported (62), there is little information about O-linked carbohydrate in the ß chain.

In this study and the previous study, several pieces of information suggest that the B44 epitope is an O-linked carbohydrate dependent epitope. These include: 1) Screening human β_1 integrin NovaTope libraries with B44 failed to find any positive clone although B44 recognized a linear epitope on human β_1 chain with high affinity. This library was established in *E.coli*, carbohydrates can not be expressed properly in this system. 2) The B44 epitope is resistant to N-glycosidase F but is sensitive to TMSF, which removes both N and O-linked carbohydrates (Li A and Wilkins JA unpublished observation). 3) The B44 epitope is sensitive to O-sialoglycoprotein endopeptidase that specifically digests O-linked carbohydrate protein although the cleavage site may be several amino acids away from the O-glycosylation site (***Figure 3.25f***). 4) The B44 epitope has been localized between residues 355 and 425 with human/mouse chimeric integrins, however, neither B44 recognize the peptides derived from the region 355-425 nor do these peptides block B44 recognition of the native β_1 chain. These peptides cover all immunogenic sites in this region (***Figure 3.25e***). Thus, an O-glycosylation site may exist in this ligand induced binding site. However, these results cannot rule out other possibilities: 1) The repertoire of NovaTope libraries was not big enough to cover the B44 epitope. 2) Peptide-borne B44 epitope was chemically modified during TMSF deglycosylation so that B44 can not recognize it. TMSF may oxidize some groups of amino acid during deglycosylation. 3) Although O-sialoglycoprotein endopeptidase is highly specific for O-sialoglycoproteins and no significant cleavage has been seen in hundreds of non-glycoproteins, we cannot rule out that it could cleave the B44 binding site on human β_1 integrin through the enzyme itself or traces of bovine serum proteases in the product. 4) That the linear peptide PKKDSD, which is located in residue 418 to 423 of human β_1 chain, might be

B44 epitope cannot be ruled out. This hexameric peptide was not included in the previous used two overlap peptides (see Figure 3.25e). In order to further confirm that B44 did not recognize linear peptide epitope, two more experiments need be done: 1) ELISA to test whether B44 binds synthetic peptide PKKDSD. 2) ELISA or western-blot to test whether B44 binds recombinant protein that contains the amino acids of 355-425 of the human β_1 chain.

It has been suggested that carbohydrate moieties play important roles in glycoproteins including stabilization of specific protein conformations, protection against proteolytic degradation and modulation of biological activity. However at this point, it is not clear as to what the role of O-linked carbohydrates might be in this ligand induced binding site. It may mediate intramolecular interaction or mediate interaction with the α chain or other integrin associated proteins. These interactions may be necessary to keep integrin in inactive form. It will be interesting to examine $\beta_1$integrin function and B44 epitope expression after the cell O-glycosylation pathway is inhibited. It will be also interesting to determine whether this O-glycosylation site exists in all other integrins and plays similar roles.

4.4 Phorbol ester PMA induces β_1 integrin loss from the cell surface.

Integrin internalization and shedding haven't been well studied although there are a few publications (65,66). This mechanism may be important during cell migration. In this study, I found dramatically reduced β_1 integrin levels on K562 cell surface after PMA treatment in 37°C 30 minutes. Further investigation this phenomenon may provide some valuable information for cell adhesion and migration.

Summary: This study has localized several regulatory epitopes in human $\beta_1$chain. The roles of long range disulfide bonds, divalent cations and O-linked carbohydrate in control of integrin function have been investigated. A model that suggests NH2 terminal cysteine rich domain (N domain) is the "door" of integrin family has been proposed. However, this study has not obtained direct evidence that the N domain does interact with the I-like domain. Besides, B44 epitope and the locations of free –SH groups in $\beta_1$chain are still not clear. The mechanism of rapid down regulation of integrin on K562 cell surface by PMA has not been addressed. All of these should be interesting for future investigation.

Like many other investigators, we have used high concentrations of divalent cations or the reducing agent DTT to induce cell adhesion. These reagents have provided some useful information in integrin conformations that can bind ligand. However, integrin structures induced by these reagents may differ from their natural conformations in physiological and pathological environments in vivo. That multiple stimuli and inhibitors exist at the same time and the dynamic nature of these regulators allows for responsive means of regulating integrin activities. There is a clear need for the development of better systems to monitor integrin conformational transition in vivo.

CHAPTER 5. REFERENCES

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 271, No. 6, Issue of February 9, pp. 3046–3051, 1996
Printed in U.S.A.

Control of β_1 Integrin Function

LOCALIZATION OF STIMULATORY EPITOPES*

(Received for publication, July 3, 1995, and in revised form, October 13, 1995)

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The β_1 integrins can be expressed on the surface of cells in a latent form, which is activated by a variety of stimuli. As an approach to examining the transition to an active receptor, a panel of stimulatory antibodies to β_1 were produced and characterized. These antibodies induced adherence of the T-leukemic cell line Jurkat to collagen and fibronectin. Competitive antibody binding assays indicated the existence of at least three distinct epitope clusters A (B3B11, JB1B, 21C8), B (B44, 13B9), and C (N29) defined by the indicated antibodies. Two antibodies to the A site, JB1B and B3B11, were shown to localize to positions 671–703 and 657–670, respectively, of the β_1. This region is located in an area encompassing a predicted disulfide bond between linearly distant cysteines in β_1 (Cys^{415}-Cys^{671}). The homologous region of the β_3 integrin (490–690 and 602–690) has been shown to be one of the sites recognized by stimulatory antibodies to ligand-induced binding sites. The present results indicate the existence of multiple stimulatory regions and suggest considerable homology between the locations of β_1 and β_3 regulatory sites.

Members of the integrin family mediate cellular adherence to a variety of extracellular proteins (*e.g.* collagen, fibronectin, vitronectin, laminin) or to cell surface-associated molecules through homotypic or heterotypic (1–3) interactions. Several cell types (*e.g.* platelets, leukocytes) express some of the integrins on their surfaces in latent forms. It is only subsequent to cell activation that these molecules display binding potential for their cognates (4–7).

The mechanisms responsible for the transition to an activated state are unknown. However, it is apparent that receptor conformational charges are associated with the generation of a functional complex (8–10). A number of antibodies that can activate receptors have been described for the integrins (11–18). In the case of $\alpha_{IIb}\beta_3$, a number of the stimulatory antibodies (anti-LIBS1, D3GP3 (14), anti-LIBS2 (15), anti-LIBS3, anti-LIBS6 (16), and AP5 (17)) recognize epitopes that are expressed on ligand occupancy of the receptor. Some of these anti-LIBS reagents have been particularly useful in probing aspects of cation requirements for receptor function and for the localization of possible regulatory regions of the molecule (10, 17). However, there is considerably less information about regulatory sites on other members of the integrin family.

The β_1 integrins play a critical role in the functional activity of lymphocytes by influencing cellular distribution patterns and by functioning as costimuli for differentiation or proliferation induction (20, 21). Similar to the $\alpha_{IIb}\beta_3$ system, the functional status of the β_1 integrins is stringently controlled with activation being induced by a variety of stimuli including antibodies to the β_1 chain (11–13).

Two groups have reported on the locations of some of the regulatory regions of the β_1 integrins using monoclonal antibodies to the β_1 chain (22, 23). The result of one study suggested that there may be multiple regulatory regions (23), while a second indicated that the majority of stimulatory and inhibitory antibodies reacted with a very restricted region of the β_1 molecule (22). Studies from the β_3 integrin system would seem to support the existence of multiple distinct regions defined by regulatory antibodies.

As an approach to defining the number and location of potential regulatory sites on the β_1 chain, we have identified and characterized six stimulatory antibodies. The relative positions of the epitopes recognized by these antibodies were determined by competitive binding studies. Furthermore, the location of one set of such epitopes in the β_1 molecule was determined.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated, all chemicals were purchased from Sigma. Media, fetal bovine serum, and fibronectin were obtained from Life Technologies, Inc. Custom synthesized peptides were purchased from Chiron Mimotopes Peptide Systems.

Antibodies—Mice were immunized with Jurkat-derived β_1 integrins, which had been affinity purified with the anti-human β_1 monoclonal antibody JB1 (24). Hybridomas were produced and enzyme-linked immunosorbent assay screened for reactivity with purified β_1 (25, 26). Positive clones were screened for reactivity with electroblotted proteins of SDS, 7% polyacrylamide gel electrophoresis-separated Jurkat proteins and their abilities to immunoprecipitate β_1 integrins from ^{125}I surface-labeled Jurkat cells (7). Antibodies were purified from the 30–50% ammonium sulfate fractions of ascitic fluid on a protein A-Sepharose column (26).

Biotinylation—Protein A-purified antibodies, 10 mg/ml, were suspended in 50 mM sodium bicarbonate, pH 8.5, and reacted with *N*-succinyl LC biotin, 400 μg/ml (Pierce), for 30 min at room temperature. The buffer was exchanged with PBS,[1] 0.1% sodium azide using Centricon-30 centrifuge column (Amicon). The final sample was adjusted to 0.5–1.0 mg/ml.

Cells and Culture—The human T cell leukemia Jurkat was grown in RPMI 1640 supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol.

CHO cells transfected with human α_5, provided by Dr. R. Juliano (27), were cotransfected by electroporation with pFnRβ, a construct containing the full-length human β_1 gene provided by Dr. E. Ruoslahti (28), and pREP4, a vector carrying the hygromycin reductase marker, provided by Dr. M. Tykocinski (29). The cells were cultured for 48 h in

* This research was supported by grants from the Manitoba Health Research Council, the Medical Research Council, and the Canadian Arthritis Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; BSA, bovine serum albumin; bp, base pair(s).

RPMI with fetal bovine serum containing G418 (80 μg/ml), after which hygromycin B was added (300 units/ml). Transfectants reacting with the anti-human β_1 monoclonal, JB1 (24), were selected by fluorescence activated cell sorter and cultured in the selection media. This cycle was repeated three times to achieve stable lines. It was found to be necessary to use the CHO α_5 as the host cell for β_1 expression, as in our hands the wild type CHO cells did not efficiently express the human β_1 gene product. Specificity of the transfectants were confirmed using a panel of monoclonal antibodies to β_1 including JB1A (30), 3S3 (31), and JB1 (24).

Cell Binding Assay—Binding assays were performed as described previously (12). Non-tissue culture microtiter wells were coated with purified plasma fibronectin (5 μg/ml) or type I collagen (20 μg/ml). The wells were washed and blocked with 1% BSA in RPMI. Jurkat cells prelabeled with ^{51}Cr were preincubated with the indicated antibodies for 30 min and then added (10^5/well) to fibronectin, collagen, or BSA-coated wells and incubated for 1 h at 37 °C. The nonadherent cells were removed by three washes with PBS, 1% BSA. The bound cells were solubilized in 1% SDS, and the levels of radioactivity were quantitated. Each assay was performed in triplicate, and the experiments were repeated at least three times. The S.E. of the replicates was less than 15%.

Competitive Binding Assay—Cells were suspended (5×10^4) in PBS, 1% BSA in a V-bottom 96-well microtiter tray and incubated in the presence of the indicated concentration of unlabeled antibody for 1 h at 4 °C. The cells were washed with PBS-BSA, after which biotinylated reporter antibody (0.2 μg/ml) was added for 30 min at 4 °C. The cells were washed, reacted with a horseradish peroxidase-conjugated avidin complex, washed again, and the color was developed with substrate 400 μg/ml *O*-phenylenediamine. The reaction was stopped by the addition of an equal volume of 10 M H_2SO_4 and quantitated at 492 nM.

Epitope Library Production and Screening—Libraries were constructed using the NovaTope system (Novagen Inc.) according to the supplier's instructions. The method based on the use of modified pET expression vectors consisted of digesting the full-length pFnRβ with DNase I in the presence of Mn^{2+} and size fractionating the random fragments by electrophoresis in 1.2% agarose gels. The 50–150-bp or 150–300-bp fragments were flush ended with T4 DNA polymerase, single dA-tailed, and ligated into the *Eco*RV site of the pTOPE-1b(+) plasmid. Novablue (DE3) cells were transformed with the plasmid, and colonies were immunoscreened with a panel of anti-β_1 monoclonal antibodies and an alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (26). Positive colonies were subcloned and examined for reactivity with the antibodies. The inserts from individual clones were sequenced using a T7 gene 10 primer.

Enzyme-linked Immunosorbent Assays and Blocking Assays—Peptides were suspended at 10 μg/ml in 0.1% dimethyl formamide in water and allowed to dry overnight, 0.5 μg/well, in Nunc Maxisorb plates. The plates were washed three times with 0.5% Tween 20 in PBS and blocked for 2 hours at room temperature with 2% BSA in PBS. The indicated antibodies (5 μg/ml) were added to the wells, and the binding was quantitated using a peroxidase rabbit anti-mouse IgG conjugate and developed with 400 μg/ml *O*-phenylenediamine as substrate.

Blocking assays were performed by preincubating antibodies (150 ng/ml) with the indicated peptide concentrations for 2 h. The antibodies were then added to Nunc plates coated with affinity-purified placental β_1 integrin (32). The color was developed after reaction of the wells with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin and substrate.

RESULTS

Anti-β_1 Antibodies—The characteristics of the antibodies used in this study are listed in Table I. The specificity of the antibodies for the β_1 integrin chain were demonstrated by several methods. The antibodies specifically stained CHO cells stably cotransfected with human α_5 and β_1 cDNAs (Fig. 1). In contrast, neither the wild type cells nor those transfected with only the α_5 chain showed any binding with these antibodies. Each of the antibodies was also shown to immunoprecipitate all of the β_1 species (*i.e.* α_2, α_4, and α_5) on Jurkat cells (Refs. 8 and 19 and data not shown).

Previous studies had demonstrated that Jurkat cells display a low level of adherence to immobilized collagen (7). However, following stimulation with phorbol 12-myristate 13-acetate (7, 12) or some antibodies to the α_2 or β_1 chains (12), there was a marked increase in adherence. When a panel of anti-β_1 reagents was analyzed for the effects on Jurkat adherence, six stimulatory antibodies were identified (Fig. 2). This stimulation was detectable within 30 min of exposure and lasted for several hours (Ref. 12 and data not shown). This effect was not a property of all antibodies to β_1 as 3S3 (31) and JB1 (24) did not induce adherence. In the case of 3S3, there was inhibition of the low level of spontaneous binding of these cells to collagen.

TABLE I
Antibody properties

Antibody	Specificity	IP[a]	Blot[b]	Activity[c]	Reference
JB1	β_1	+	−	NULL	24
JB1A	β_1	+	+	Inhibitory	30
3S3	β_1	+	−	Inhibitory	31
B3B11	β_1	+	+	Stimulatory	This study
N29	β_1	+	+	Stimulatory	This study
JB1B	β_1	+	+	Stimulatory	12
21C8	β_1	+	−	Stimulatory	This study
13B9	β_1	+	−	Stimulatory	This study
B44	β_1	+	+	Stimulatory	This study

[a] Antibody is active in immunoprecipitation.
[b] Antibody is active in immunoblot of reduced antigen.
[c] Effect of antibody on adherence to fibronectin and collagen.

Competitive Binding of Stimulatory Antibodies to Jurkat Cells—The identification of several monoclonal antibodies with similar biological activities raised the possibility that they were reacting with a common region of the β_1 chain. To address this question, competitive blocking studies were performed in which cells were preincubated with an unlabeled competitor antibody. A biotinylated reporter antibody was then added, and the level of binding was assessed relative to that in the absence of competitor.

Fig. 3 gives an example of the type of results obtained using JB1B. There was a dose-dependent inhibition by the unlabeled JB1B. There was also competition by B3BII and 21C8. In contrast, another stimulatory antibody (N29) and an inhibitory antibody (3S3) failed to influence JB1B binding. Similar experiments were performed with each of the antibodies, and a summary of the results is given in Table II. Based on these results, it appears that there are at least three regions of the β_1 integrin that can act as targets for antibodies which activate integrin function. These are designated group A (B3BII, JB1B, 21C8), group B (B44, 13B9), and group C (N29).

The Localization of JB1B and B3B11 Stimulatory Epitopes—As JB1B, B3B11, and 21C8 appeared to recognize a related region of the β_1 molecule, it was decided to focus on these epitopes for further studies. Preliminary results had indicated that the epitopes recognized by JB1B and B3B11 were likely to be linear sequences of the β_1 chain since reduction did not influence antibody binding to β_1 integrins on immunoblot. In contrast, the 21C8 epitope was most probably a discontinuous one, as it was reduction sensitive. To increase the likelihood of identifying the locations of both continuous and discontinuous epitopes, a random β_1 epitope fusion protein library containing large β_1 inserts (150–300 bp) was constructed.

The library was screened with a pool of JB1B, B3B11, and 21C8, and five reactive colonies were identified. Assay of the individual clones with each of the antibodies indicated that JB1B and B3B11 reacted with all five clones; in contrast, 21C8 failed to react with these clones. Immunoblot analysis of SDS-polyacrylamide gel electrophoresis-separated lysates of one of the clones, B, under reducing conditions indicated that JB1B and B3B11, reacted with a 46-kDa band corresponding to a fusion protein (Fig. 4*B*), containing an insert of approximately 65 amino acids. In contrast, an inhibitory anti-β_1, JB1A, failed to react with this band, thus indicating the specificity of the

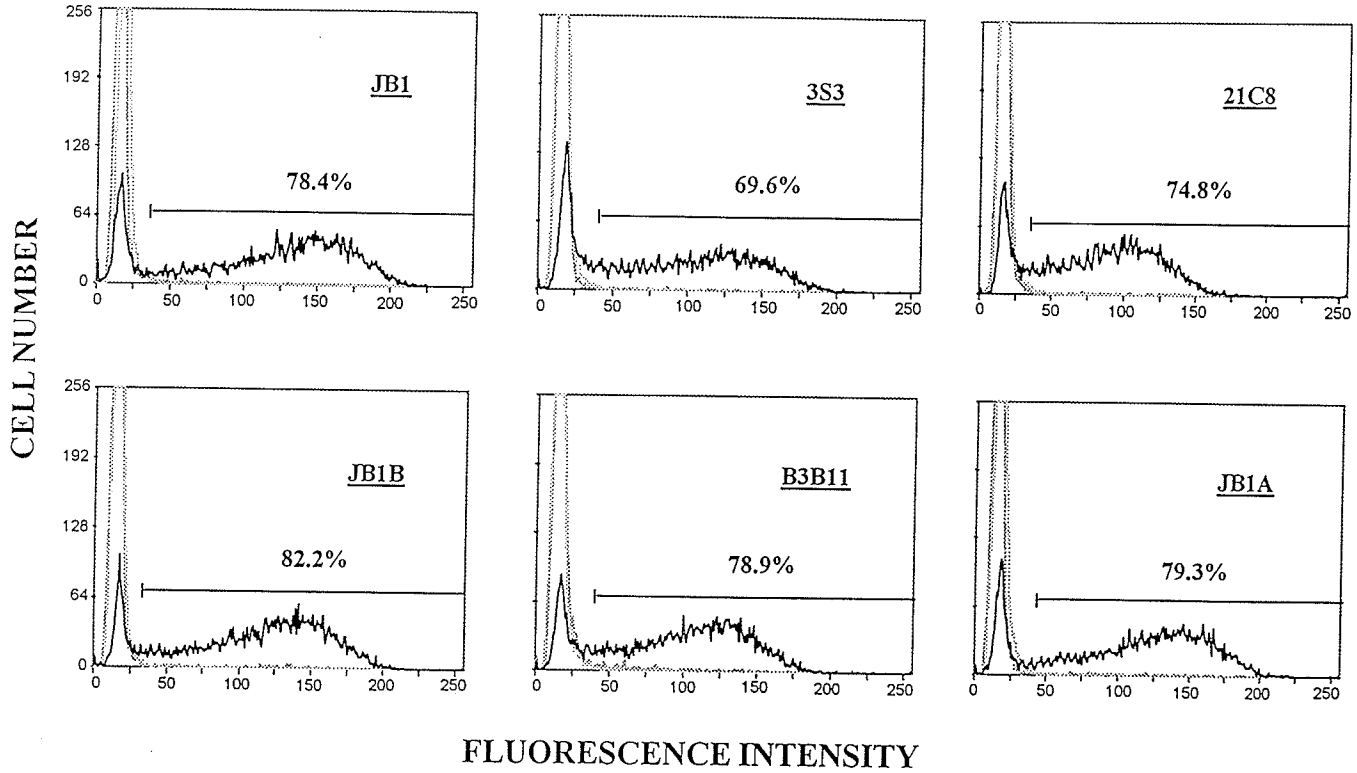


FIG. 1. **Specific binding of antibodies to CHO cells transfected with human β_1 integrin.** The binding of β_1 antibodies to CHO cells coexpressing human α_5 and β_1 (*solid line*) but not to CHO wild type or those expressing only human α_5 (*dotted lines*) indicates the specificity of these antibodies for human β_1.

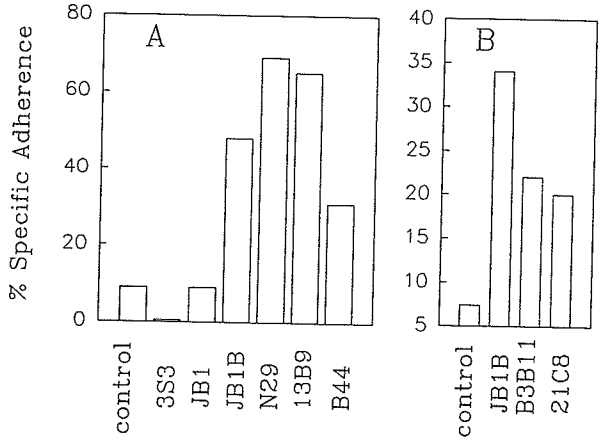


FIG. 2. **The induction of Jurkat cell adherence to type I collagen.** *A*, Jurkat cells were pretreated with the indicated anti-β_1 monoclonal antibodies for 1 hour, after which adherence to immobilized collagen was determined. *B*, a comparison of JB1B, B3B11, and 21C8 induced adherence. Values represent the mean of sextuplicate assays. All values were within 10% of the mean.

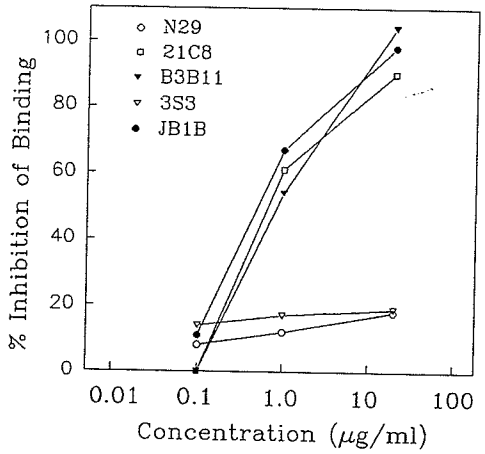


FIG. 3. **Competitive blocking of JB1B binding to Jurkat cells by monoclonal antibodies to β_1 integrin chain.** Blocking studies were performed as described under "Experimental Procedures." The stimulatory antibodies JB1B, B3B11, and 21C8 inhibit JB1B binding. Another stimulatory anti-β_1, N29, and an inhibitory antibody, 3S3, do not affect JB1B binding. Results are expressed as the percent inhibition of JB1B binding relative to that of JB1B to untreated cells.

reaction. It was noteworthy that 21C8 gave a very weak reaction with reduced proteins from this clone (Fig. 4*B*, *lane 3*) but not with unreduced fusion protein (data not shown).

The screening of a second epitope library containing smaller inserts (50–150 bp) identified a single colony, which reacted with the antibody pool. The product of this clone, B3, was found to react with B3B11 but not 21C8 or JB1B (Fig. 4*A*). Based on these results, it is clear that these antibodies recognize distinct epitopes. Furthermore, as predicted by the competitive binding studies, the JB1B and B3B11 epitopes are in close proximity to one another on the β_1 chain.

The analysis of the DNA sequences of the inserts in those clones containing both the B3B11 and JB1B epitopes (A, B, D, G, E) indicated that these epitopes were located in a peptide containing a predicted amino acid sequence (33) corresponding to residues 636–705 of the mature β_1 chain (Fig. 5). The B3 insert sequence further served to localize the B3B11 epitope to amino acids 648–670. Based on the overlap of the clone sequences, the JB1B epitope would appear to be contained in the peptide spanning residues 671–703.

The sites of reactivity of B3B11 were further examined using

TABLE II
Summary of competitive antibody blocking studies

Each experiment was performed three times with triplicate samples in each assay. +, >80% and −, <10% inhibition of binding of labelled antibody by 20 μg/ml of competitor antibody.

	A			B		C		
	JB1B	B3B11	21C8	B44	13B9	N29	JB1	3S3
JB1B	+	+	+	−	−	−	−	−
B3B11	+	+	+	−	−	−	−	−
21C8	+	+	+	−	−	−	−	−
B44	−	−	−	+	+	−	−	−
13B9	−	−	−	+	+	+	−	−
N29	−	−	−	−	−	+	−	−
JB1	−	−	−	−	−	−	+	−
3S3	−	−	−	−	−	−	−	+

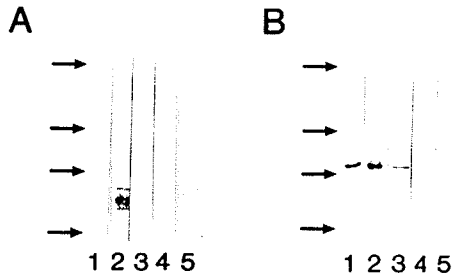
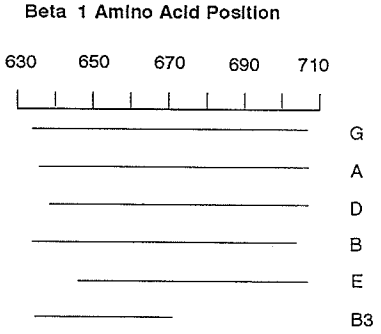


FIG. 4. **Localization of JB1B and B3B11 epitopes on β_1 fusion proteins.** Bacterial lysates of clones expressing β_1 integrin fusion proteins were screened with 1) JB1B, 2) B3B11, 3) 21C8, 4) JB1A, and 5) no first antibody. Molecular mass markers indicated by the *arrows* are 30, 46, 69, and 98, respectively. *A*, reactivity of clone B3 with B3B11; *B*, reactivity of clone B with JB1B and B3B11. Weak reactivity with 21C8 was also noted under reducing conditions.



636 EKKDTCTQECSYFNITKVESRDKLPQPVQPDPVSHCK 672

673 EKDVDDCWFYFTYSVNGNNEVMVHVVENPECPT 705

FIG. 5. **Predicted amino acid location of the inserts in the mature β_1 chain.** Fusion proteins from clones G, A, B, D, and E react with both JB1B and B3B11. The product of clone B3 reacts with B3B11 but not JB1B. The sequence of positions 636–705 of the β_1 chain is given in the lower portion of the figure. This corresponds to the region spanned by the insert in clone G.

three overlapping peptides, which spanned the predicted sequence of the B3 fusion protein. B3B11 reacted specifically with the peptide P3, which corresponded to residues 657–670 of the β_1 sequence (Fig. 6). In contrast, neither of the other two peptides, P1 and P2, spanning the remainder of the B3 insert was bound by B3B11. There was also no reactivity of the other two competing antibodies, 21C8 or JB1B, with any of the peptides.

Further support for P3 being the location of the B3B11

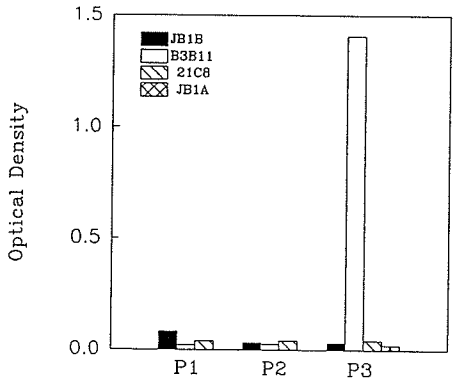


FIG. 6. **The reaction of anti β_1 antibodies with synthetic peptides corresponding to the B3B11 epitope of β_1.** Three overlapping peptides corresponding to residues 636–649 (P1), 646–659 (P2), and 657–670 (P3) were examined for their reactivities with JB1B, B3B11, and 21C8. These fragments spanned the entire sequence of the B3 clone isolated from the epitope fusion library (Fig. 5).

epitope was obtained with the demonstration that only this peptide inhibited the binding of B3B11 to purified β_1 integrin (Fig. 7). Neither P1 or P2 peptides blocked B3B11 binding. Furthermore, the P3 effect appeared to be specific as it did not influence the binding of an unrelated antibody to β_1 JB1A. These results indicate that the B3B11 epitope is contained in residues 657–670 and that it lies in close proximity to the JB1B epitope.

DISCUSSION

The results of the present study provide several new pieces of information: 1) the characterization of a panel of antibodies to β_1 that induce Jurkat adherence to collagen; 2) the demonstration of the presence of at least three distinct β_1 sites, which stimulatory antibodies can react with, *i.e.* group A (B3BII, JB1B, 21C8), group B (B44, 13B9), and group C (N29); 3) the localization of the epitopes detected by some of group A antibodies (JB1B, B3B11) to a membrane proximal region (648–705) of the β_1 integrin. The biological activities of the antibodies described in this study are not unique as a number of stimulatory antibodies to both β_1 (11–13), β_2 (18), and β_3 (14–17) integrins have been described. However, it was possible through the use of this panel to begin to address the location and existence of multiple regions of the integrin, which could act as targets for stimulatory antibodies.

In two previous studies relating to this point, there were somewhat divergent views presented on the question of the number of regulatory sites (22, 23). Takada and Puzon (22) observed that the region 207–218 of the β_1 appeared to be critical for the generation of epitopes, which were recognized by a number of inhibitory and stimulatory antibodies. These re-

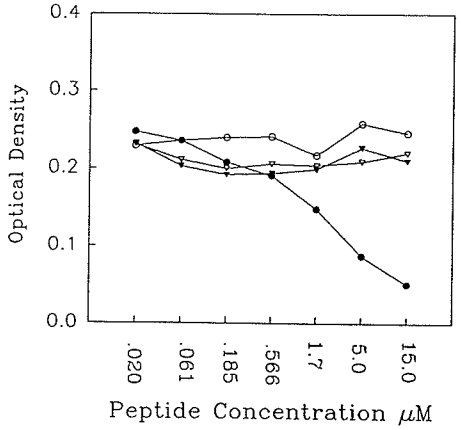


FIG. 7. **Competitive inhibition of B3B11 binding to purified β_1 by P3.** B3B11 was preincubated with peptides 1 (▼), 2 (▽), or 3 (●) and assayed for residual binding to immobilized purified β_1 integrin. The effects of P3 on JB1A (○) were also determined.

sults were based on the observations that the expression of human regulatory epitopes on interspecies β_1 chimeras required inclusion of this region of the human β_1 integrin. It was also possible to demonstrate that the introduction of multiple point mutations (S207H, E208K, N210E, K211V) into the highly conserved homologous region of the chicken integrin resulted in the generation of epitopes recognized by anti-human β_1 monoclonal antibodies. Thus, it appeared that a single region of the β_1 was capable of modulating β_1 function in either a positive or a negative fashion. The authors did, however, note that there might be other regulatory regions, as it was not possible to localize the epitopes of all regulatory antibodies.

Shih *et al.* (23) used a similar approach but found that the expression of regulatory epitopes mapped to two distinct regions using chicken mouse chimeric β_1 molecules. Epitopes related to the interference with ligand binding appeared to localize to the first 260 residues of the molecule. In contrast, those antibodies that altered ligand specificities or interfered with α-β_1 association were dependent upon the more membrane proximal parts of the integrin. Their results were interpreted to indicate the presence of multiple regulatory regions with the inhibitory and stimulatory epitopes mapping to different sites on the β_1 chain.

Two antibodies of the group A cluster, JB1B and B3B11, localize the membrane proximal region of the β_1 (648–705). The third antibody in this cluster, 21C8, reacts with a discontinuous epitope, which is not detectable on Western blot. The B3B11 (657–670) and JB1B (671–705) flank Cys^{671}, which is predicted to be involved in a disulfide bond with Cys^{415} (34). Such a bond would bring sequentially distant regions of the β_1 chain into close spatial proximity. The predicted β_1 disulfide bonding pattern is based upon those of the β_3 chain, as both integrins have an identical number of cysteines, and a homologous pairing pattern has been proposed (33, 35). However, the sequences in this region do not show significant homology. Thus, while the disulfide bond locations may be similar, the intervening sequences are completely different.

It is noteworthy that several anti-LIBS to β_3 chain, which activate adhesion, have been suggested to map in the 490–690 using interspecies β_3 chimeras (17). In the case of anti-LIBS2, the reduction-sensitive epitope has been localized to 602–690 (10). Thus, a number of stimulatory monoclonal antibodies appear to bind to the sterically constricted region of β_3, which is homologous to the A region on β_1.

The results of our competitive binding assays support the existence of at least three β_1 regions, which are the targets of stimulatory antibodies. The presence of multiple sites is consistent with the results of the β_3 chain, where three regions have been identified as sites of stimulatory anti-LIBS activity (14–17). It has been suggested that these sites may be in close proximity in the intact molecule as a result of long range disulfide bonds (17). By analogy, it would appear that the A region of β_1 corresponds to the LIBS2, LIBS3, LIBS6, P41 site of β_3 (10, 17). A second site in β_3 (1–6) recognized by AP5 encompassed a Cys^6 involved in a long range disulfide bond (17). Preliminary data suggest that N29 reacts with an epitope within the first 100 amino acids of β_1.[2] It will be of interest to determine the relationship between the N29 epitope and that of AP5 on β_3. The group B epitopes have not been localized to date.

In summary, it would appear that there is homology between the sites recognized by the group A antibodies and some of the β_3 stimulatory epitopes. It remains to be determined if these antibodies are similar to the LIBS-type reagents of the β_3 system. Preliminary data do indicate differences in expression levels on adhesion-competent and latent cells.[3] However, the stringency of expression on the lymphoid cells does not appear to be as great as for the LIBS.

We have previously demonstrated that several of the antibodies in this panel can have a number of effects on adhesion (12), intracellular signaling (36), and the association of cytosolic proteins (37). It will be of interest to determine whether interactions with the different stimulatory regions will differentially influence these other parameters of integrin activation.

Acknowledgments—We appreciate the helpful discussions of Drs. Mike Doyle (Chiron Corp.) and Bosco Chan (Robarts Research Institute, London, Ontario) in these studies. We also thank Drs. E. Ruoslahti, R. Juliano, and M. Tykocinski for the provision of constructs and plasmids. The excellent technical assistance of Nina Hunt and Sherri Gregorish is also gratefully acknowledged.

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Cell Adhesion and Communication, 1998, Vol 5. pp. 257–271
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Localisation of a Novel Adhesion Blocking Epitope on the Human β_1 Integrin Chain

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(Received 28 May 1997; Revised 16 July 1997; In final form 16 July 1997)

Members of the β_1 integrin family mediate cellular adherence to a wide range of extracellular and cell surface associated ligands. Conformational changes have been shown to be associated with integrin activation and ligand binding. Some studies suggest that there may be a restricted region of the β_1 integrin that serves as the target for regulatory antibodies which can inhibit or stimulate integrin function. Here we identify an inhibitory epitope that is located at a distinct sight from that suggested for other inhibitory antibodies. Three different adhesion blocking antibodies, JB1A, C30B, and D11B bind to a peptide corresponding to residues 82-87 of the mature β_1 chain. Mn^{++} inhibited the binding of JB1A to purified β_1 integrin. In contrast the binding of several other antibodies to β_1 were not influenced by these conditions. JB1A binding to purified peptide was also inhibited by Mn^{++} suggesting that it related to interference with the antibody function rather than a cation dependent change in the epitope. Our data 1) directly demonstrates the peptide sequence recognised by three adhesion blocking antibodies to the human β_1 integrin chain 2) identifies a novel epitope located at residues 82-87, distinct from that of previously described regulatory epitopes 3) characterises a Mn^{++} sensitive antibody integrin interaction. Collectively, these results indicate the existence of multiple regulatory sites on the β_1 integrin molecule.

Keywords: β_1 Integrin, blocking, epitope, human

INTRODUCTION

The integrins represent one of the major families of adhesion structures expressed on eukaryotic cells [17]. They mediate interactions with components of the extracellular matrix [31], cell surface proteins [4] and infectious agents [18]. Ligand contact can also lead to phenotypic changes in the cells which express them such as the induction of proliferation [21,28], the generation of apoptotic signals [14,41] or the activation of gene expression [37,40]. Integrin dependent activation of a number of signalling pathways such as phospholipid metabolism [7], Ca^{++} and protein phosphorylation [25,26] have also been

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observed. Thus substrate interactions via integrins can provide both positional and response pattern cues to cells which express them.

The integrins are heterodimeric transmembrane proteins in which each subunit consists of a large extracellular domain, a single membrane spanning region and usually a short cytoplasmic region [16]. The α β receptor pair defines the ligand binding specificity of each integrin. Thus the association of one of several α chains with a given β chain leads to the final specificity of the receptor complex. While the molecular mechanisms responsible for ligand recognition and specificity are unknown, it is clear that both α and β chains are involved in ligand contact.

Peptides containing Arg-Gly-Asp (RGD), a sequence recognised by many but not all integrins [3,23] or KQAGGDV [19], a sequence from the carboxyl terminus of the γ chain of fibrinogen which is recognised by $\alpha_{IIB}\beta_3$, have been used in crosslinking studies to localise the ligand contact sites of the β_3 integrins [9,30]. The KQAGGV peptide was found to be associated with residues 294-314 of the β_3 chain [9]. While RGD peptides were crosslinked to β_3 regions containing residues (109-171) and (66-203) in $\alpha_{IIB}\beta_3$ [9] and $\alpha_v\beta_3$ [30] respectively. Further support for the potential involvement of this region in ligand binding derives from the observations in $\alpha_{IIB}\beta_3$ that antibodies that react near this region block integrin binding to fibrinogen [1]. A naturally occurring point mutation in β_3 (119 D → Y) or site directed mutagenesis of homologous residue of the β_1 (130 D → Y) chain resulted in the loss of ligand binding potential [20,35]. More recently it has been demonstrated that this region of the β_3 chain is involved in both ligand and cation binding [10]. This may serve to explain some aspects of cation dependency of integrin function.

The overall amino acid homology of the β subunits (45-50%) as well as the presence of selected areas with very high levels of homology (80%) in β subunits suggests that there may be considerable similarity in the regulation and binding mechanisms of the different integrins [15]. However, there is relatively little known about these aspects of the functioning of non-β_3 integrins. Epitope mapping studies of the β_1 integrins using interspecies β_1 chimeras have provided some insight as to the possible locations of inhibitory and regulatory epitopes [27,36]. In one case the expression of the epitopes recognised by several regulatory antibodies were found to be sensitive to the amino acid changes in residues 207-218 of the β_1 subunit [36]. This has lead to suggestions that a single region may be the target of all known regulatory antibodies to the human β_1 integrin chain.

The present study identifies and localises a novel epitope that is recognised by several antibodies to the β_1 chain that block adhesion. This epitope is distinct from previously described regulatory sites. Evidence is also presented for a Mn^{++}-sensitive interaction of one of these antibodies.

MATERIALS AND METHODS

Materials

Unless otherwise indicated all chemicals were purchased from Sigma Chemicals. Media, fetal bovine serum, and fibronectin were obtained from Gibco Life Sciences. Custom synthesised peptides were purchased from Chiron Mimotopes Peptide Systems, CA and from Research Genetics, AL. The purity of all peptides was greater than 85%.

Monoclonal Antibodies

The antibodies JB1A [33], JB1 [6] 3S3 [11] and B3B11 [39] have been described previously. Antibodies, AP-138, and AIIB2 [5] were provided by Dr. Andrew Shaw, Cross Cancer Research Institute, Edmonton, AB and Dr. Caroline Damsky, Dept. of Anatomy, UCSF respectively. A summary of the properties and specificities of the antibodies used in this study is provided in Table I. The specificities of the antibodies for β_1 were confirmed as previously described using CHO cells transfected with the human β_1 gene [39].

TABLE I Properties of Antibodies used in this Study

Antibody	IP[a]	Blot[b]	Activity[c]	Ref.
JB1	+	−	weak stim.	[6]
JB1A	+	+	inh.	[33]
C30B	+	+	inh.	(This study)
D11B	+	+	inh.	(This study)
3S3	+	−	inh.,agg.	[29]
AP-138	+	−	inh.,agg.	−
AIIB2	+	−	inh.,agg.	[5]
B3B11	+	+	stim.	[39]

[a]ability to immunoprecipitate β_1; [b]reaction of antibodies with reduced β_1 on immunoblot; [c]effects of antibodies on cellular adherence to type I collagen and fibronectin; inh.-inhibits adherence; stim.-stimulates adherence; agg.-induces homotypic aggregation of Jurkat cells.

Cells and Culture

The human lymphoid cell lines Jurkat and IM9 (ATTC) was grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum.

Cell Binding Assay

Non-tissue culture treated microtiter wells were coated with purified plasma fibronectin, 5μg/ml (Gibco Life sciences) or collagen, 20 μg/ml, as previously described [38]. The wells were washed and blocked with 1% BSA in RPMI.

Cells were normally suspended in RPMI-1640. However for the Mn^{++} studies, they were resuspended in Puck's saline alone or with the indicated cation concentration and then added to fibronectin or BSA coated wells (2×10^5/well) and incubated for 60 minutes at 37°C. The non-adherent cells were removed by centrifugation of the inverted plates for 5 minutes at $70 \times g$ and the supernatants were removed. The adherent cells were fixed and stained for 30 minutes with 0.5% crystal violet in a 30% solution of methanol in water. The plates were washed with water to remove unbound dye and air dried. The residual dye was solubilised in methanol and the absorbance at 550 nm was determined. Binding to collagen was induced in Jurkat cells by stimulating the cells with PMA, 20 ng/ml, for 30 minutes prior to assaying for adherence [38]. In all assays the adherence to BSA was subtracted from the values obtained for the fibronectin coated wells. All experiments were performed at least three times in sextuplicate. All values were within 15% of the mean.

Epitope Library Production and Screening

Libraries were constructed using the NovaTope system (Novagen Inc., Wisconsin) according to the supplier's instructions. The method based on the use of modified pET expression vectors [32] consisted of digesting pFnRβ with DNAse I in the presence of Mn^{++} and size fractionating the random fragments by electrophoresis in 1.2% agarose gels. The 50-150 bp or 150-300 bp fragments were flush ended with T4 DNA polymerase, single dA tailed and ligated into the EcoR V site of the pTOPE-1b (+) plasmid which had been tailed with single 3′dT residues. Novablue (DE3) cells were transformed with the plasmid, and colonies were immunoscreened with a panel of anti-β_1 monoclonals and an alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin. Positive colonies were subcloned and examined for reactivity with the antibodies. The inserts from individual clones were sequenced using a T7 gene 10 primer.

Random Phage Peptide Display Library

An M13 phage based random peptide display library, CMCC #3858, was provided by Chiron Corporation. In this library random 15mer peptides are displayed on the phage, M13LP67, as a gene III fusion proteins [8].

Positive clones were selected by incubating 10^{10} pfu in 1 ml of PBS with 1μg of biotinylated JB1A for 15 minutes with shaking after which 20μl of streptavidin conjugated magnetic beads (Dynal Inc., NY) were added to the phage antibody mixture and incubated for an additional 30 minutes. The beads were magnetically collected and washed 8 times, 1 ml per wash, with PBS containing 0.1% BSA, 0.5% Tween 20. The phage were eluted from the beads in 100μl of 0.1M glycine-HCl, pH2.2, and neutralised with 9μl of 2M Tris pH 8.0. The eluted phage were amplified in competent MV1190 cells on LB agar plates. The phage were purified by PEG NaCl precipitation and tittered [29].

The sequences of the inserts were determined using double stranded DNA sequencing system (BRL/GIBCO) and the oligonucleotide primer ACAGACAGCCCTCATAGTTAGCG [8].

Purification of β_1 Integrin

Integrin was isolated from human placenta using a modification of the method described by Smith and Cheresh [30]. Briefly, 300g of washed placenta was homogenized in 300 ml of 50 mM n-octylglucopyranoside in 25 mM Tris pH 7.6, 150 mM NaCl, 2 mM $CaCl_2$ and 1 mM PMSF. The homogenate was centrifuged 10,000 × g for 1 hr at 4°C after which the supernatant was collected and passed sequentially through an ovalbumin sepharose 4B and a JB1A sepharose 4B column at a rate of 1 ml/minute. The column was washed sequentially with 20 column volumes each of 1) 0.1% NP-40 in 25 mM Tris pH 7.6, 150 mM NaCl, 2 mM $CaCl_2$, 2) 0.1% NP-40 in 0.01 M sodium acetate buffer pH 4.5. The β_1 integrin was eluted from the JB1A column in 0.1% NP-40 10 mM sodium acetate buffer pH 3.6 and 3 ml fractions were collected into tubes containing 0.5 ml of 3M Tris pH 8.8.

The purity of the fractions were assessed by SDS-PAGE and Coomassie blue staining. The fractions containing β_1 also had a mixture of the associated α chains. However, the α and β_1 chains collectively represented greater than 85% of the total stained proteins. The presence of β_1 in the fractions was confirmed by western blot with JB1A and B3B11.

Antibody Binding to Peptides and Purified β_1

For capture of biotinylated peptides on microtiter trays, avidin was suspended at 5 μg/ml in water and allowed to dry overnight, 0.5μg/well, in Nunc Maxisorb plates. The plates were washed three times with 0.5% Tween 20 in TBS and blocked for two hours at room temperature with 1% BSA in TBS. Biotinylated peptides, 100 pM, were added to each well, washed and incubated with the indicated monoclonal antibody at 150 ng/ml after which the binding was quantitated using an alkaline phosphatase conjugated rabbit anti-mouse IgG and substrate.

In the case of nonbiotinylated peptides, they were dissolved in water and added to Nunc Maxisorb plates at 100 pM each/well and allowed to dry overnight. Following three washes with TBS 0.5% Tween 20, the indicated antibodies were added at 5 μg/ml and the ELISA performed as described above.

Cation Effects on Antibody Binding to peptides and purified β1 integrins were assessed by mixing the antibodies with the indicated cations in Puck's saline. The binding of the antibodies to peptides and purified β_1 were then quantitated by an ELISA as described above.

Peptide Blocking of Antibody Binding to Purified β_1 Integrin

The indicated antibodies (150 ng/ml) were pre-incubated with the indicated concentrations of peptide for 18 hours at 4°C. The antibodies were then added to Nunc Maxisorb plates that had been precoated with affinity purified β_1 integrin. The level of binding was quantitated using an alkaline phosphatase conjugated anti-mouse IgG as described above. The experiments were carried out three times with triplicates in each assay.

Flow Cytometry

Washed cells were resuspended in Puck's saline with the indicated divalent cations and saturating concentrations of antibody for 30 minutes. The cells were washed three times in the corresponding buffer and reacted with FITC labelled goat anti-mouse IgG. The cells were washed and the level of staining was determined using a BD FACScaliber. Gating was on live cells and 10,000 events were analysed for each sample. Representative data is provided for one of three replicate experiments.

RESULTS

Characterisation of Antibodies

The antibodies used in the present study were selected for their abilities to block integrin dependent adher-

ence of Jurkat cells. As predicted from previous studies, the antibodies AIIB2, JB1A, and 3S3 blocked Jurkat binding to fibronectin [5,11,33] (Figure 1). AP-138 also caused almost complete inhibition of adherence. The antibodies D11B and JB1A inhibited adherence 80-85%, while C30B was consistently observed to cause a lower level of inhibition that varied between 30-50% inhibition in different experiments. The specificity of the antibody effects was demonstrated by the fact that another antibody to β_1, B3B11, [39] did not interfere with cell binding (Figure 1).

Jurkat cells displayed a low level of $\alpha_2\beta_1$ dependent adherence to collagen that could be enhanced by treatment with the phorbol ester PMA or with stimulatory antibodies to the α_2 or the β_1 integrin chains [34,38,39]. The antibodies 3S3, AP-138, JB1A, and AIIB2 caused an 85%-90% inhibition of adherence of PMA treated cells to collagen (Figure 1). The adherence was also markedly inhibited (i.e. >70%) by C30B and D11B. As previously reported, B3B11 further enhanced the binding of Jurkat cells to collagen [34]. These results indicated that the antibodies JB1A, 3S3, AIIB2, AP-138, C30B and D11B

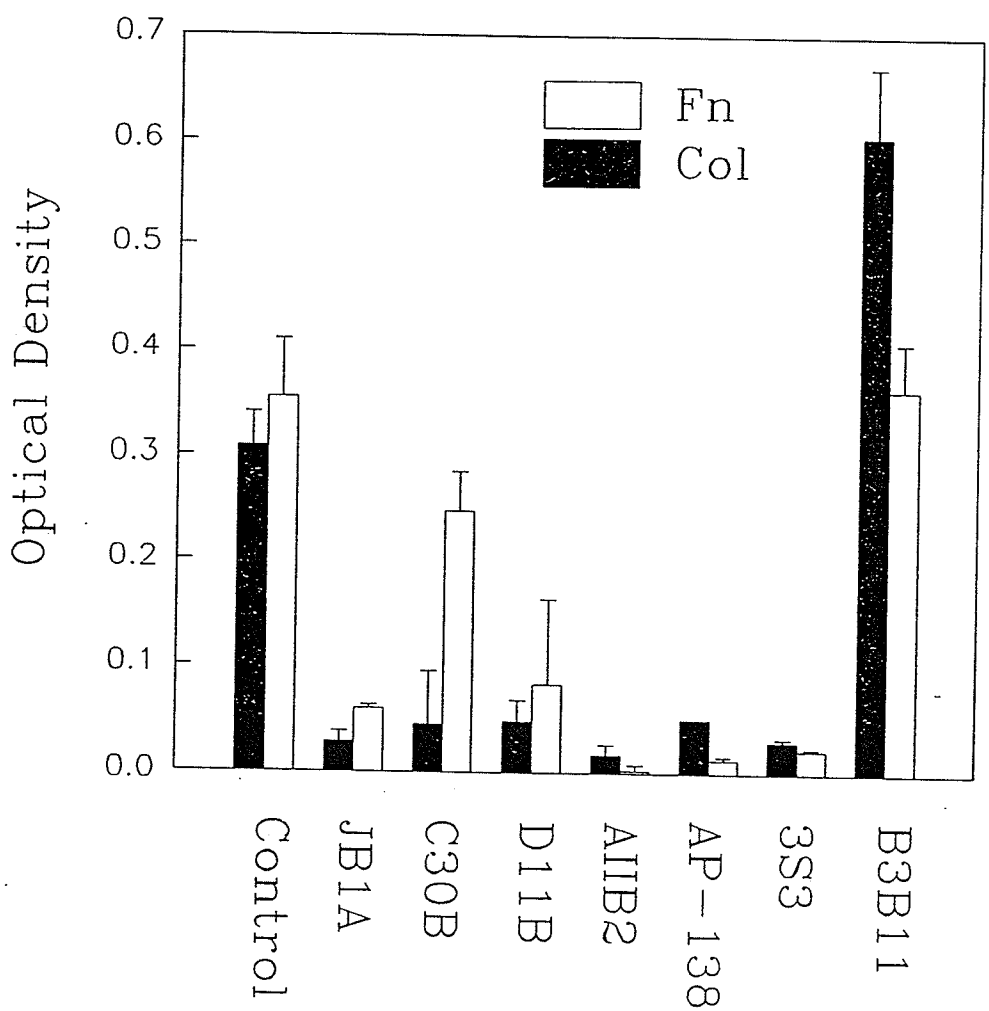

FIGURE 1 The effects of antibodies to β_1 on the adherence of Jurkat cells to fibronectin or collagen. Adherence to collagen was assessed following PMA stimulation of the cells as described in Materials and Methods. Note the anti-β_1 monoclonal, B3B11, stimulates adherence to collagen as previously described [39].

could inhibit β_1 integrin mediated adherence to both collagen and fibronectin further supporting the β_1 specificity of these antibodies.

Identification of the JB1A Binding Region of β_1 Integrin

Preliminary studies had shown that JB1A, C30B and D11B reacted with β_1 integrin chains in western blots under reducing conditions, while AIIB2, 3S3, and AP-138 did not. These results suggested that the former group detected linear epitopes on the β_1 molecule. Based on this assumption, a fusion library containing 50-150 bp inserts of the β_1 cDNA was constructed in an effort to localise the epitopes recognised by these antibodies.

Two clones, A and C, were found to react with JB1A, C30B and D11B but not with the other inhibitory antibodies. The predicted amino acid sequences of the inserts of these clones indicated that they coded for amino acid residues that corresponded to positions clone A, 55-93, and clone C, 61-105 of the mature β_1 (Figure 2). These results suggested that the JB1A, C30B and D11B epitopes were located at positions 61-93 of the mature β_1 chain. However, it was not possible with this strategy to determine the exact location of the epitope recognised by this antibody.

As a complementary approach to defining the JB1A epitope, a random 15mer peptide phage display library was screened for possible mimetics of the natural epitope recognised by the antibodies. Phage were selected using biotinylated JB1A and streptavidin dynabeads. The recovery rose from 10^{-6} to 10^{-1} of initial input (i.e. 10^{10} phage) respectively, for the first and third rounds of selection. Nineteen plaques were randomly selected from the third round for further characterisation. A comparison of the predicted amino acid sequences of the clones indicated that there was consensus TxxKLK in seven clones (Figure 3). An additional five had a related sequence S/GxxKLK. Three other clones contained a common sequence of TxxKLR where a semiconservative substitution of arginine for lysine had occurred. A comparison with the β_1 amino acid sequence suggested that the consensus sequence TxxKLK approximated that of β_1 residues 82-87 TAEKLK. This sequence was also present in both of the fusion protein clones, A and C, isolated from the β_1 epitope library. Thus lending further support to this sequence being the epitope recognised by these antibodies. A minor consensus sequence TxxKLR was also observed in three clones. This sequence approximates that of β_1 residues 179-184 (TPAKLR).

The antibodies JB1A, D11B and C30B were each found to react with the peptide biotin-SGSGTAEKLK while they displayed little reactivity to the peptide SGSGTPAKLR, implying that the former was the epitope recognised by the antibody (Figure 4). In contrast B3B11 which recognises a β_1 epitope at

Clone A

^{55}CPPDD**IENPRGSKDIKKNKNVTNRSKGTAEKLK**PEDIHQ^{93}

Clone C

^{61}**ENPRGSKDIKKNKNVTNRSKGTAEKLK**PEDIHQIQPQQLVLRLRS^{105}

FIGURE 2 The predicted amino acid sequences of the two JB1A, C30B, D11B reactive clones, A and C, isolated from the Novatope based β_1 epitope libraries. The superscripts indicate the residue positions of the mature human β_1 integrin. The letters in bold identify the common sequence between the two clones.

Predicted Amino Acid Sequences of JB1A Phage Isolates

Clone No.	Predicted Amino Acid Sequence
1	STS F **KLKHPPTTLSP**
4,7,9,14	QSSTWA**KLK**NTLIST
5	LLARPSSTSH **KLK**WQ
19	LPRNTAY **KLK**NSIPS
15	LFQQPFASAD **KLK**PI
8,11	LSGTS **KLK**FWHETSH
12	MSTHTERYGSM **KLK**S
18	PNHGSQ **KLK**NWSLHT
Predicted Consensus Sequence	**S/T x xKLK**
Corresponding ß$_1$ Sequence	T AEKLK
13	MMTIYSHAT TGKLRS
3	NVHLPHAT SSKLRSS
6	FFKHDSTTCKLRSCH
Minor Consensus Sequence	**Tx xKLR**
Corresponding ß$_1$ Sequence	**TPAKLR**
10	LQLSSHFSGTTQRLK
16	PHSERLGTI DKFLKP
2,17	QLQSPCKTRDKLLFC

FIGURE 3 The predicted amino acid sequences of the pIII inserts of JB1A isolates from the random peptide phage display library, CMCC#3858. The consensus residues are highlighted in bold. The major consensus sequence is provided with the corresponding predicted region of the mature β_1. A minor consensus sequence identified by clones 3, 6, and 13 is also provided.

residues 657-670 did not bind to either of these peptides [39]). The specificity of this peptide was further confirmed by its concentration dependent inhibition of JB1A, C30B, and D11B binding to purified β_1 (Figure 5). The binding of B3B11 to the purified β_1 integrin was not influenced by the 82-87 peptide under the same conditions. Similarly the peptide 82-87 did not influence the binding of several other inhibitory antibodies, AIIB2, AP-138, or 3S3 to the purified β_1 implying that they detected different epitopes (Figure 6). Thus a peptide corresponding to residues 82-87 of the β_1 integrin specifically inhibited binding of the three adhesion blocking antibodies JB1A, C30B, and D11B.

The Effects of Mn^{++} on JB1A Binding to β_1 Integrin

During the course of studies on the Mn^{++} induced adherence of IM9 cells to fibronectin it was noted that

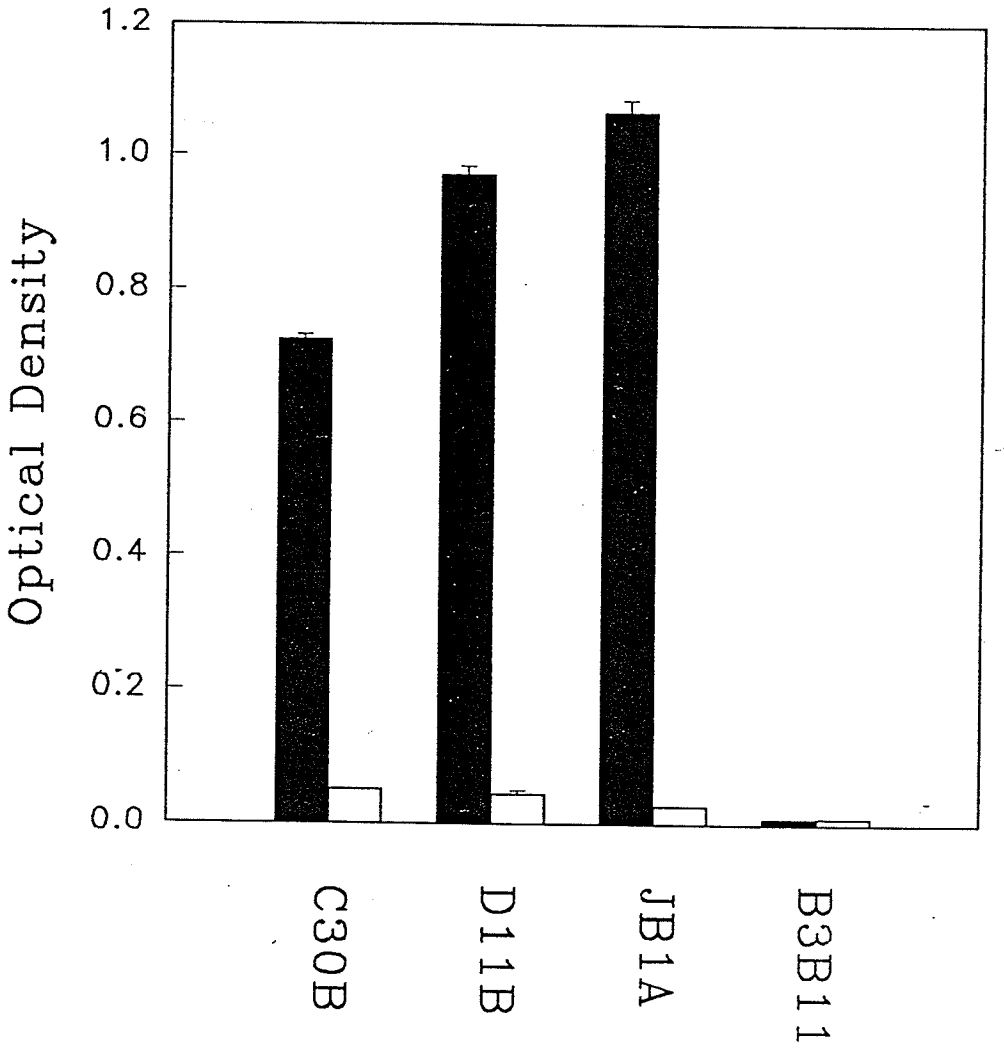

FIGURE 4 The binding of anti-β_1 monoclonal antibodies to peptides containing the predicted JB1A epitopes. The binding of JB1A, C30B, D11B, and B3B11 to the peptides, TAEKLK (82-87) (solid bar), and TPAKLR (179-184) (open bar) identified respectively by the major and minor consensus sequences in the random peptide phage library isolates. Antibody B3B11 has previously been shown to bind to a peptide corresponding to residues 657-670 [39].

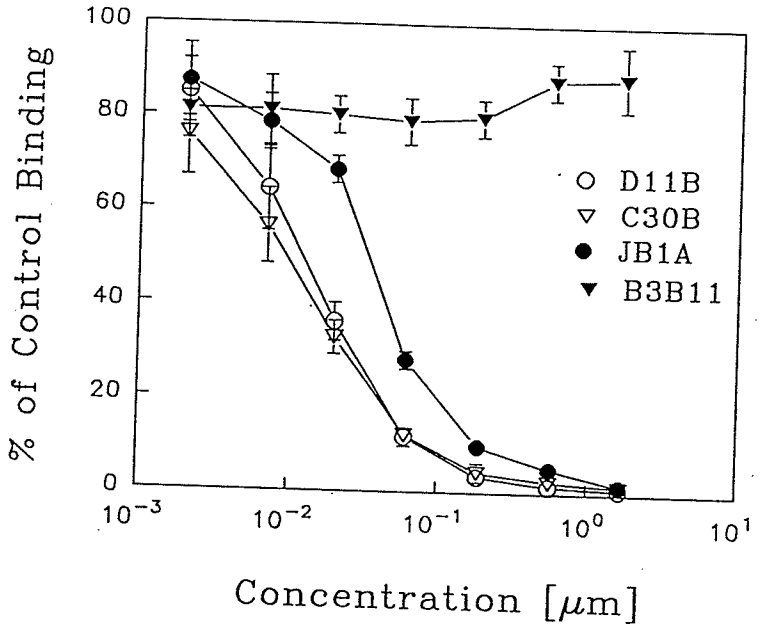

FIGURE 5 The effects of β_1 peptide 82-87 on antibody binding to purified β_1 integrin. Antibodies were mixed with the indicated concentrations of peptide, incubated and assessed for their residual binding to immobilized purified β_1 integrin. The antibodies JB1A, C30B, and D11B were inhibited from binding to the integrin, in contrast B3B11 binding was not influenced.

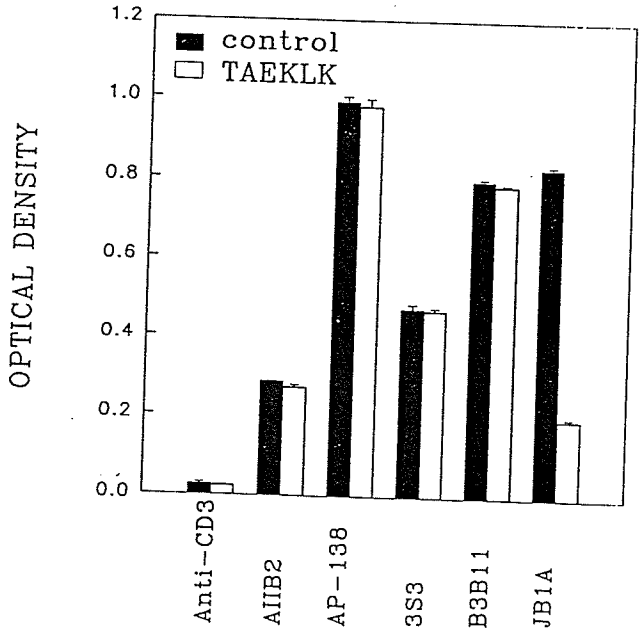

FIGURE 6 A comparison of the effects of β_1 peptide 82-87 on the binding of other antibodies to purified β_1. Antibodies to β_1 were incubated with the peptide TAEKLK and assessed for binding to purified β_1. The anti-CD3 monoclonal was included as a specificity control for antibody binding to integrin.

there was a marked reduction in JB1A binding to cell associated β_1 in the presence of 8 mM Mn^{++} (Figure 7). Under identical conditions the binding of other antibodies to β_1, C30B, D11B, and 3S3 were not influenced (Figure 7). These effects appeared to be Mn^{++} specific as neither Ca^{++} nor Mg^{++} influenced antibody binding at similar concentrations (data not shown). The inhibition of antibody binding was concentration dependent with maximal inhibition observed at 8 mM of Mn^{++}, the highest concentration tested. These same conditions did not influence 3S3 staining of the IM9 cells (Figure 8). Thus it appeared that this effect was Mn^{++} dependent and specific for JB1A binding.

In order to address directly the mechanism of action of Mn^{++} on JB1A binding, the effects of Mn^{++} on the binding of B3B11 and JB1A purified β_1 and to the β_1 peptide 82-87 were compared. The binding to either of these antigens by JB1A were inhibited by Mn^{++} in a concentration dependent fashion (Figure 9). Although the effects of Mn^{++} on peptide binding appeared to be detectable at lower concentrations than those observed with purified β_1. The binding of B3B11 to β_1 and to a peptide corresponding to the site of the β_1 epitope (residues 657-670) recognised by this antibody [39] was not influenced by Mn^{++} (Figure 9). Similar to the situation observed with IM9 cells the binding of C30B and D11B to purified β_1 and to

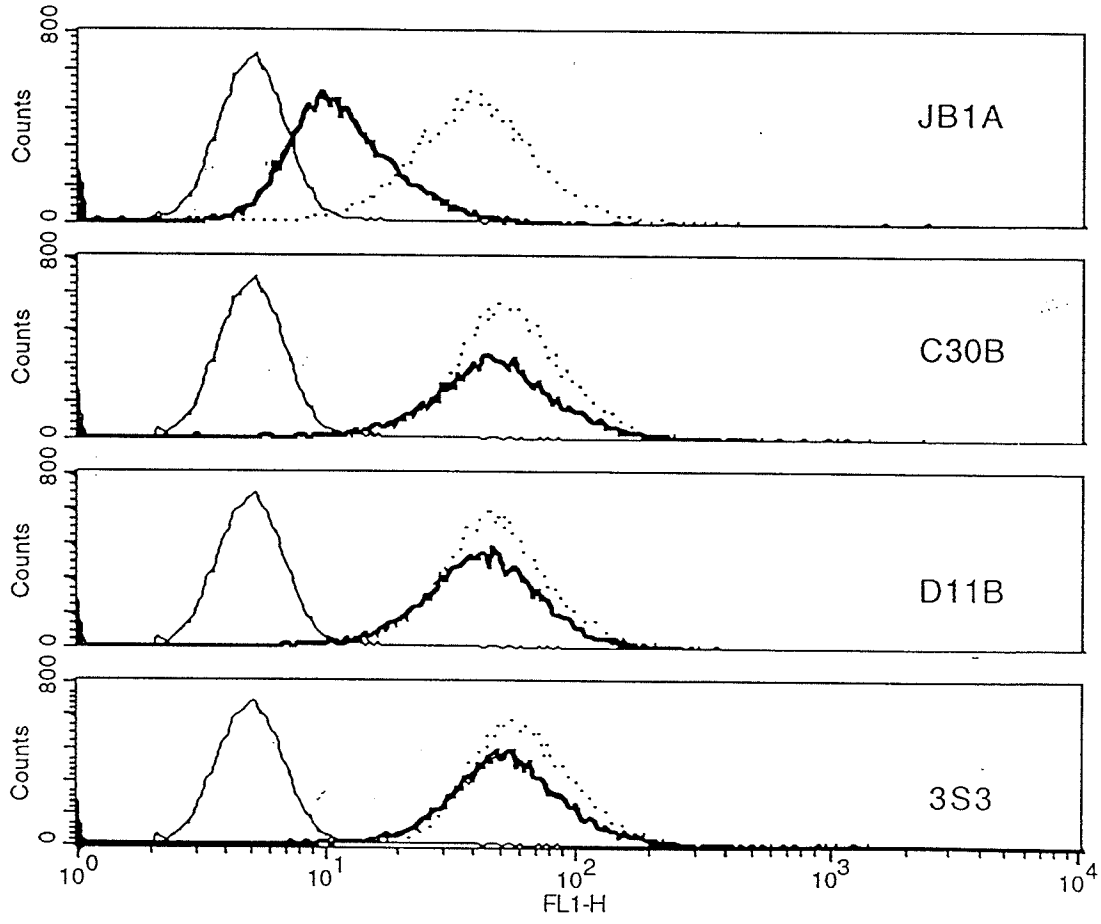

FIGURE 7 The effects of Mn^{++} on JB1A binding to IM9 cells. IM9 cells were washed in Puck's saline and resuspended in the same in absence (dotted line) or presence (solid line) of 8mM Mn^{++}. The cells were stained with the indicated antibodies and analyzed by flow cytometry. The negative control (light solid line) is included with each histogram.

peptides containing the JB1A epitope were unaffected by the presence of Mn^{++} (Figure 10). Collectively these results would seem to indicate that these effects were specific for JB1A. They would also seem to suggest that the Mn^{++} dependent inhibition of JB1A binding relates to a direct effect on the antibody binding to the integrin rather than to alterations of the target epitope on the β_1 chain.

DISCUSSION

The significant results of the present study are: 1) The direct demonstration of the peptide sequence recognised by three adhesion blocking antibodies to the human β_1 integrin chain; 2) The identification of a novel epitope location, distinct from that of previously described regulatory epitopes; and 3) The

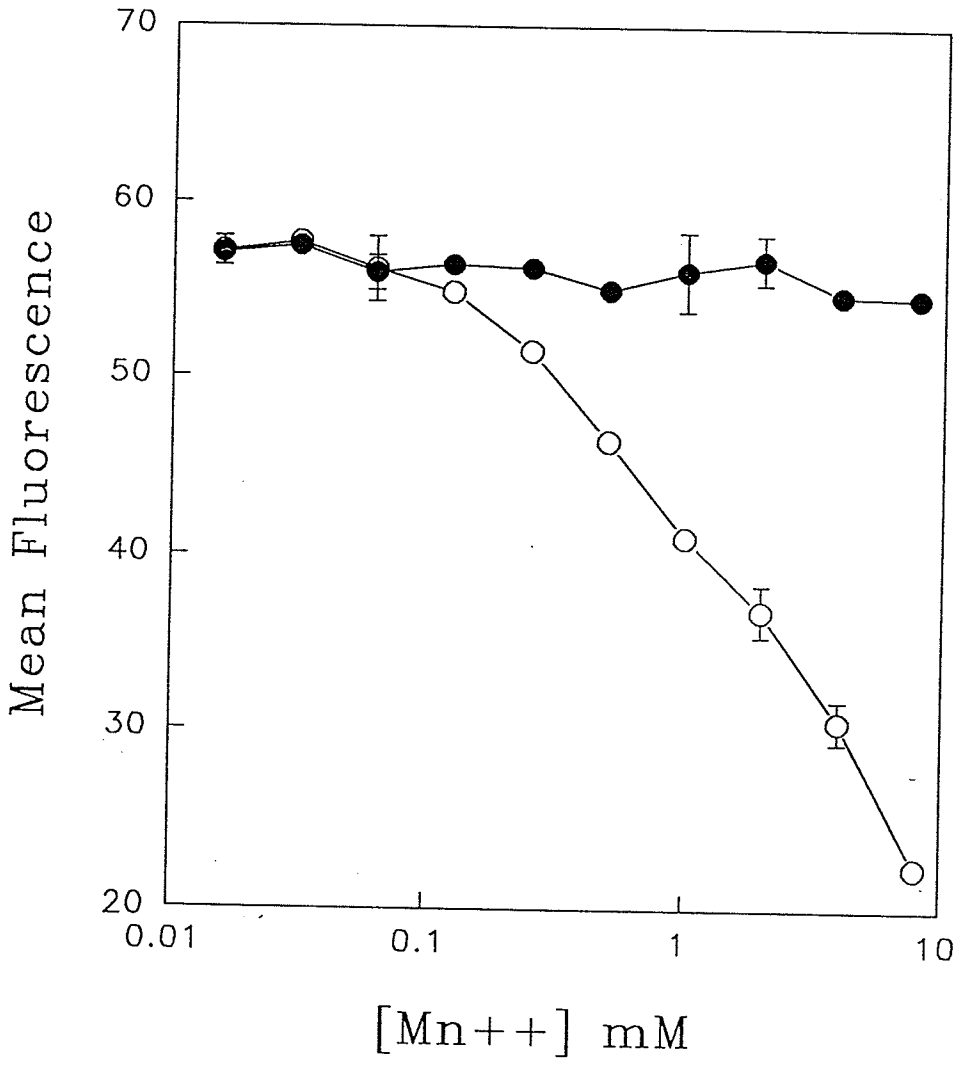

FIGURE 8 The concentration dependence of Mn^{++} mediated inhibition of JB1A binding. IM9 cells were pretreated with the indicated concentration of Mn^{++} and reacted with either JB1A (open circles) or 3S3 (solid circles) and the mean fluorescence intensity of each sample was determined by flow cytometry.

characterisation of a Mn^{++} sensitive antibody integrin interaction.

The epitope recognised by JB1A, C30B and D11B contains residues 82-87 of the human β_1 integrin chain. This conclusion is based on several pieces of evidence. Each of these antibodies binds to fusion proteins that contain fragments of β_1 that span this region of the integrin. A decapeptide, SGSGTAEKLK, containing residues 82-87 is selectively bound by these three antibodies. The binding of these same antibodies to purified β_1 is specifically inhibited by this peptide.

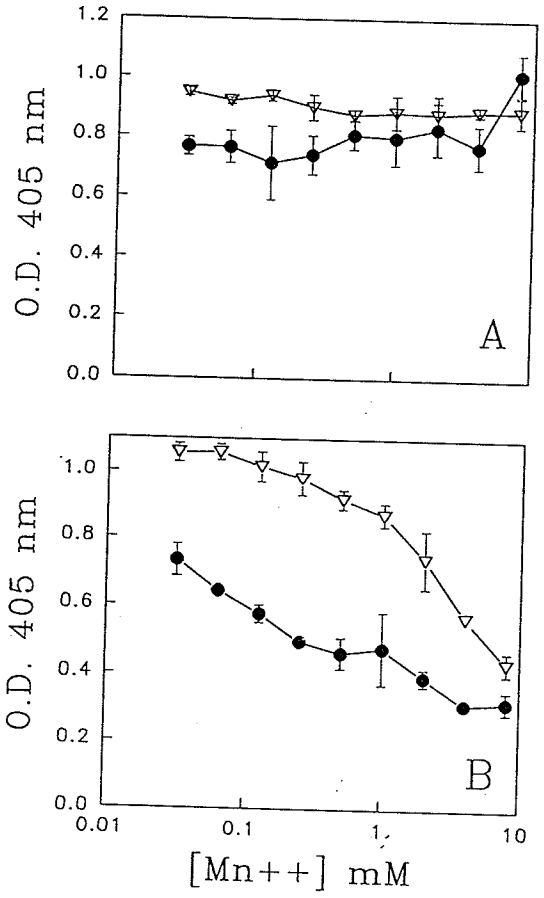

FIGURE 9 The effects of Mn^{++} on antibody binding to purified β_1 and peptides. (A) B3B11 or (B) JB1A were mixed with the indicated concentrations of Mn^{++} and assessed for their binding to β_1 (open triangles) or β_1 peptides (solid circles) 658-666 for B3B11 (A) or 80-90 for JB1A (B).

The location of the JB1A epitope appears to be distinct from that described for a number of other adhesion regulating antibodies [36]. Takada and Puzon reported that residues 207-218 of the β_1 subunit were critical for the binding of a large number of integrin inhibiting or activating antibodies. This sequence is located between two highly conserved regions that are homologous to the putative ligand binding site of β_3 [9,30]. The 207-218 region is also predicted to be part of a β bend in the integrin chain. Antibodies to this region might then be expected to influence integrin function as they could either induce or prevent the appropriate conformational changes involved in the acquisition of ligand binding potential. Shih et. al. also used interspecies chimeric integrins to map β_1 epitopes [27]. In their case, there appeared to be a differential localisation of regulatory epitopes with inhibitory antibodies recognising residues in the first 260 residues while other function modifying antibodies were dependent on the membrane proximal and cysteine rich regions of the β_1 chain. Although the results of each of these studies demonstrated quite conclusively that the expression of certain epitopes were dependent on residues in a given region of the integrin, there was no direct evidence that any of the antibodies actually bound to residues within these regions. This situation arises as a consequence of the fact that the majority of antibodies that were examined in these studies appear to detect conformational epitopes. Thus precluding the detailed direct localisation of their corresponding epitopes.

In a previous study, we have shown that there are at least three distinct loci of epitopes that are recognised by antibodies that can stimulate integrin function [39]. This conclusion was based on the results of competitive antibody binding assays and physical mapping of several of these epitopes. A subset of these antibodies was shown to bind to residues located in the membrane proximal region (657-703) of β_1. These data would seem to collectively indicate that there are multiple regions of the β_1 that contain regulatory epitopes. Such an interpretation appears to be compatible with epitope mapping results in the β_3 integrin were multiple regulatory sites have been identified [13].

It is important to keep in mind that the apparently limited number of regulatory epitopes, which have been described to date, may be a reflection of the high levels of sequence identity observed between human and murine β integrins [12]. Thus it might be anticipated that the antibody data represents a minimal estimate of the number of such epitopes, as many potential sites would be expected to be immunologically silent in this species pair.

The location of the JB1A epitope does not correspond to the homologous ligand contact sites proposed for the β_3 chain [1,9,30]. This may indicate that direct inhibition of ligand binding is not the mode of action of this antibody. A recent study with antibody 13, demonstrated that this antibody interfered with integrin ligand binding by a non-competitive mechanism [24]. It was therefore proposed that the inhibition was a result of allosteric changes

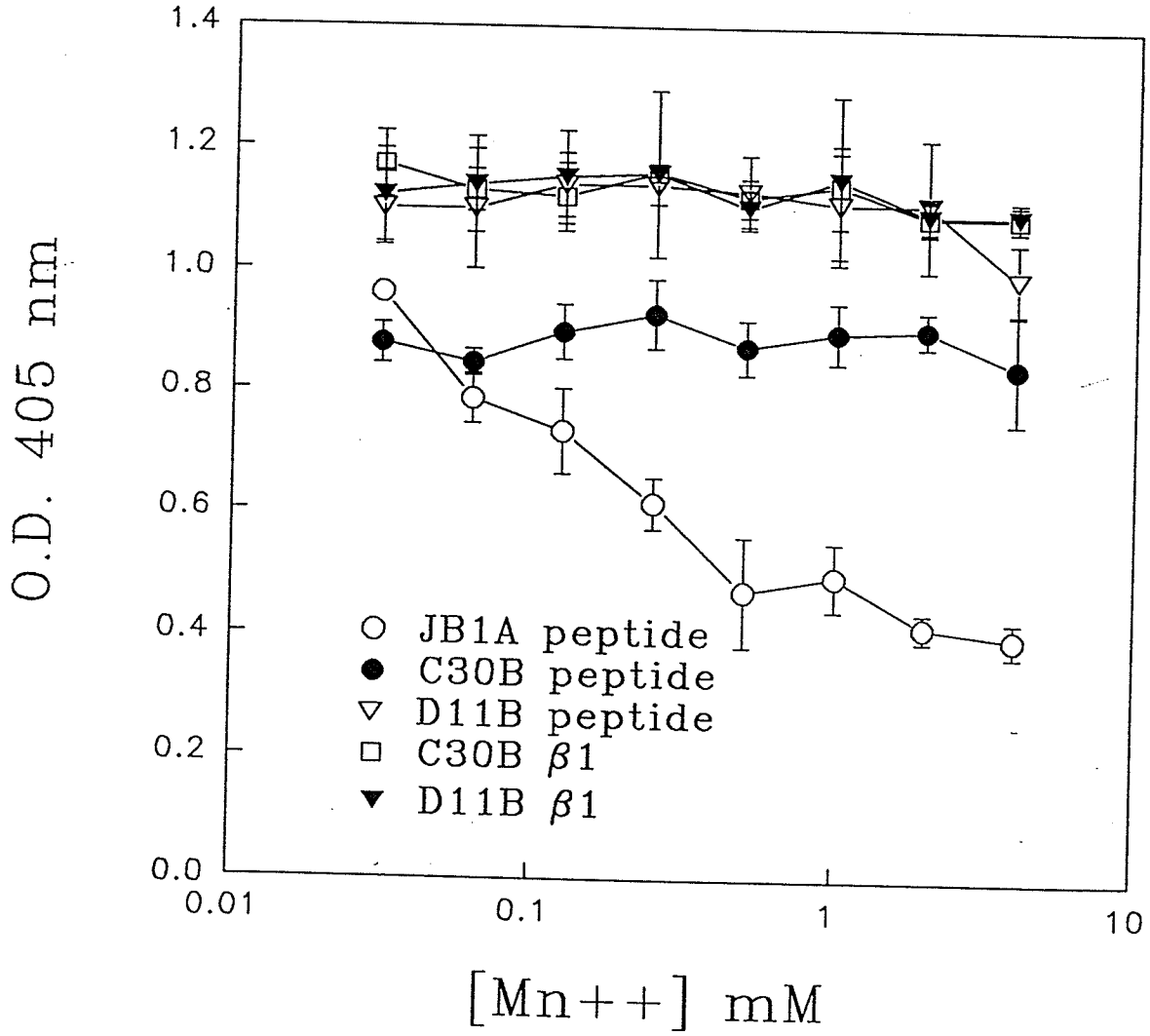

FIGURE 10 A comparison of the effects of Mn^{++} on the binding of JB1A, D11B and C30B to purified β_1 and to β_1 peptide 82-90. Antibodies were mixed with the indicated concentrations of Mn^{++} and assessed for binding to peptide or to β_1.

induced in the β_1 molecule by this antibody. It may be that such a mechanism is operative in the case of the inhibition observed by antibodies of the JB1A group.

An alternative explanation for the antibody inhibition by the JB1A group of antibodies may be that the corresponding epitope is located at a secondary site involved in the stabilisation of ligand contact. Inhibition would then arise from direct steric hindrance of ligand binding. To date there is no evidence to suggest such a role for the JB1A epitope. In fact preliminary studies have failed to demonstrate the direct binding of peptides containing the 82-87 region of the β_1 chain to fibronectin (HN, JW unpublished data). While these results do not rule out a role in ligand binding, they do suggest that any such interactions would be of a low affinity.

The initial observations that JB1A binding to cell associated integrins was Mn^{++} sensitive raised the possibility that there was a relationship between the expression of the JB1A epitope and functional state of the integrin. However, there are several observations that strongly argue against such a relationship. 1) Neither C30B nor D11B, which recognise to the same core epitope as JB1A, were inhibited in their binding to cell associated or purified integrin by Mn^{++}. 2) The binding of JB1A to a biotinylated octapeptide, biotin-SGTAEKLK, or to an undecapeptide, KGTAEKLKPED, was inhibited by Mn^{++}, while those of C30B and D11B are not. Furthermore, preliminary infrared spectroscopic data did not provide any evidence of cation dependent spectral changes (unpublished data JAW, MJ). Thus it seems highly unlikely that the cation effects are a result of conformational changes in the peptide or of interactions between the peptide and cations. There have been a number of antibodies that have been reported to detect cation sensitive epitopes on the integrin β chains [2,13,22]. In some, but not all, of these cases other stimuli have been shown to alter antibody binding implying that conformational changes in the antigen are involved in the altered antibody binding properties. The results with JB1A should serve as a cautionary note regarding the interpretation of cation effects on antibody binding to their antigens. This data also points to the potential value of direct epitope mapping in the resolution of such issues.

Acknowledgements

We thank Dr. Mike Doyle, Chiron Corp. for providing the random peptide phage libraries and advice in their use; Dwayne Stupack and Caixia Shen for their roles in the production and characterisation of several of the monoclonal antibodies; Dr. Guangming Zhong for his advice and assistance with the flow cytometry. This research was supported by grants from the Medical Research Council of Canada, the Canadian Arthritis Society and the Manitoba Health Research Council. Heyu Ni is the recipient of a Manitoba Health research Council Studentship Award.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 273, No. 14, Issue of April 3, pp. 7981–7987, 1998
Printed in U.S.A.

Integrin Activation by Dithiothreitol or Mn^{2+} Induces a Ligand-occupied Conformation and Exposure of a Novel NH_2-terminal Regulatory Site on the β_1 Integrin Chain*

(Received for publication, October 30, 1997, and in revised form, December 17, 1997)

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Integrins can be expressed in at least three functional states (*i.e.* latent, active, and ligand-occupied). However, the molecular bases for the transitions between these states are unknown. In the present study, changes in the accessibility of several β_1 epitopes (*e.g.* N29, B44, and B3B11) were used to probe activation-related conformational changes. Dithiothreitol or Mn^{2+} activation of integrin-mediated adhesion in the human B cell line, IM9, resulted in a marked increase in the exposure of the B44 epitope, while N29 expression levels were most sensitive to dithiothreitol treatment. These results contrasted with the epitope expression patterns of spontaneously adherent K562 cells, where N29 was almost fully accessible and B44 was low. Addition of a soluble ligand resulted in a marked increase in B44 levels, suggesting that this antibody detected a ligand-induced binding site. The N29 epitope was mapped to a cysteine-rich region near the NH_2 terminus of the integrin chain, thus defining a novel regulatory site.

These studies indicate that the activation of integrin function by different stimuli may involve related but nonidentical conformations. Both Mn^{2+} and dithiothreitol appear to induce localized conformational changes that mimic a ligand-occupied receptor. This differs from the "physiologically" activated integrins on K562 cells that display a marked increase in overall epitope accessibility without exposure of the ligand-induced binding site epitopes. The increased exposure of the N29 site on K562 cells may indicate a role for this region in the regulation of integrin function.

Members of the integrin family mediate cellular interactions with elements of their microenvironment (1–3). These contacts can lead to cellular adhesion, migration, and activation (4–6). In a number of cell types, such as platelets and leukocytes, the activities of integrins are tightly regulated such that host cell activation is required before cell binding can proceed (7–9). This prerequisite ensures that integrin function is operative only at the appropriate anatomical or pathological sites.

Although the structural basis for the underlying changes associated with the acquisition of integrin functionality is unknown, data from a number of different biochemical and immunological approaches clearly demonstrate activation-associated alterations in integrin conformation (10–12). Antibody-binding studies and protease-susceptibility studies have shown that there are activation-associated changes in the accessibility of regions of the complex (10, 11). Fluorescent energy transfer studies on $\alpha_{IIb}\beta_3$ have also demonstrated that there are alterations in the spacing and interaction of α_{IIb} and β_3 in the activated integrin structure (12). Changes in epitope expression are also observed following receptor occupancy (13–16). Collectively the data suggest that the activated integrin complex acquires a more open conformation than is observed in the latent structure.

Recently models of integrin activation have been proposed that involve allosteric mechanisms for the acquisition of an adhesion-competent conformation (17, 18). Support for such a model derives from the observations that the binding of ligand to purified integrin inhibits the binding of an inhibitory antibody to the β_1 chain (19). The pattern of inhibition displays characteristics that are most compatible with an allosteric mechanism. However, as pointed out by Mould (17), the situation with the integrins is more complex than a classical allosteric mechanism, as the "active" integrin does not necessarily acquire a conformation that approximates the ligand-bound receptor. Thus the existence of multiple intermediate conformations have been suggested.

Activation of integrin function can be achieved by a variety of stimuli (20–26). Mn^{2+} and the bifunctional reducing agent, DTT,[1] have been shown to activate integrin binding in a number of systems (22–26). Since both of these agents activate purified integrins, it would appear that their effects on adhesion might be directly on the receptor complex (19, 24). These agents may provide useful probes for the analysis of the changes associated with integrin activation and ligand binding.

We have previously described a panel of regulatory antibodies to the human β_1 integrin chain and localized their continuous epitopes (27–29). Three noncompeting groups of antibodies were identified, and one set of antibodies was shown to react with the membrane proximal β_1 region (28). The present study localizes a novel stimulatory region to the cysteine-rich amino-terminal portion of the β_1 chain. Furthermore, it is demonstrated that it is possible to generate functionally "activated" integrins with overlapping but nonidentical conformations.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated, all chemicals were purchased from Sigma. Media, fetal bovine serum, and GRDS/GRES peptides were obtained from Life Technologies, Inc. Purified human plasma fibronectin was obtained from Chemicon Intl., Temecula, CA. Custom-synthesized peptides were purchased from Research Genetics, Huntsville, AL.

* This work was supported by grants from the Medical Research Council and the Canadian Arthritis Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviation used is: DTT, dithiothreitol.

TABLE I
Antibodies

Antibody	Activity	Epitope location	Reference
JB1A/C30B/D11B	Inhibitory	82–87	30
3S3	Inhibitory	Discontinuous?	31
B3B11	Stimulatory	660–668	28
N29	Stimulatory	15–54	28, this paper
B44	Stimulatory	Linear?	28

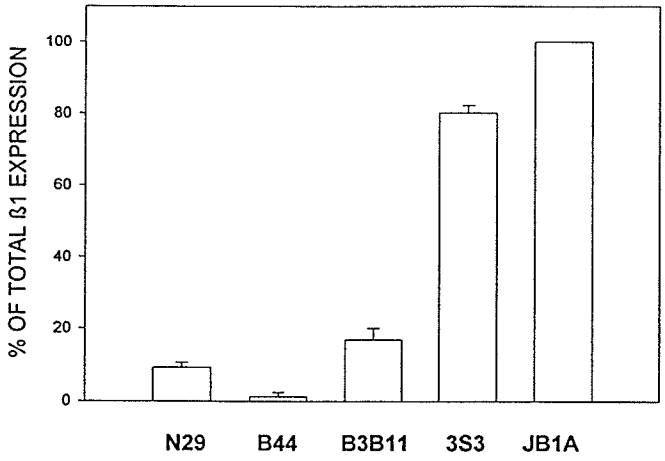

FIG. 1. **The relative levels of β_1 epitope expression on IM9 cells.** IM9 cells were stained with the indicated antibodies and their relative levels of binding expressed as a percentage of JB1A MFI levels. The average and ranges of two representative experiments are provided.

Antibodies—The production, properties, and purification of the antibodies to β_1 (Table I), JB1A (30), B3B11, B44, N29 (28), and 3S3 (31) have been previously described in detail. Dr. C. Damsky provided the anti-β1 AIIB2 (32).

Cells and Culture—The human cell lines IM9 (B cell), Jurkat (T leukemia), and K562 (erythroleukemia) were obtained from the ATCC. They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Cell Binding Assay—The assays were performed as described previously (28). Nontissue culture treated microtiter wells were coated with purified plasma fibronectin (5 μg/ml) in bicarbonate buffer at 4 °C overnight. The wells were washed and blocked with 1% bovine serum albumin in RPMI. In studies involving Mn^{2+}, the cells were washed and resuspended in Puck's saline A alone or in the presence of the indicated concentration of cation. When cells were pretreated with DTT, they were washed to remove the DTT prior to their addition to the binding assays.

Cells were preincubated with the indicated stimuli for 30 min at room temperature and then added (2×10^5 cell/well) to the coated wells and incubated for 60 min at 37 °C. The nonadherent cells were removed by centrifugation of the inverted plates for 5 min at 70 × *g*, and the supernatants were removed. The adherent cells were stained for 60 min with 0.5% crystal violet in a 30% solution of methanol in water. The plates were washed with tap water to remove unbound dye. The residual dye was solubilized in methanol, and the absorbance at 550 nm was determined. In all assays the adherence to bovine serum albumin (OD < 0.1) was subtracted from the values obtained for the fibronectin or antibody coated wells. Unless indicated otherwise, all assays were performed at least three times in sextuplicate.

Flow Cytometry Analysis—Cells were preincubated with the indicated stimuli at room temperature and then incubated with the indicated antibody (5 μg/ml) for 30 min at 37 °C. The cells were washed twice with phosphate-buffered saline and incubated for 60 min at 4 °C with a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (Chemicon). All assays included cells incubated with the second antibody alone as a control for nonspecific binding. Fluorescence analysis was performed with a BD FACScaliber.

For the studies involving ligand binding to K562, the cells were preincubated with the indicated peptides (1 mM) or fibronectin (100 μg/ml) for 1 h at room temperature. Antibodies were then added to this mixture for 30 min at 37 °C, and the cells were processed for fluorescence-activated cell sorter analysis as described above.

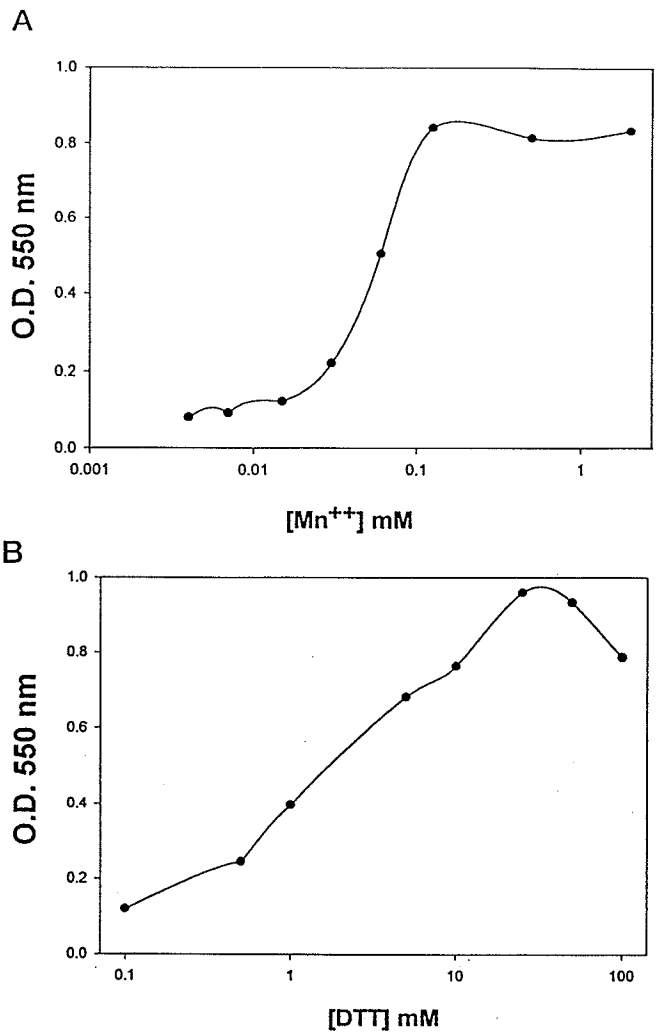

FIG. 2. **The induction of IM9 adherence to fibronectin following treatment with either Mn^{2+} (*A*) or DTT (*B*).** IM9 cells were incubated with the indicated concentration of stimulus and tested for adherence to immobilized fibronectin. Representative results of one of four independent experiments are shown. The standard errors for all samples were less than 15%.

Epitope Library Production and Screening—Libraries were constructed using the Novatope System (Novagen Inc.) according to the manufacturer's instructions. The method based on the use of modified pET vectors for the expression of β_1-T7 gene 10 fusion proteins consisted of digesting pFnRβ (33) with DNase I in the presence of Mn^{2+} and size fractionating the random fragments. The 250–350-base pair fragments were flush ended with T4 DNA polymerase, single dA tailed and ligated into the *Eco*RV site of the pTOPE-1b(+) plasmid. Novablue (DE3) cells were transformed with the plasmid, and colonies were immunoscreened with anti-β_1 monoclonal antibodies and an alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin. Positive colonies were subcloned and examined for reactivity with the antibodies. The inserts from individual colonies were sequenced using T7 gene 10 primers as described previously (28).

Expression of β_1 Chain NH_2-terminal 57-Amino Acid-containing Peptide—Polymerase chain reaction amplifications were performed with the β_1 chain primer pair GTGAATTCATATGCAAACAGATGAAAAT-AGATG/GAGGATCCATATGTCATGGAGGGCAACCCTTCTTTT using a plasmid isolated from the above library containing a β_1 integrin 5′ 315-base pair fragment. The products were digested with *Eco*RI and *Bam*HI, ligated into pBS(+) phagemid to introduce an *Nde*I site (Stratagene, La Jolla, CA). The recombinant phagemid was expanded, purified, and digested with *Nde*I. The β_1 fragment was purified and ligated to the expression vector pET-14b (Novagen). The resulting insert was predicted to code for residues Gln^1 through Pro^{57} of the mature β_1 chain. The corresponding fusion protein was expressed in competent *Escherichia coli* BLR(DE3)plyss strain, purified with Ni^{2+} columns, and

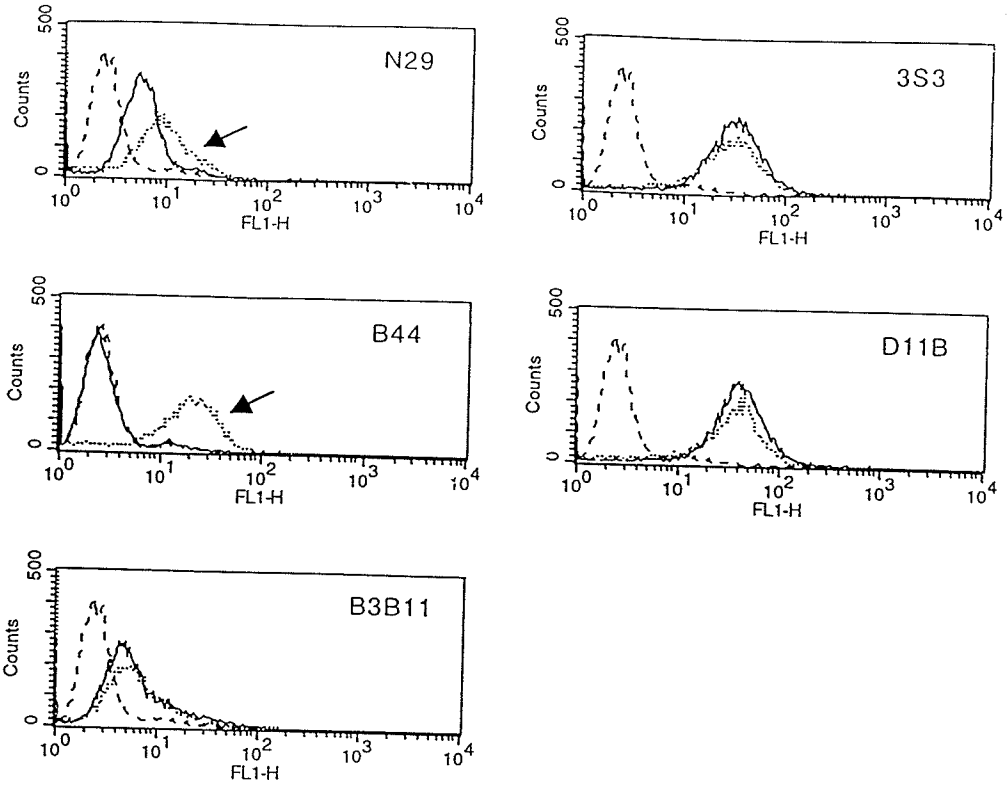

FIG. 3. **The effects of Mn^{2+} treatment of IM9 cells on β_1 epitopes expression.** Cells were treated with Mn^{2+} (1 mM), stained with the indicated antibodies and analyzed by flow cytometry. The control, untreated, and Mn^{2+}-treated cell profiles are represented by the *dashed*, *solid*, and *dotted* lines, respectively. The *arrows* indicate the profiles of the Mn^{2+}-treated cells.

visualized on 15% SDS-polyacrylamide gel electrophoresis gel by Coomassie Blue staining or immunoblots with the indicated antibodies.

Peptide Enzyme-linked Immunosorbent and Blocking Assays—The purified fusion protein or a peptide corresponding to the first 14 residues of the mature β_1 were suspended at 10 μg/ml in distilled water and allowed to dry overnight, 0.5 μg/well, in Nunc Maxisorb plates. The plates were washed three times with 0.5% Tween 20 in Tris-buffered saline and blocked for 2 h at room temperature with 1% bovine serum albumin in Tris-buffered saline. The indicated antibodies (5 μg/ml) were added to the wells, and the binding was quantitated with rabbit anti-mouse IgG alkaline phosphatase conjugate and developed with pNPP as substrate.

Blocking assays were performed by preincubating antibodies (1 μg/ml) with the indicated peptides (10 μM) at 4 °C overnight. The antibodies were then added to the wells of Nunc plates precoated with affinity-purified placental β_1 integrin (28). The color was developed as per the peptide enzyme-linked immunosorbent assay.

RESULTS

Differential Expression of β_1 Epitopes on IM9 Cells—A comparison of the binding levels of a panel of anti-β_1 monoclonals to IM9 cells indicated that there were marked differences in their levels of expressions (Fig. 1). A calculation of their expression levels relative to the total β_1 expression detected by JB1A or C30B indicated that B3B11, B44, and N29, respectively, were present on 18, 2, and 10% of the integrins. Previous studies had determined that these antibodies recognized continuous epitopes in the nonpolymorphic extracellular domain of the β_1 chain (28). Thus it appeared that their low expression levels were indicative of a sequestration of the regions containing these epitopes. As IM9 cells express $\alpha_4\beta_1$ but do not spontaneously adhere to fibronectin, it was speculated that the negative correlation of expression of B44, B3B11, and N29 epitopes with adhesive function might indicate that they were reporters of integrin activity.

Treatment of the cells with Mn^{2+} or DTT resulted in a marked increase in adherence. The half-maximal stimulatory concentration for Mn^{2+} was 70 μM (Fig. 2*A*). The situation with DTT was somewhat more complex with half-maximal activity at 2–5 mM and a loss in adhesion at concentrations in excess of 50 mM (Fig. 2*B*). The adhesion induced by both stimuli was inhibited by more than 60% by anti-α_4 and anti-β_1, suggesting that $\alpha_4\beta_1$ was mediating a significant proportion of the induced binding (data not shown). Neither of the stimuli caused any change in total β_1 levels, indicating that the adhesive changes related to altered integrin activity rather than increases in expression levels (Figs. 3 and 4).

Cells treated with Mn^{2+} displayed a 40–50-fold increase in the levels of B44 expression such that 30–40% of the β_1 displayed this epitope (Fig. 3). Although there was a doubling of the N29 levels, the majority of integrins did not express this epitope. The binding of B3B11 and 3S3 were relatively unaffected under these conditions. DTT treatment caused a comparable increase in the level of B44 binding (Fig. 4). However, unlike the case for the Mn^{2+}- treated cells, there was almost 100% exposure of the N29 epitope and a small increase in B3B11 binding.

β_1 Epitope Expression Patterns on K562 Cells—The results of the above studies supported the concept that the expression of the B44 and possibly of the N29 epitope might relate to the activational status of the integrin. As an approach to testing this possibility K562 cells were examined for their antibody binding patterns. These cells spontaneously adhere to fibronectin, and their receptors have been shown to be in an intermediate affinity state (15). There were increases in the proportions of N29 and B3B11 expressed on these cells (Fig. 5). However, there was almost a complete absence of B44 binding, suggesting that this antibody was not a marker of integrin functionality.

Preliminary studies had indicated that Mn^{2+} induced the expression of the B44 epitope on K562 cells. Since it had been

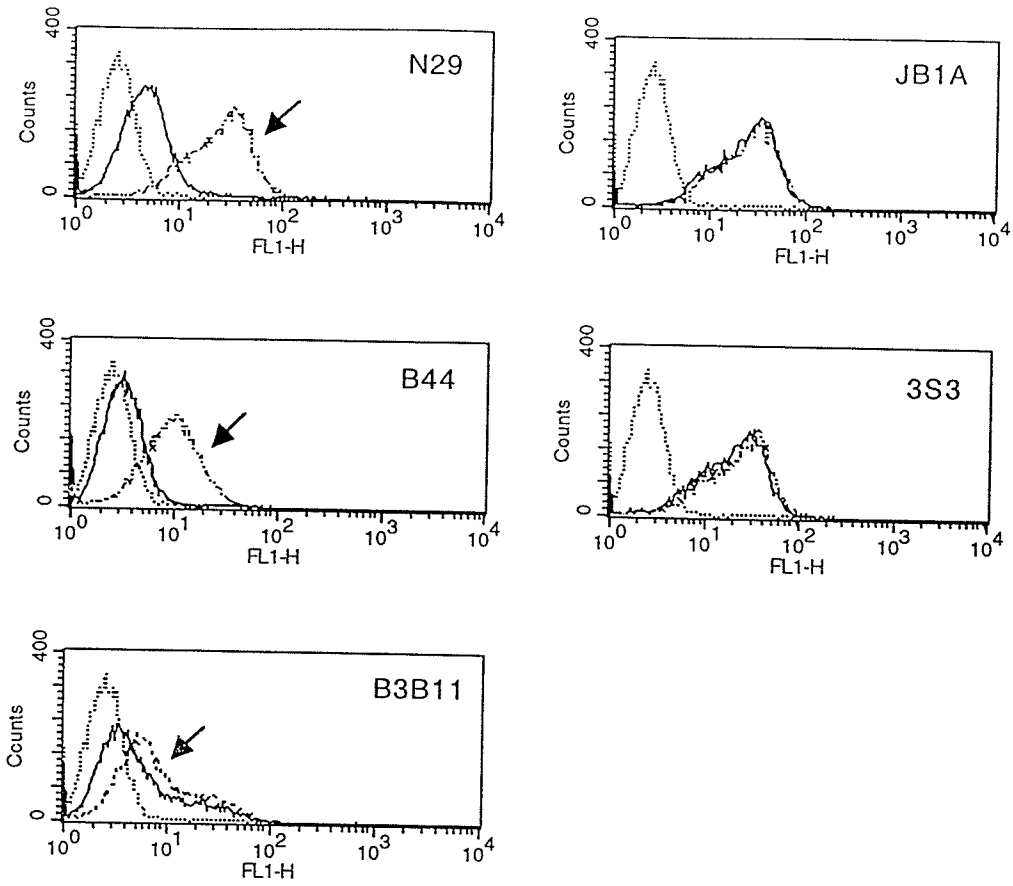

FIG. 4. **The effects of DTT treatment of IM9 cells on β_1 epitopes expression.** Cells were treated with DTT (10 mM) and stained with the indicated antibodies. The control, untreated, and DTT cell profiles are represented by the *dotted*, *solid*, and *dashed lines*, respectively. The *arrows* indicate the profiles of the DTT-treated cells.

observed that this cation could induce conformational states which resembled those of a ligand-occupied integrin, the effects of ligand binding on B44 expression were examined. Treatment of the cells with fibronectin or RGDS-containing peptides resulted in a 2–3-fold increase in the B44 levels (Fig. 5). The expression levels of the other epitopes were not significantly changed by this treatment. Furthermore, the control peptide RGES did not induce these changes, indicating that the effects were specific to integrin ligands.

Location of the N29 Epitope—A β_1 epitope library was screened with N29 and B44 and a single N29 reactive clone, B105, was identified. DNA sequencing of B105 indicated that this clone contained the first 105 residues of the β_1 chain (data not shown). Previous studies had determined that the JB1A epitope consisted of residues 82–87 and that a panel of monoclonal antibodies to the β_1 chain including N29 did not react with fusion proteins containing a fragment spanning residues 55–105 (29).

Expression of β_1 residues 1–57 as a fusion protein resulted in a product that was reactive with N29 under reducing conditions (Fig. 6). In contrast, N29 did not react with a gonococcal porin (1b) fusion protein expressed in the same vector system. The specificity of the reaction was also demonstrated by the fact that N29 but neither B3B11 nor JB1A reacted with the fusion protein (Fig. 7*A*). Furthermore, preincubation of N29 with the purified 1–57 fragment specifically inhibited the binding of N29 to purified β_1 (Fig. 7*B*).

Honda *et al.* (40) have described a stimulatory antibody to β_3, AP-5, which recognized a cation-sensitive epitope containing residues 1–6 of the β_3 integrin (34). It was therefore questioned if the N29 epitope might be located in a homologous region of the β_1 chain. Pretreatment of N29 with a synthetic peptide containing residues 1–14 did not influence the ability of the antibody to bind to purified β1 (Fig. 7*B*). These results indicate that the N29 epitope was located between residues 15 and 54 of the β_1 chain.

DISCUSSION

The present studies provide several new pieces of data relevant to integrin activation. 1) The stimulatory antibody, N29, recognizes a new regulatory region located near the NH_2 terminus of the β_1 molecule. 2) The stimulatory antibody, B44, identifies an epitope, which is exposed on ligand binding. 3) Mn^{2+} and DTT induce changes in β_1 epitope accessibility, which resemble those observed in the ligand occupied receptor. 4) The overall accessibility of epitopes in physiologically active integrins is increased relative to those on nonadherent cells or on Mn^{2+}- and DTT-activated cells.

The initial assumption that N29 might identify an activation epitope does not appear to be fully supported by the results of this study. In the case of DTT-treated cells, there was an almost total exposure of the N29 epitope associated with activation of adhesion. However, the N29 levels on spontaneously adhesive cells such as K562 and Jurkat[2] or following treatment with Mn^{2+} were elevated 2–4-fold, such that 20–30% of the integrins displayed this epitope. There were also low but detectable levels of N29 exposure on nonadherent cells. Thus the correlation between integrin functional status and N29 accessibility appeared to be semiquantitative rather than a qualitative one.

[2] H. Ni and J. A. Wilkins, unpublished results.

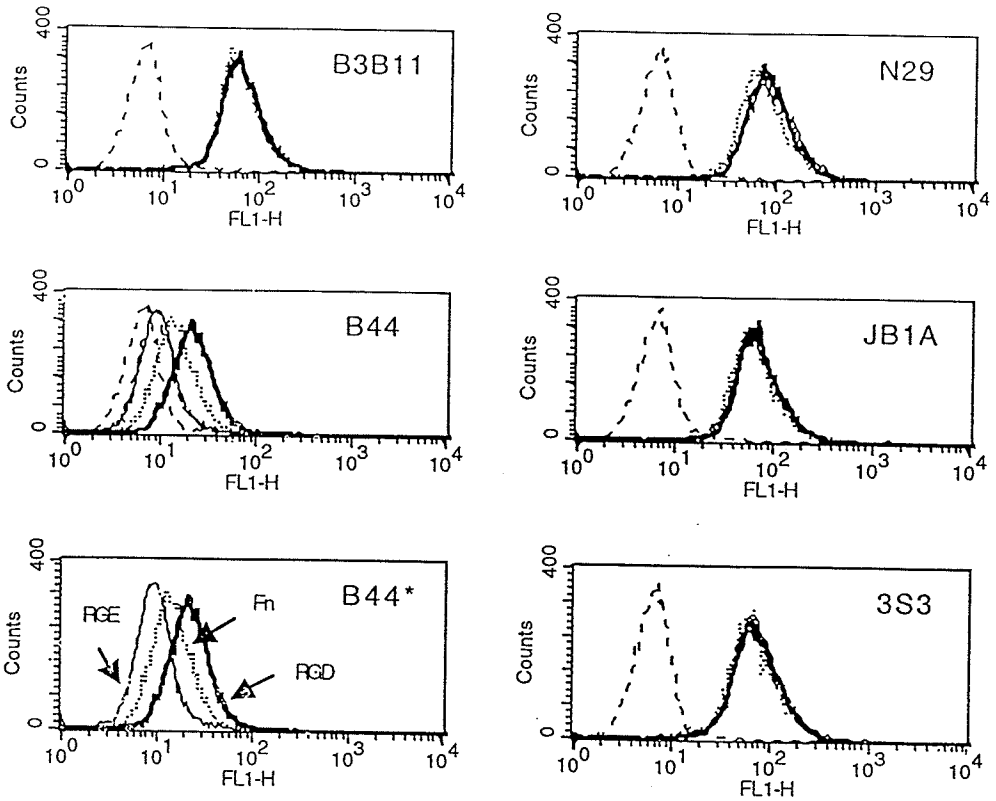

FIG. 5. **The effects of ligand binding on the β_1 epitope expression of K562 cells.** Cells were treated with fibronectin or RGDS/RGES peptides, and stained with the indicated antibodies. The panel labeled *B44** indicates the line patterns used for each treatment group. Note that the negative control cells are omitted from this panel for clarity. Control binding is indicated in all other panels by the line with *large dashes*.

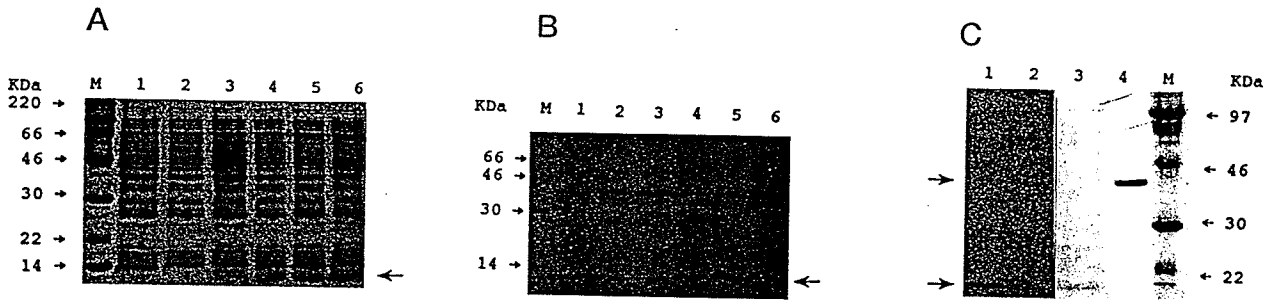

FIG. 6. **The expression and reactivity of the β_1 1–57 fusion protein.** *Panel A*, Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis gel; *lanes 1*, no vector; *2*, vector only; *3*, vector plus insert uninduced; *4–6*, isopropyl-1-thio-β-Dgalactopyranoside-induced cells containing the β1–57 insert at 1, 2, and 3 h postinduction. The *arrow* indicates the location of the fusion protein. *Panel B*, a Western blot of a replicate of *panel A* stained with N29. *Panel C*, *lanes 1* and *3* contain purified β1–57 fusion protein, *lanes 2* and *4* contain a gonococcal porin (1b) fusion protein produced in the same vector as a control. *Lanes 1* and *2* were reacted with N29, *lanes 3* and *4* were stained with Coomassie Blue.

The antibody B44 identifies an epitope, which under normal conditions appears to be of very limited accessibility. Thus the expression levels of this epitope on adhesion competent cells such as Jurkat and K562 are significantly lower than the total integrin levels. However, occupancy of integrin by ligand or by an RGD-containing antagonist results in a marked increase in B44 expression. The B44 epitope is reduction resistant under SDS-polyacrylamide gel electrophoresis conditions, implying that the antibody detects a continuous peptide sequence. It appears that ligand binding exposes the cryptic epitope to the solvent and renders it antibody accessible. However, it is unlikely that this epitope represents a ligand contact site as B44 binding has been shown to induce adherence in Jurkat cells (28). The properties of B44 most resemble those of two other antibodies, 15/7 (35) and HUTS-21 (36), which detect integrins in a ligand-occupied or high affinity state. These antibodies have been shown to react with epitopes that are located in the cysteine rich region of the β_1 (residues 355–425). However, to date it has not been possible to determine the location of the B44 epitope.

Treatment of IM9 cells with Mn^{2+} induces B44 epitope expression. The implication is that the Mn^{2+} induces alterations that resemble those caused by ligand binding to a competent integrin. It has been suggested that Mn^{2+} may stimulate adhesion by forming a co-ordination complex with residues in the cation binding domains of the integrin and the aspartate residue of the ligand (37, 38), or by facilitating the ligand entry to the binding site via an exchange mechanism (18). Recently it has been proposed that Mn^{2+} may induce a conformation resembling the ligand occupied receptor thus permitting ligand access to the binding region of the integrin (17). The binding pattern of B44 is compatible with the latter explanation of Mn^{2+} action. However, it does not address the issue of the relative contributions of Mn^{2+} to cation-facilitated exchange and ligand co-ordination.

Activation of adhesion by reducing agents has also been

described in several systems. Edwards *et al.* (25) noted that there was an obligate requirement for a bifunctional thiol with a minimal spacing of four carbons between the two –SH groups.

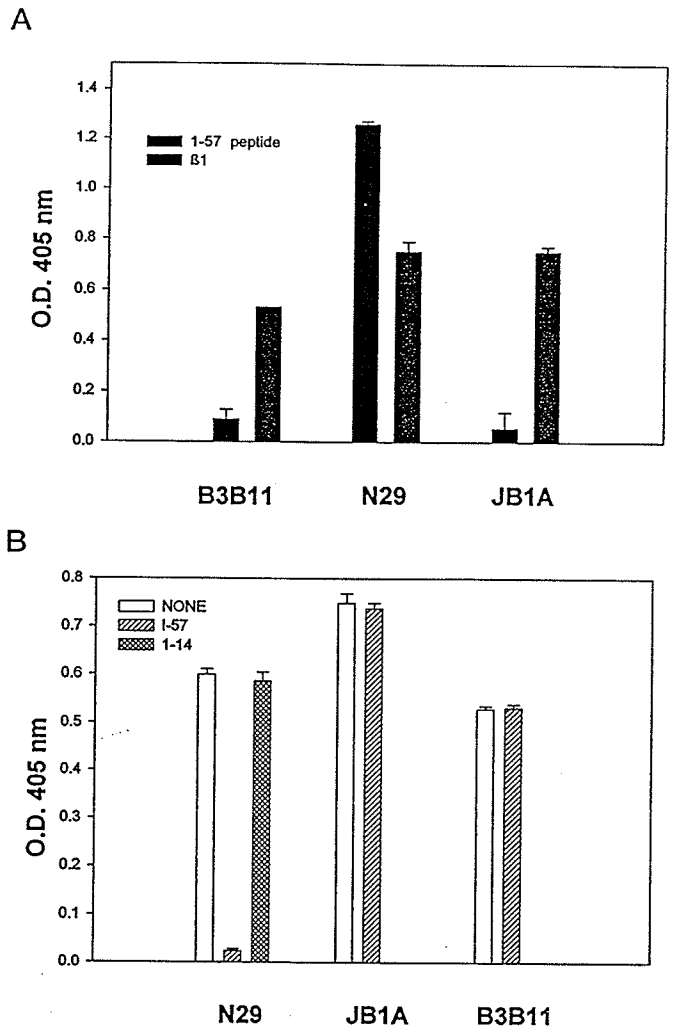

FIG. 7. **The specificity of antibody binding to β1–57 fusion protein.** *A*, the binding of B3B11, JB1A, and N29 to immobilized β1–57 fusion protein or to purified β_1 integrin were compared in an enzyme-linked immunosorbent assay. *B*, the capacity of β1–57 fusion protein to block the binding of the indicated antibodies to immobilized purified β_1 integrin. The effects of a synthetic peptide containing β_1 residues 1–14 on N29 binding were also determined.

Early studies on the activation of platelet adhesion by DTT indicated that there were changes in $\alpha_{IIb}\beta_3$ electrophoretic mobility associated with activation by this agent (26). The DTT-dependent activation of mutant $\alpha_{IIb}\beta_3$ in platelets from a patient with Glanzmann's thrombasthenia by DTT was shown to be associated with the appearance of activation epitopes (39). However, DTT-induced activation of $\alpha_L\beta_2$ mediated adhesion of natural killer cells to intercellular adhesion molecule 1-expressing target cells failed to reveal conformational changes using two reporter antibodies (25). Furthermore, these authors could not demonstrate the appearance of free thiol groups in the $\alpha_L\beta_2$ complex, implying that the integrin chains were not directly modified by DTT treatment (25). The present data clearly indicate that significant conformational changes are induced by DTT as access to the B44 and the N29 epitopes are markedly increased.

The increased B3B11 and N29 expression on K562 cells implies that physiologically activated integrins undergo changes that allow an increased accessibility to the membrane proximal and NH_2-terminal regions of the molecule. Although the integrins on these cells are in an adhesion competent state, ligand binding is required for B44 epitope expression. These results would seem to suggest that there is an intermediate conformation in which the integrin is adhesion-competent but unoccupied. The fact that agents such as Mn^{2+} and DTT induce conformations that resemble the ligand-occupied state suggests that they stimulate adhesion competence by generating integrin intermediates that are distinct from the native active forms observed in K562. Although different functional forms of integrins have been described or postulated (16, 17, 40), it is unclear at this point whether active forms such as those induced by DTT or Mn^{2+} are representative of physiological integrin intermediates. These observations suggest that caution should be exhibited when attempting to correlate competent states induced by these agents with those found in physiologically activated integrins.

The localization of the N29 epitope between residues 15 and 54 places it in a highly conserved cysteine-rich region (41). This area has not previously been identified as a regulatory site, although it is adjacent to region that has been shown to be a cation and ligand sensitive in the β_3 chain (34). Unlike the β_3 situation, the binding of N29 is relatively insensitive to the cationic composition of the extracellular milieu. Thus if a homologous region exists in the β_1 chain it would appear that it is not located in the N29 reactive 15–54 sequence of the molecule.

The antibodies N29, B44, and B3B11/JB1B were originally identified because of their abilities to stimulate Jurkat adher-

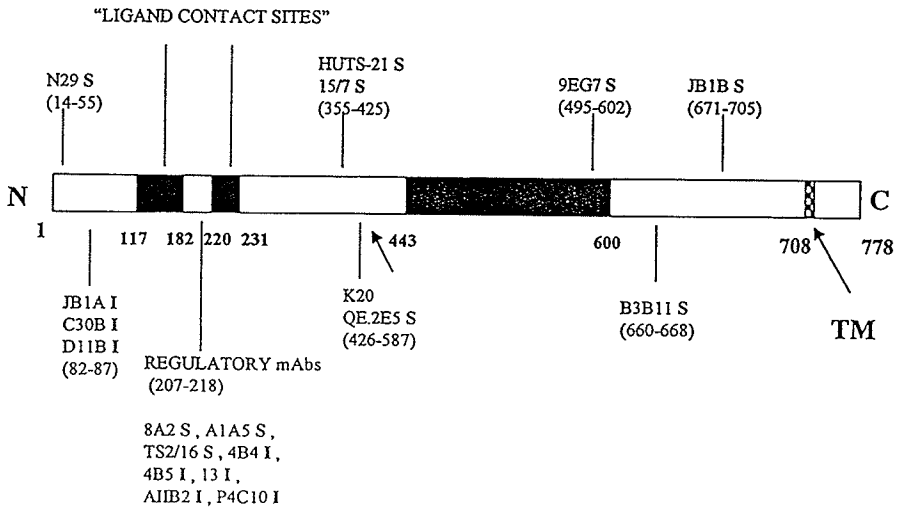

FIG. 8. **The locations of regulatory epitopes on human β_1.** The relative positions of the epitopes recognized by regulatory antibodies (*i.e.* stimulatory (*S*) and inhibitory (*I*)). The positions of the two putative ligand contact sites (*solid area*), the cysteine-rich repeat region (*gray area*), and the transmembrane region (*checkered area*) are indicated. The relevant references are provided in the text.

ence to collagen and fibronectin (28). It is noteworthy that in those cases where their corresponding epitopes have been identified (28, 29), the stimulatory epitopes map to regions that are in close proximity to residues that are predicted to be involved in disulfide bonds between sequentially distant cysteines (*i.e.* Cys^7–Cys^{415} and Cys^{444}–Cys^{671}). The present results extend those of others employing interspecies β_1 chimeras (16, 17, 42–44) and expands the locations of regulatory sites to include both the membrane proximal and the distal regions of the β_1 (Fig. 8).

It might be speculated that the NH_2-terminal region of the β_1 chain is involved in the normal control of integrin function. The increased N29 expression on K562 could reflect a situation in which physiologically activated integrins undergo a conformational change to expose this site. The exposure may indicate accessibility to the ligand-binding site. However, it seems unlikely that N29 contact is required for binding as Mn^{2+} induces adhesion competence with minimal effects on N29 exposure. Subsequent to ligand binding, the B44 epitope is expressed, and this presumably reflects a secondary change in the integrin conformation, perhaps as a consequence of ligand displacement of previously buried residues. It is important to bear in mind that, although the results of the antibody studies indicate changes in the accessibility of β_1 integrin epitopes following activation, the basis for these changes are unknown. They could relate to integrin conformational changes, to alterations in the patterns of integrin-associated proteins, or to both of these mechanisms. Studies with purified integrin may permit the differentiation of these possibilities.

Acknowledgments—We thank Drs. E. Ruoslahti and C. Damsky, respectively, for providing the pFnRβ and AIIB2 antibody and Dr. Guangming Zhong for assistance with the fluorescence-activated cell sorter analysis.

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The Selective Inhibition of β_1 and β_7 Integrin-Mediated Lymphocyte Adhesion by Bacitracin[1]

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Integrins play an important role in lymphocyte adhesion to cellular and extracellular components of their microenvironment. The regulation of such adhesion often involves changes in the functional state of the integrins rather than alterations in their expression levels. Although the functional basis for such transitions is unknown, a possible role for disulfide exchange might be postulated based on the observations that integrin function can be activated by bifunctional reducing agents or by Abs that react with areas adjacent to predicted long-range disulfide bonds in integrins. Recently, it has been reported that enzymes that catalyze disulfide exchanges such as protein disulfide isomerase (PDI) are present on the surface of lymphoid cells, raising the possibility that such enzymes might be involved in the control of lymphocyte adhesion. A number of inhibitors of PDI function were examined for their effects on integrin-mediated adherence of T cells. The results did not support role for PDI in the regulation of integrin function, as the inhibitors somatostatin A, tocinoic acid, dithiobisnitrobenzoic acid, and anti-PDI mAb did not interfere with adherence. However, one of the PDI inhibitors, bacitracin, selectively interfered with the β_1 integrin-mediated adherence of lymphoid cells to collagen, fibronectin, laminin, and VCAM-1, and with $\alpha_4\beta_7$-dependent adherence to fibronectin and to VCAM-1. In contrast, $\alpha_v\beta_3$- and $\alpha_L\beta_2$-mediated adherence were not inhibited. Thus, it appears that bacitracin may be a selective inhibitor of β_1 and β_7 integrin functions by an as yet unknown mechanism. *The Journal of Immunology,* **1998, 161: 6323–6329.**

Lymphoid cells display marked variations in their requirements to interact with other cells and with extracellular matrix components of their microenvironment. These interactions are essential for the appropriate recruitment to and retention of cells at sites of immunologic responsiveness (1). Thus, it is important for lymphocytes to acquire adhesive potential at the appropriate tissue sites. Agents such as Ags, mitogenic stimuli, chemokines, and cytokines have been shown to induce lymphocyte adhesion, thus providing a basis for the selection of the appropriate cells from the circulating lymphocyte pool (2–4).

Integrins represent one of the major adhesive systems employed by lymphoid cells for extravasation, migration, and adherence to the extracellular matrix. Integrins are expressed on the surface of leukocytes in a latent form that can be activated by a variety of stimuli (1–4). Often the acquisition of adhesion competency is not associated with changes in the levels of integrin expression, implying that the surface-expressed molecules undergo conformational changes (5). Although there is considerable evidence from a variety of biochemical and immunologic approaches to support such a contention, the molecular basis for the transition to an active state is unknown (6).

A number of stimuli, including divalent cations such as Mn^{2+} and Mg^{2+}, have been shown to activate integrin function (7–9). In many cases, it has been possible to demonstrate that the integrin complex acquires a conformation that resembles that of a ligand-occupied structure (10–12), suggesting that these agents may promote binding by stabilizing an integrin conformation that facilitates ligand-receptor interactions (6). Collectively, these observations raise the possibility that physiologic mechanisms might also generate such a conformational transition.

The β_1, β_2, and β_3 integrins can be activated by bifunctional reducing agents such as DTT (13–16). There are structural constraints on the distance separating the sulfydryl groups of the reducing agent, implying that there may be a minimal distance that must be spanned to allow for simultaneous $-SH$ exchange on the target molecule (15). We have demonstrated that the induction of β_1 adhesion by DTT is associated with conformational changes in the integrins, suggesting that the acquisition of adhesion competence may be the result of the direct actions of the reducing agent on the integrin (13).

It has recently been reported that the enzyme protein disulfide isomerase is located on the surfaces of a variety of cell types, including lymphocytes (17–21). The distribution of this enzyme was previously thought to be restricted to the endoplasmic reticulum, where it plays an essential role in the exchange of disulfide bonds and in the proper folding of newly synthesized protein (20). Surface-associated thiol disulfide transferase activity has been shown to be necessary for diphtheria toxin activation and for the infection of lymphoid cells with HIV (22, 23). Activation of platelet adhesion has also been shown to lead to a marked increase in PDI expression on the surface of these cells. It was therefore questioned whether cell surface PDI[3] might play a role in integrin activation, possibly by facilitating intramolecular disulfide exchanges. As an approach to examining this possibility, the effects of a number of inhibitors of PDI function were examined for their effects on integrin-mediated adherence of lymphoid cells to a number of ligands.

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Received for publication February 27, 1998. Accepted for publication August 6, 1998.

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[1] The Medical Research Council of Canada and the Canadian Arthritis Society supported this research. H.N. and Y.M., respectively, are recipients of studentships from the Manitoba Health Research Council and the Faculty of Graduate Studies, University of Manitoba.

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[3] Abbreviations used in this paper: PDI, protein disulfide isomerase; DTNB, dithiobisnitrobenzoic acid.

0022-1767/98/$02.00

The present studies demonstrate that an inhibitor of PDI activity, bacitracin, inhibits β_1 integrin-mediated adhesion of lymphoid cells to collagen, fibronectin, laminin, and VCAM-1, and β_7 to fibronectin and VCAM-1. Bacitracin interferes with binding of soluble fibronectin, implying that the inhibition is due to a direct effect on integrin-ligand interactions. These effects appear to be selective, as β_2 and β_3 integrin-dependent adherence was not inhibited by bacitracin. The fact that these effects were not observed with other inhibitors of PDI activity suggests that the mechanism of bacitracin action does not involve interference with surface thiol-reductase activity.

Materials and Methods

Materials

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO). Media and FBS were obtained from Life Technologies (Gaithersburg, MD). Purified human plasma fibronectin and FITC goat anti-mouse IgG were obtained from Chemicon (Temecula, CA).

Antibodies

The Abs to β_1, JB1A (24), B3B11, N29 (25), and 3S3 (26) have been described previously. Anti-PDI, RL 77 (27), was purchased from Affinity Bioreagents (Golden, CO). The hybridoma was subsequently obtained through Dr. Charlotte Kaetzel (University of Kentucky). A rat anti-mouse β_7 integrin chain that cross-reacts with human β_7 integrin, FIB 504 was purchased from PharMingen (San Diego, CA). Anti-β_2 and anti-$\alpha_v\beta_3$, LM-609, were purchased from Chemicon.

Cells and culture

PBMCs were isolated from normal healthy volunteers on Ficoll-Hypaque and cultured for 72 h in RPMI 1640 containing 10% FBS and 10 μg/ml of phytohemagglutinin-P (PHA-P). Human rIL-2 (25 U/ml) was added to the culture, and the cells were maintained by dilution every 2 to 3 days in fresh media containing IL-2 until day 12, after which time they were used for analysis.

IL-2-dependent T cells were washed twice in RPMI and resuspended in media containing bacitracin (0.1–10 mM), tocinoic acid (0.5 mM), or anti-PDI (800 μg/ml) for 30 min before activation for adherence. Cells were stimulated with PMA (50 ng/ml), or anti-β_1 (10 μg/ml) or cross-linked anti-CD3 (150 ng/ml) and added at 2×10^5 cells/well to microtiter wells coated with fibronectin (1 μg/well) (28). The plates were incubated at 37°C for 10 min. The wells were then filled with 0.15 M saline, sealed with an adhesive plate sealer, inverted, and centrifuged at $70 \times g$ for 5 min. The supernatant was aspirated, and the adherent cells were stained with crystal violet for at least 30 min with 0.5% crystal violet in a 30% solution of methanol in water. The plates were washed with tap water to remove unbound dye. The cell-bound dye was dissolved in methanol, and the absorbance at 550 nm was determined. In all assays, the adherence to BSA (OD < 0.1) was subtracted from the values obtained for the fibronectin-coated wells. Unless indicated otherwise, all assays were performed at least three times in pentuplicate.

The human lymphoid cell lines Jurkat and RPMI 8866 were examined for adhesion, as described above. In some cases, the adherence to fibronectin fragments, 120 or 40 kDa (Life Technologies), was determined by substituting the purified fragments for fibronectin in the coating of the wells.

Ab blocking of cell adhesion was achieved by preincubating the cells with the indicated Abs for 30 min before the addition to the cell-binding assays.

Homotypic aggregation assays

JY cells were treated with 50 ng/ml PMA with or without 3.5 mM bacitracin for 3 h and microscopically examined for homotypic aggregation. Ab inhibition studies were performed by incubating the cells with 10 μg/ml of blocking anti-β_2.

Flow cytometry analysis

Cells were preincubated with the indicated stimuli at room temperature and then incubated with the indicated Ab (5 μg/ml) for 30 min at 37°C. The cells were washed twice with PBS and incubated for 60 min at 4°C with a FITC-labeled goat anti-mouse Ig (Chemicon). Fluorescence analysis was performed with a BD FACScaliber.

The direct binding of soluble fibronectin was monitored by incubating control or bacitracin-treated (3.5 mM) cells with 50 μg of biotinylated plasma fibronectin for 30 min at 37°C. The cells were washed three times with PBS and incubated with FITC-conjugated avidin at 4°C for 30 min. The cells were washed and examined for fluorescence by FACS, as described above.

Purification of β_1 integrin

Integrin was isolated from human placenta, used as previously described (13). Briefly, 300 g of washed placenta was homogenized in 300 ml of 50 mM *n*-octylglucopyranoside in 25 mM Tris, pH 7.6, 150 mM NaCl, 2 mM $CaCl_2$, and 1 mM PMSF. The homogenate was centrifuged $10,000 \times g$ for 1 h at 4°C, after which the supernatant was collected and passed sequentially through an OVA Sepharose 4B and a JB1 Sepharose 4B column at a rate of 1 ml/min. The column was washed sequentially with 20-column volumes each of 1) 0.1% Nonidet P-40 in 25 mM Tris, pH 7.6, 150 mM NaCl, and 2 mM $CaCl_2$, and 2) 0.1% Nonidet P-40 in 0.01 M sodium acetate buffer, pH 4.5. The β_1 integrin was eluted from the JB1 column in 0.1% Nonidet P-40, 10 mM sodium acetate buffer, pH 3.6, and 3-ml fractions were collected into tubes containing 0.5 ml of 3 M Tris, pH 8.8.

The purity of the fractions was assessed by SDS-PAGE and Coomassie blue staining. The fractions containing β_1 also had a mixture of the associated α-chains. However, the α- and β_1-chains collectively represented greater than 85% of the total stained proteins. The presence of β_1 in the fractions was confirmed by Western blot with JB1A and B3B11.

Fibronectin binding to purified β_1 integrin

Purified integrin was diluted in PBS, and microtiter wells were coated for 18 h at 4°C with 100 μl (50–100 ng) of the dilute integrin preparation. The plates were washed with PBS and blocked with 2% BSA in PBS, and 1 μg of biotin-labeled fibronectin (10 μg/ml) in RPMI was added in the presence or absence of 3.5 mM bacitracin. The plates were incubated at 37°C for 3.5 h and subsequently washed five times with PBS containing 0.05% Tween-20. Alkaline phosphatase-labeled avidin was added to the wells, and the plates were then incubated at 37°C for 30 min. The plates were washed five times and substrate was added. The plates were incubated at 37°C for 30 min, and the OD was determined at 405 nm. The experiment was performed twice in replicates of five each time.

Results

Effects of PDI inhibitors on β_1-mediated T lymphocyte adherence

Several inhibitors on PDI activity were examined for their effects on the adhesion of IL-2-dependent T cells to fibronectin. We and others have demonstrated previously that the adherence of these cells to fibronectin is mediated by $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins (29). Bacitracin, tocinoic acid, and dithiobisnitrobenzoic acid (DTNB) have previously been reported to interfere with cell surface thiol-reductase activity and with PDI function (22, 23, 30, 31). Treatment with 1.5 mM bacitracin resulted in a >80% inhibition of PMA-induced adherence (Fig. 1*A*). In contrast, exposure to 5 mM DTNB, a concentration greater than those previously reported for inhibition enzyme activity, did not influence adhesion (Fig. 1*A*). Similarly, pretreatment of the cells with concentrations of tocinoic acid reported to block PDI activity did not affect the induction of adhesion (Fig. 1*B*). Another inhibitor of PDI activity, somatostatin A (0.2 mM), also had no effect on cell adhesion (data not shown).

As an alternative approach to determining a possible role for PDI in lymphocyte adhesion, cells were treated with concentrations of a function-blocking anti-PDI mAb, RL-77 (22). Exposure to Ab concentrations up to 800 μg/ml (the highest concentration tested) failed to influence adherence, while bacitracin caused a concentration-dependent inhibition of the adherence with a 50% inhibition at 1–2 mM (Fig. 2).

Bacitracin effects on T cell adhesion

The above results suggested that PDI was not directly involved in the induction of β_1 integrin-mediated adhesion to fibronectin. This raised the question of the mechanism(s) of bacitracin inhibition of adherence.

Stimulation of T cell adherence by PMA or anti-CD3 treatment was inhibited by pretreatment with 3.5 mM bacitracin (Fig. 3).

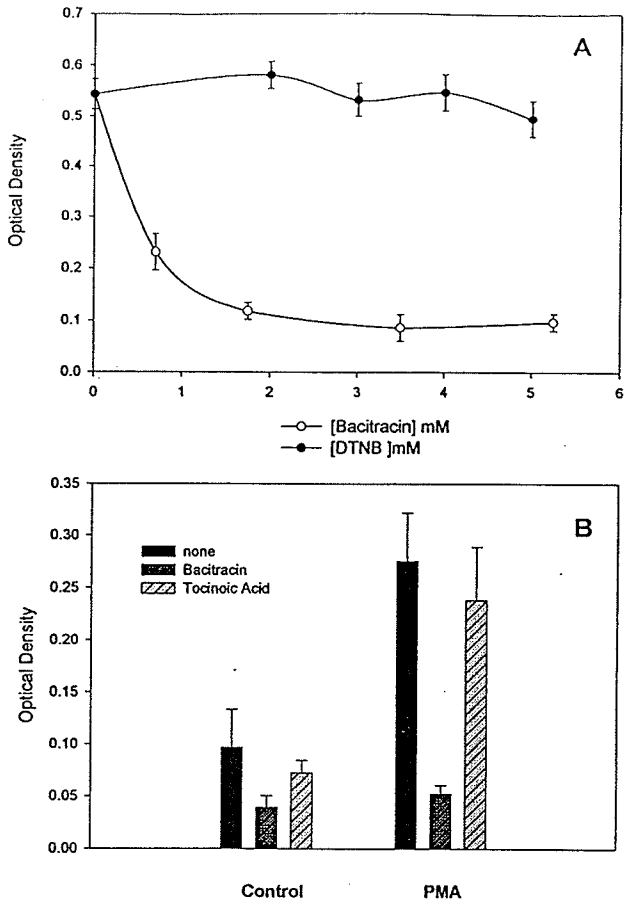

FIGURE 1. The effects of inhibitors of PDI and disulfide exchange on lymphocyte adherence to fibronectin. *A*, IL-2-dependent T cells were pretreated with the indicated concentrations of bacitracin or DTNB and assayed for adherence to immobilized fibronectin following PMA stimulation. *B*, Cells were untreated or pretreated with tocinoic acid (0.5 mM) or bacitracin (3.5 mM), and either stimulated with PMA or untreated and assessed for binding to fibronectin. The results for all experiments are the mean of sextuplicate assays that have been repeated at least three times.

Under these conditions, the adherence of the non-PMA-treated cells was also inhibited by bacitracin, implying that the inhibition was occurring at a postactivation event. To test this possibility, cells were treated with PMA for 30 min to activate adhesion, exposed to bacitracin for 30 min, and assayed for adherence. The control cells that were activated by PMA displayed strong binding to fibronectin. This contrasted with cells activated with PMA and treated with bacitracin, in which almost complete inhibition was observed. It would appear that bacitracin inhibits adherence either by reversing adhesion competence or by interfering with the integrin function because it can block preactivated integrin function.

The activation of adhesion by PMA and stimulatory Abs has been shown to have different biochemical requirements, as only the former was staurosporine H sensitive (32). The adhesion induced by the stimulatory anti-β_1, B44, was also fully inhibited by bacitracin (Fig. 4). The induction of adherence by Mn^{2+}, anti-β_1, TS2/16, or DTT was also inhibited by bacitracin (data not shown). These results suggested that the site of action was at a postactivational β_1 step.

The possibility that the action of bacitracin was due to a toxic effect on the cells was examined by exposing the cells to bacitracin, and removing the bacitracin from one set of cells. The spontaneous and PMA-induced adhesion was then compared with either cells that were nonbacitracin treated or with cells that had bacitracin present throughout the incubation period. Within 30 min of removal of bacitracin, the cells displayed a 40–45% recovery of adhesive potential (Fig. 5). These results, in conjunction with direct viability measurements, using trypan blue staining of the cells (data not shown), suggested that the bacitracin effects were at least partially reversible and they were not a result of direct cytotoxicity for the cells.

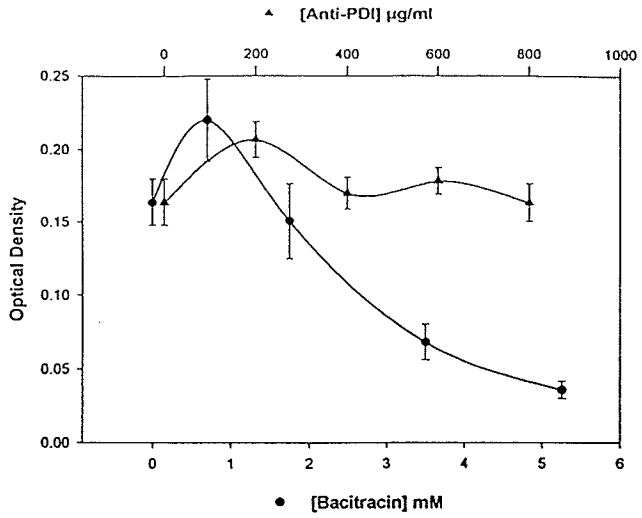

FIGURE 2. The effects of anti-PDI on lymphocyte adherence. Cells were pretreated with the indicated concentrations of anti-PDI or bacitracin and assessed for PMA-induced binding to fibronectin in the presence of the indicated inhibitors.

The effects of bacitracin exposure on integrin expression levels were determined by comparing the staining patterns of treated cells with those of control cells. Four Abs were used for the analysis. JB1A inhibits adhesion and it detects an epitope (residues 82–87) that is normally accessible on the β_1-chain (28). N29 stimulates adhesion and it recognizes an epitope near the $-NH_2$ terminus of the β_1-chain (13). Access to this epitope appears to be related to functional status of the integrin. The stimulatory Ab B3B11 reacts with a membrane-proximal epitope (666–668) (25). The Ab 3S3 blocks adhesion and it recognizes a discontinuous epitope (26). The level of staining of cells with these Abs was not influenced by

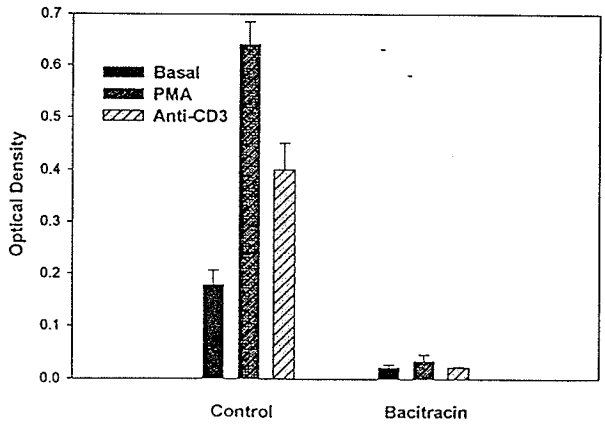

FIGURE 3. The effects of bacitracin on PMA- or anti-CD3-activated adherence of lymphocytes. Cells were untreated or stimulated with anti-CD3 or PMA in the presence or absence of bacitracin (3.5 mM) and assessed for binding to fibronectin.

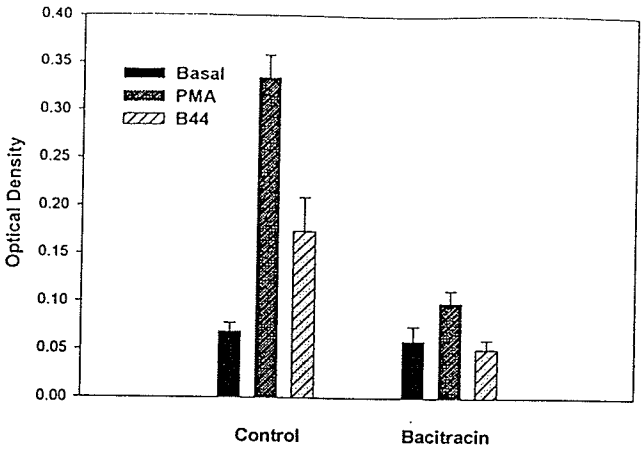

FIGURE 4. The effects of bacitracin on activated cell adhesion. Cells were activated with the indicated stimuli and then either exposed to bacitracin (3.5 mM) or untreated and assayed for binding to fibronectin.

the presence of 3.5 mM bacitracin (Fig. 6). These results suggested that the integrin levels and conformations were not altered by bacitracin.

The ability of cells to bind soluble fibronectin was assessed as a direct test of integrin competence. Treatment of the cells with bacitracin resulted in a complete inhibition of fibronectin binding, suggesting that the integrin function was the site of action rather than postreceptor occupancy events required for cell adherence (Fig. 7).

As a direct test of the above prediction, the effects of 3.5 mM bacitracin on the binding of fibronectin to purified β_1 integrin were examined. Coincubation of biotinylated fibronectin with bacitracin resulted in >80% inhibition of fibronectin binding (Fig. 8). In contrast, the background binding of fibronectin to BSA-coated plates was relatively unaffected by this treatment. These results suggest that bacitracin can directly interfere with fibronectin binding to purified β_1 integrin.

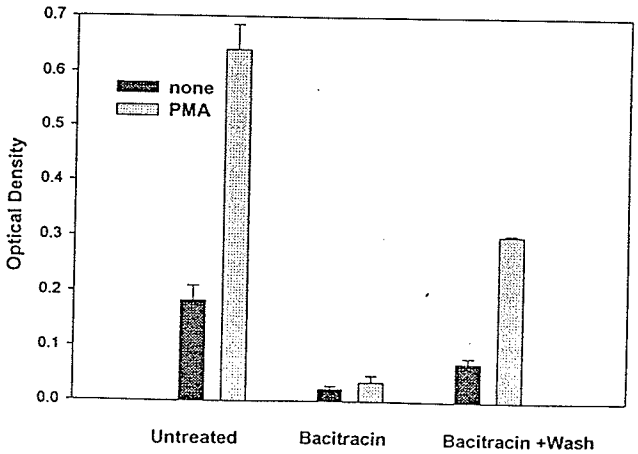

FIGURE 5. The reversibility of bacitracin effects on cell adhesion. Cells were treated with bacitracin (3.5 mM) for 30 min and the bacitracin was removed. The cells were then incubated for 30 min in fresh media and assessed for spontaneous and PMA-induced adherence to fibronectin. The adhesion was compared with control cells that had been handled in a similar fashion, but not exposed to bacitracin, or with cells that had been maintained in the presence of bacitracin.

Integrin inhibition patterns of bacitracin

Jurkat cells have previously been shown to employ both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ to bind to fibronectin (7). The former receptor binds to a 40-kDa chymotryptic fragment of fibronectin, while the latter recognizes a 120-kDa fibronectin fragment (33, 34). The use of fragments that contain only one of the binding sites allowed for the analysis of cell binding by one receptor type in isolation of possible contributions by occupancy of the other.

Jurkat cells were incubated with 3.5 mM bacitracin and examined for adherence to either 40- or 120-kDa fragments of fibronectin. The adherence to both fragments was markedly inhibited by this treatment (Table I). In both cases, the inhibition was comparable with that seen with the inhibitory anti-β_1, 3S3 (data not shown). Thus, it appears that both $\alpha_4\beta_1$- and $\alpha_5\beta_1$-mediated binding to fibronectin are inhibited by bacitracin.

The binding of IL-2-dependent cell lines to laminin and collagen was also blocked by bacitracin (Table I). These results indicated that at least $\alpha_2\beta_1$-, $\alpha_4\beta_1$-, $\alpha_5\beta_1$-, and $\alpha_6\beta_1$-mediated adherence were inhibited by bacitracin. It was noteworthy that inhibition of collagen adherence required a much higher concentration of bacitracin than was required for fibronectin and laminin.

The B cell line, RPMI 8866, displays spontaneous and PMA-inducible adherence to the 40-kDa fragment of fibronectin (35, 36). This adherence was fully inhibited by an Ab to the β_7-chain, FIB 504, but not by the anti-β_1, 3S3 (Fig. 9*A*). The RPMI 8866 adhesion to this fragment of fibronectin was fully inhibited by bacitracin, indicating that adhesion by the $\alpha_4\beta_7$ complex was also sensitive to bacitracin (Fig. 9*A*).

RPMI 8866 cells can also be induced by PMA to adhere to fibronectin via $\alpha_v\beta_3$. This integrin complex binds to the same region of the 120-kDa fibronectin fragment as $\alpha_5\beta_1$ (35). Pretreatment with these cells with 3.5 mM bacitracin (Fig. 9*B*) or even up to 8 mM did not inhibit their adherence to this fragment. Since this concentration fully blocked the $\alpha_4\beta_7$-dependent binding of these same cells to the 40-kDa fragment, these results further exclude cytotoxicity as the mechanism of bacitracin action.

The cell line JY undergoes PMA-induced aggregation that is inhibited by Abs to $\alpha_L\beta_2$ (37). As a test of the range of bacitracin effects, these cells were pretreated with up to 7 mM of the antibiotic and examined for their capacities to aggregate. The bacitracin treatment did not inhibit adherence; in fact, it may have enhanced the aggregation (data not shown).

Discussion

The present studies were initiated to examine the possible role of protein disulfide isomerase-like activity in the control of cellular adhesion. The rationale for this proposal was 1) the demonstration of immunochemical and biochemical PDI-like activity on the surface of lymphoid cells (17, 38); 2) the capacity of bifunctional reducing agents to activate adhesion (15); and 3) the localization of the epitopes of a number of stimulatory Abs to the integrin β-chains to regions adjacent to predicted long-range disulfide bonds (13, 25, 39–41). Collectively, these observations suggest that changes in disulfide bond pairing or associated regions can influence integrin function, and that the necessary enzyme activities to facilitate such reactions are present on the surface of lymphocytes.

Cell surface-associated thiol-reductase activity has been demonstrated on a number of cell types, including lymphocytes (17–19, 38). The infection of T cells by HIV has been shown to require a cell surface-associated thiol-reductase activity (23). In the case of B cells, the activity appears to have immunologic differences with conventional PDI (17). Inhibition of this activity results in a

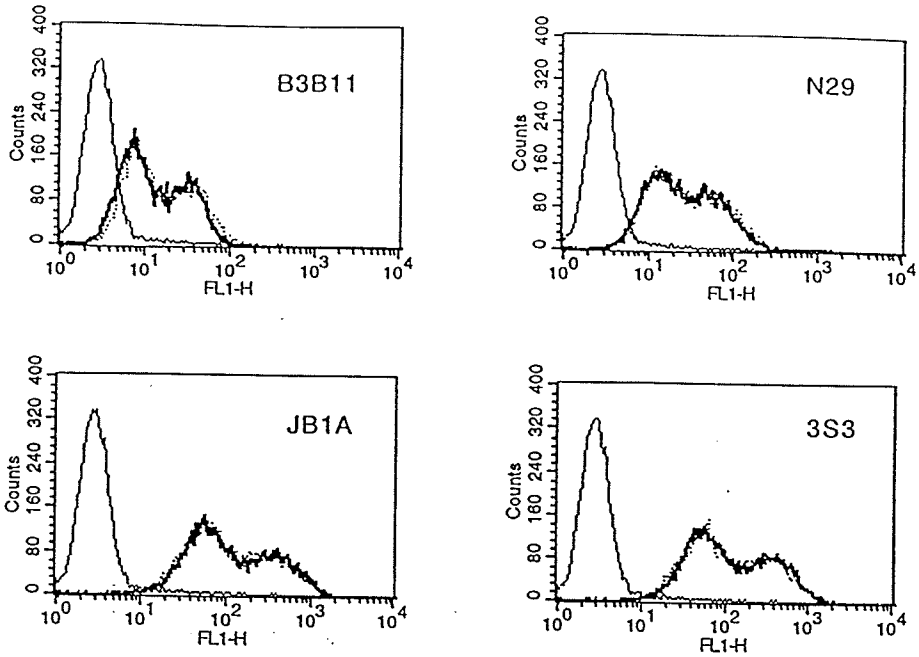

FIGURE 6. The effect of bacitracin on B_1 integrin expression levels and conformation. Cells were incubated with the indicated Abs in the absence or presence of bacitracin (3.5 mM). The expression levels and patterns were examined by flow cytometry. The light line in each histogram represents the negative control. Note the staining patterns of each of the Abs in the presence of bacitracin are totally superimposed on their corresponding untreated counterparts.

marked increase in the levels of free thiol groups on the surface of CLL, suggesting that related enzymes play a role in regulating the oxidation status of membrane proteins (38).

The results of our studies do not support a role for PDI activity in the regulation of integrin-mediated adhesion. Treatment with chemical or immunologic inhibitors of PDI did not influence adhesion. The concentrations used were in excess of the reported K_I values of the various compounds, and they were equal to or exceeding those reported to block T cell membrane thiol-reductase activity. The addition of exogenous functional PDI did not modify the adhesive properties of a number of cell lines that displayed distinctive binding phenotypes (data not shown). These results suggest that PDI activity is not involved in the acquisition or maintenance of T cell adhesion competence. However, the endoplasmic reticulum-associated form of the enzyme undoubtedly plays a critical role in the proper folding of newly synthesized integrins.

The anti-adhesive effects of bacitracin are dependent upon continuous presence of the inhibitor, and the effects on integrin function are reversible. Bacitracin blocks the activity of functional integrins rather than the induction of integrin activity. The FACS results suggest that the mode of inhibition does not involve gross changes in integrin conformation or expression levels. Bacitracin has been shown to be an inhibitor of purified and cell-associated PDI (22, 23, 30, 31). However, as discussed above, it seems unlikely that this is the mode of action in the current studies. The direct inhibition of the binding of soluble fibronectin to cells suggests that the integrins may be the targets for bacitracin effects. This point was further supported by the fact that the binding of fibronectin to purified β_1 was almost fully inhibited by 3.5 mM bacitracin. It should be kept in mind that bacitracin has also been reported to inhibit a broad range of proteases, including members of the aspartic, serine, and cysteine proteases, and metalloproteinases (42). It is not possible to exclude a role for this activity in the observed anti-adhesive effects observed with whole cells. This

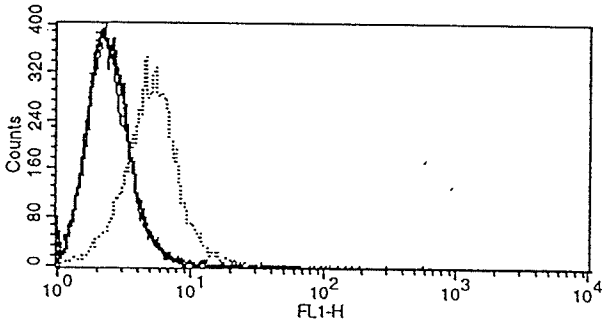

FIGURE 7. The effect of bacitracin on the binding of soluble fibronectin to cell surface $\alpha_5\beta_1$. Cell were incubated with biotinylated fibronectin in the presence or absence of 3.5 mM bacitracin for 30 min and washed. The cells were then reacted with FITC-labeled avidin and examined for staining by FACS. The control binding of fibronectin is marked with the dashed lines; the binding in the presence of bacitracin is indicated by the solid dark line; and the negative control binding is indicated by the solid light line.

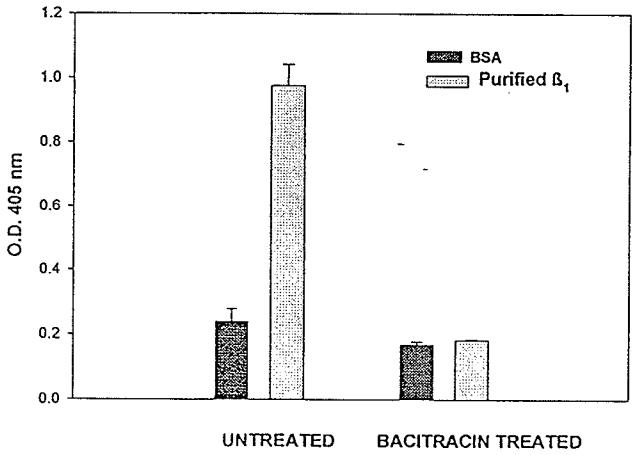

FIGURE 8. The effect of bacitracin on ligand binding to purified fibronectin. Biotinylated fibronectin alone or the presence of 3.5 mM bacitracin was added to the microtiter wells coated with BSA or purified β_1 integrin. The level of bound fibronectin was then determined following reaction with alkaline phosphatase-conjugated avidin and substrate. The experiment was performed twice in replicates of five each time. The mean and the SE of one such experiment is given.

Table I. *The effects of bacitracin on integrin binding of selected ligands*

Cell	Integrin	Ligand	IC_{50}[a]
PBL	$\alpha_4/\alpha_5\beta_1$	Fibronectin	500–1000 μM
PBL	$\alpha_6\beta_1$	Laminin	500–1000 μM
PBL	$\alpha_2\beta_1$	Collagen	5–6 mM
PBL	$\alpha_4\beta_1$	VCAM-1	500–1000 μM
Jurkat	$\alpha_4\beta_1$	Fibronectin, 40 kDa (CS-1)	500–1000 μM
Jurkat	$\alpha_5\beta_1$	Fibronectin, 120 kDa (CBD)	500–1000 μM
RPMI 8866	$\alpha_4\beta_7$	Fibronectin, 40 kDa (CS-1)	500–1000 μM
RPMI 8866	$\alpha_4\beta_7$	VCAM-1	500–1000 μM
RPMI 8866	$\alpha_V\beta_3$	Fibronectin, 120 kDa (CS-1)	> 10 mM

[a] IC_{50} concentration required to induce 50% inhibition of cell adhesion to immobilized ligand.

question warrants further investigation, and biochemical studies are in progress to address this issue.

The full ranges of integrin sensitivities to bacitracin have yet to be determined. To date we have demonstrated that β_1- and β_7-mediated adherence to collagen, fibronectin (both CS-1 and CBD domains), laminin, or VCAM-1 is inhibited by bacitracin. Although the sequences of the β_1 and the β_7 subunits display considerable sequence homology, they are clearly antigenically and functionally distinct with the latter, displaying greater sequence homology with β_2 than β_1 (43). However, it may be that there are critical shared regions of homology between β_1 and the β_7 that are the targets of bacitracin action. The fact that neither β_2 nor β_3 functions are inhibited by bacitracin suggests that these effects do not relate to alterations in central processes that are shared in all adhesive interactions, but rather to aspects of the ligand interaction that are unique to at least the β_1- and β_7-chains. A direct test of this point will require the direct analysis of bacitracin binding sites on purified integrins.

The fact that $\alpha_v\beta_3$ is not inhibited by bacitracin at concentrations that are 10-fold higher than those required to inhibit β_7 function on the same cell indicates that the inhibition is not due to direct cytotoxic effects of the treatment. In preliminary studies, it has been determined that cells can be grown in the presence of 5 mM bacitracin for at least 72 h without any effect on viability. The removal of the bacitracin at this point leads to a complete recovery of cell adherence and growth properties (unpublished data, J.A.W. and M. A. Miranda).

While the mechanism of bacitracin action remains to be elucidated, the selectivity of the effects suggests that this agent may be a useful probe for the analysis of integrin function. It may afford a means to identify functionally homologous regions on distinct integrin families. Also, because of its previous history as a safe therapeutic agent, bacitracin may offer a useful adjunct for the selective modification of adhesion in vivo.

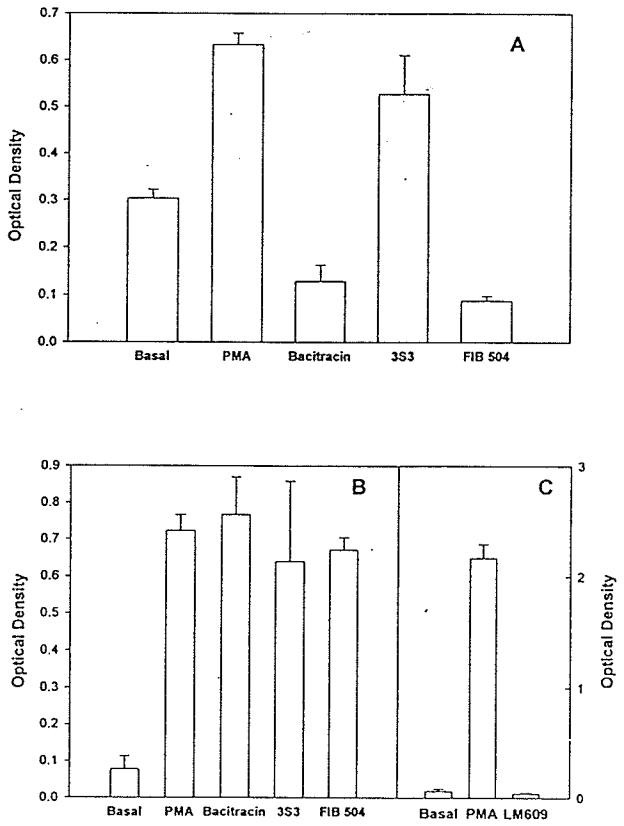

FIGURE 9. The effects of bacitracin on RPMI 8866 adherence to the 40- and 120-kDa fragments of fibronectin. PMA-stimulated RPMI 8866 cells were treated with bacitracin (3.5 mM), anti-β_7 (FIB 504), anti-β_1 (3S3), or anti-$\alpha_v\beta_3$ (LM-609) (*c*) and examined for binding to the 40-kDa (*A*) or 120-kDa (*B* and *C*) fragments of fibronectin.

Acknowledgments

We thank Dr. Charlotte Kaetzel (University of Kentucky) for making the R77 hybridoma available to us, and Dr. Dianne Dottaviao (Leukosite, Boston, MA) for providing us with recombinant VCAM-1. A special thanks to Dr. Hiram Gilbert (Baylor College of Medicine) for his patient and helpful discussions and for providing the purified protein disulfide isomerase.

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